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Dynamic Properties of Gramicidin A in Phospholipid Membranes†

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ABSTRACT: The flexibility of the tryptophan side chains of gramicidin A and the rotational diffusion of the peptide in methanolic solution and in three membrane systems were studied with deuterium nuclear magnetic resonance (NMR). Gramicidin A was selectively deuteriated at the aromatic ring systems of its four tryptophan side chains. In methanolic solution, the tryptophan residues remained immobile and served as a probe for the overall rotation of the peptide. The experimentally determined rotational correlation time of $\tau_c = 0.6 \times 10^{-9}$ s was consistent with the formation of gramicidin A dimers. For gramicidin A incorporated into bilayer membranes, quite different results were obtained depending on the chemical and physical nature of the lipids employed. When mixed with 1-palmitoyl-*sn*-glycero-3-phosphocholine (LPPC) at a stoichiometric lipid:peptide ratio of 4:1, gramicidin A induced the formation of stable bilayer membranes in which the lipids were highly fluid. In contrast, the gramicidin A molecules of this membrane remained completely static over a large temperature interval, suggesting strong protein–protein interactions. The peptide molecules appeared to form a rigid two-dimensional lattice in which the interstitial spaces were filled with fluidlike lipids. When gramicidin A was incorporated into bilayers of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine or 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) above the lipid phase transition, the deuterium NMR spectra were motionally narrowed, indicating large-amplitude rotational fluctuations. From the measurement of the quadrupole echo relaxation time, a rotational correlation time of 2×10^{-7} s was estimated, leading to a membrane viscosity of 1–2 P if the rotational unit was assumed to be a gramicidin A dimer. For DMPC in the gel state, we observed an immobilization of the peptide molecules. The tryptophan side chains of the immobilized gramicidin A in both the DMPC membrane in the gel state and the stoichiometric gramicidin A–LPPC membrane were found to execute rapid fluctuations of small angular amplitude with correlation times $\tau_c < 10^{-8}$ s.

The functioning of membrane proteins can be influenced by the physical properties of membrane lipids [for a recent review, see McElhaney (1982)]. However, it is not yet clear just which structural features of a given protein are sensitive to lipid properties and can translate into functional changes.

^{2}H nuclear magnetic resonance (NMR)[1] offers several unique advantages for characterizing molecular structure and dynamics in membrane proteins, and a number of such studies

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[1] Abbreviations: NMR, nuclear magnetic resonance; GA, gramicidin A; LPPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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have now appeared (Oldfield et al., 1982; Opella, 1986; Pauls et al., 1985; Datema et al., 1986). Through selective isotopic labeling, different portions of a protein can be observed in a specific and nonperturbing fashion. The ^2H NMR spectrum is readily interpreted, and different molecular motions often lead to distinctively different NMR spectral line shapes. In addition, ^2H NMR is sensitive to dynamic processes occurring over an extremely wide range of time scales (Spiess & Sillescu, 1981).

We have chosen to study a model membrane protein, gramicidin A, incorporated into bilayers consisting of single lipid species. Such idealized membranes will avoid the complications inherent in the complexity of natural biological membranes and will permit systematic variations to be made within the context of an otherwise well-defined system. Gramicidin A is a hydrophobic pentadecapeptide of alternating L- and D-amino acids. Its properties include the inhibition of RNA polymerase, the formation of transmembrane ion channels, and the modulation of the long-range organization of lipids. Interestingly, the four tryptophans located in the carboxy-terminal portion of gramicidin have been shown to be absolutely essential to each to these properties (Busath & Waldbillig, 1983; Paulus et al., 1979; Killian & de Kruijff, 1986). The membrane channel forming structure, which is believed to be an amino-terminal to amino-terminal helical dimer (Wallace, 1986), has been the subject of extensive study as a model of the membrane-spanning portions of integral membrane proteins.

In the present study, we have obtained deuterium NMR spectra arising from deuterons chemically exchanged into the four tryptophan indole rings of gramicidin A. Incorporation of the deuteriated peptide into various phospholipid environments has permitted us to investigate the effects of lipid structure and lipid thermotropic phase state on the dynamics of these essential tryptophan side chains.

Materials and Methods

Peptide and Lipids. Gramicidin A from *Bacillus brevis* [actually a mixture of gramicidins A (80%), B (15%), and C (5%)] was obtained from Sigma (St. Louis, MO) and used without further purification.

1-Palmitoyl-*sn*-glycero-3-phosphocholine (LPPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were purchased from Avanti Lipids (Birmingham, AL). All were purified on silicic acid columns and checked on thin-layer plates prior to use.

Synthesis of ([$^2H_5$-Trp]$_4$)Gramicidin. The indole ring protons of the tryptophan residues in gramicidin were deuteriated by using essentially the procedure described by Bak et al. (1969) and Holt et al. (1971). Gramicidin (500 mg) was treated with deuteriated trifluoroacetic acid (10 g) for 2 h under nitrogen at room temperature. The reaction was stopped by the addition of 1 mL of D_2O. The reaction mixture was diluted with ethanol–water (1:1) to twice the initial volume and dialyzed against 5 mM sodium acetate (pH 6.0)–ethanol (1:1) for 36 h. The contents of the dialysis bag (Spectrapor 6, molecular weight cutoff 1000) were heated at 80 °C for 10 min in order to back-exchange amide protons. Finally, the gramicidin was dried by rotary evaporation and placed under high vacuum over phosphorus pentoxide to remove traces of water. Reaction yields typically exceeded 90%. Thin-layer chromatography of the product on silica gel 6 plates with isoamyl acetate–methanol–formic acid–water (65:20:5:10) (McGilveray & Strickland, 1967) gave results identical with the starting material. Both deuteriated gramicidin and nondeuteriated gramicidin were readily detected via their fluorescence under light, indicating that the tryptophan rings remained fully intact after treatment. The extent and specificity of deuterium labeling were examined by recording high-resolution ^1H NMR spectra (at 400 MHz) of deuteriated and nondeuteriated gramicidin A in perdeuteriated methanol. The extent of exchange was calculated from the relative intensities of the tryptophan resonances occurring between 7.0 and 7.8 ppm compared to the nondeuteriated resonances of the methyl region. Under the conditions employed, a 75% exchange of the tryptophan protons was achieved. Since all other resonances remained unaltered, it could be concluded that (1) selective deuteriation was limited to the four tryptophan residues and (2) the peptide remained intact by all NMR criteria. L-[$^2H_5$]Tryptophan was prepared according to Matthews et al. (1977).

Sample Preparation. Appropriate quantities of lipid and peptide were codissolved in benzene–methanol (1:1), lyophilized, and placed overnight under high vacuum to eliminate solvent traces. The mixtures were hydrated with excess deuterium-depleted buffer (100 mM NaCl–10 mM Tris-HCl, pH 7.4) using gentle vortexing in addition to warming above the lipid phase transition temperature. When equilibration was achieved, the hydrated mixtures were centrifuged and the pellets transferred to NMR sample tubes.

NMR Spectroscopy. Deuterium NMR spectra were recorded with a Bruker CXP-300 spectrometer operating at 46.063 MHz as described previously (Tamm & Seelig, 1983). The quadrupole echo technique was employed (Davis et al., 1976) in the quadrature detection mode with complete phase cycling of the pulse pairs as described by Griffin (1981). Correct data routing was achieved by coordinate cycling of the receiver phase. With properly adjusted pulse lengths and receiver phases, the quadrupole echo appeared entirely in the in-phase channel while the out-of-phase channel contained only noise. Upon Fourier transformations, symmetrical spectra were obtained requiring no further phase correction. Zeroing the dispersion signal provided an increase in the signal-to-noise ratio of $2^{1/2}$ and resulted in absolutely symmetric spectra (Davis, 1983). Particulars of 90° pulse lengths (2.5 μs), interpulse delays (50 μs), recycling delays (1 s), spectral widths (1 MHz), data size (2K), exponential line broadening (2500 Hz), and number of acquisitions (40 000) are given in the figure captions when these differ from the typical values given in parentheses.

Deuterium quadrupole echo decay time constants (T_{2e}) were determined by measuring the amplitude at the peak of the quadrupole echo as a function of the delay time, τ_A, between the 90° pulses of the quadrupole echo sequence.

The sample temperature was controlled to within ±1 °C, and at least 30-min equilibration was allowed after a temperature change prior to data acquisition.

Lipid phosphorus NMR spectra were recorded at 121.48 MHz as described previously (Seelig et al., 1981). A Hahn echo sequence with proton decoupling and cycling of the pulses was employed as described by Rance and Byrd (1983). The receiver phase was cycled coordinately. Particulars regarding 90° pulse lengths (3.5 μs) interpulse delays (50 μs), recycling delays (1 s), spectral widths (50 kHz), data size (2K), exponential line broadening (100 Hz), and number of acquisitions (1000) are given in the figure captions when these differ from the typical values given in parentheses.

Results

([$^2H_5$-Trp]$_4$)Gramicidin A in Solution and in the Solid State. The presence of deuterium label in gramicidin A could

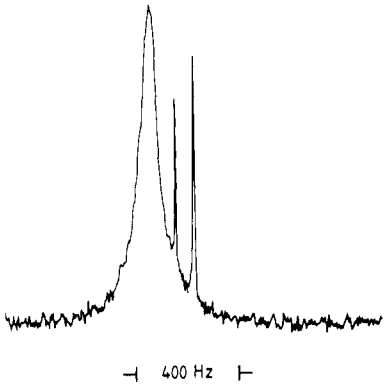

FIGURE 1: High-resolution ^2H NMR spectrum of ([$^2H_5$-Trp]$_4$)-gramicidin A dissolved in methanol. The spectrum was acquired at room temperature as described in the text. The width at half-height of the tryptophan deuteron resonance was 88 Hz. The two sharp resonances arise from natural-abundance deuterium in methanol and have a width of less than 1 Hz.

be directly demonstrated via high-resolution ^2H NMR. Figure 1 shows the ^2H NMR spectrum of deuteriated gramicidin A dissolved in methanol. The spectrum is characterized by a single broad resonance with a width of 88 Hz at half-height. Two additional sharp resonances arise from natural-abundance deuterium in methanol. By employment of standard formulas for the deuterium NMR line width [cf. Abragam (1961), p 315], a correlation time τ_c of 0.6 ns was calculated for the rotational reorientation of gramicidin A (based on e^2qQ/h = 183 kHz). The correlation time τ_c is related to the effective volume of the moving molecule (or molecular segment), V_{eff}, and the viscosity, η, of the solution according to

$$\tau_c = V_{eff}\eta/(kT) \quad (1)$$

where kT is the Boltzmann energy. As a simplest approximation, we consider the case of rigid gramicidin A with no side chain mobility of the deuteriated tryptophans. In methanol, the peptide assumes an intertwined double-helical dimer conformation (Veatch et al., 1974), the dimensions of which can be estimated from the X-ray structural analysis (Wallace, 1986). The dimer has a length of 2.5 nm and diameter of 1.5 nm, leading to a molecular volume of $V_{eff} \simeq 4.4 \times 10^{-21}$ cm^3. Methanol at 25 °C has a viscosity of 0.55 cP, and eq 1 hence predicts a correlation time of τ_c = 0.59 ns for the GA dimer, in excellent agreement with the experimental result. The correlation time of mobile tryptophan side chains, e.g., Trp-3 in bovine phospholipase A_2 (Allegrini et al., 1985), is about 2 orders of magnitude smaller. Hence, even if the close numerical agreement between the experimental and the predicted τ_c values of gramicidin A in methanol is fortuitous, it can nevertheless be concluded that all tryptophan residues in the gramicidin A molecule are strongly immobilized. Therefore, the observed line width of 88 Hz reflects essentially the overall tumbling rate of the gramicidin A dimer in methanol.

The ^2H NMR spectra of polycrystalline [$^2H_5$]tryptophan and of a dry powder of ([$^2H_5$-Trp]$_4$)gramicidin were virtually identical as demonstrated in Figure 2. The line shape of these spectra corresponds to that of a rigid solid, and the quadrupole splitting, as measured from the separation of the most intense peaks in the spectrum, was 126 kHz. The spectra can be simulated with a quadrupole coupling constant of e^2qQ/h = 183 kHz and an asymmetry parameter of η = 0.05 [cf. Kinsey et al., (1981); Figure 2], demonstrating that the tryptophan side chains of gramicidin A as well as the gramicidin A molecule as a whole are immobilized in the solid. Furthermore, we see in these spectra only limited evidence of any "finite pulse length" effects. Such effects can lead to a loss of intensity in

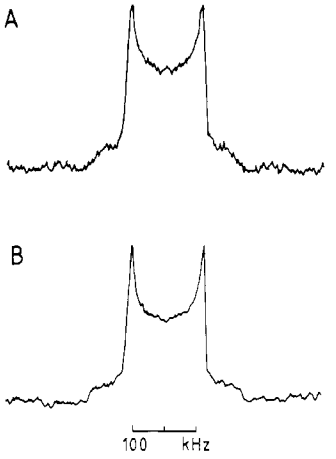

FIGURE 2: Solid-state ^2H NMR spectra of (A) amorphous crystalline [$^2H_5$]tryptophan and (B) a dry powder of ([$^2H_5$-Trp]$_4$)gramicidin A. The spectra were acquired at room temperature. The recycle delay equalled 20 s with 10 000 acquisitions. All other parameters were as described under Materials and Methods.

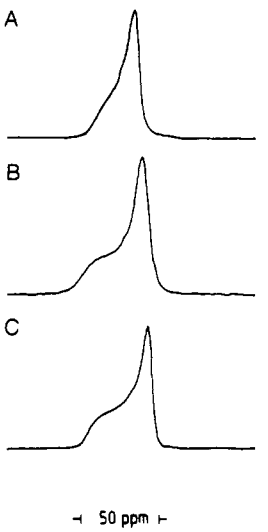

FIGURE 3: ^{31}P NMR spectra of (A) LPPC–GA (4:1 M/M), (B) DOPC–GA (10:1 M/M), and (C) DMPC–GA (15:1 M/M). All spectra were acquired at 305 K as described in the text. All samples contained 0.1 M NaCl–0.01 M Tris-HCl, pH 7.4.

the broad shoulders of the deuterium powder pattern and, in extreme cases, to severe distortions of the line shape when the pulse length used is not short enough to sufficiently excite the entire window of spectral frequencies (Bloom et al., 1980).

Phase Properties of Gramicidin–Lipid Mixtures. We have investigated three gramicidin–lipid mixtures, these being GA–LPPC (1:4 M/M), GA–DOPC (1:10 M/M), and GA–DMPC (1:15 M/M). We first studied the macroscopic phase state of the phospholipids in these mixtures by ^{31}P NMR. Since gramicidin is known to modulate the long-range organization of lipids (Killian & de Kruijff, 1986), such control experiments become essential. LPPC alone gave a narrow isotropic ^{31}P NMR signal because this lipid prefers a micellar organization. The fast tumbling of the micelles effectively averages out the phosphorus chemical shift anisotropy. However, the mixture LPPC–GA (4:1 M/M) provided a bilayer-type ^{31}P NMR spectrum (Figure 3A). Gramicidin is seen, therefore, to stabilize a bilayer organization for LPPC consistent with the results of Killian et al. (1983). This is particularly significant because the ability of GA to influence the lipid organization is dependent on the presence of intact tryptophans (Killian et al., 1985). Both DOPC and the mixture DOPC–GA (10:1 M/M) (figure 3B) gave bilayer-

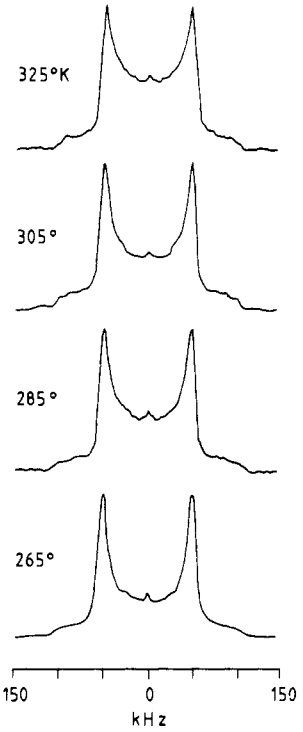

FIGURE 4: ^2H NMR spectra of LPPC–GA (4:1 M/M) at various temperatures. The spectra were acquired by using the conditions given in the text except that 10 000 acquisitions were averaged.

type ^{31}P NMR spectra as did DMPC and the mixture DMPC–GA (15:1 M/M) (figure 3C). For these two phosphatidylcholines, the addition of gramicidin apparently does not alter the macroscopic organization of the lipids under the particular conditions of lipid:peptide ratio and sample preparation employed here.

As mentioned above, gramicidin A is a mixture of the three gramicidin analogues A, B, and C which have tryptophan, phenylalanine, and tyrosine at position 11, respectively. The interaction of the individual compounds with LPPC has recently been investigated (Aranda et al., 1987). While a stoichiometry of 3.9 (LPPC:peptide) was found for the bilayer formation of the peptide mixture, the stoichiometries of the pure compounds were 4.1, 3.1, and 3.1 for gramicidin A, B, and C, respectively. Since gramicidins B and C account for only 15% of the peptide mixture, the following results are essentially determined by the A analogue.

^2H NMR of Gramicidin–Lipid Mixtures. Figure 4 shows the deuterium spectra obtained with the mixture LPPC–([$^2H_5$-Trp]$_4$GA (4:1 M/M) over the temperature range 265–325 K. At each temperature, an approximately axially symmetric line shape was observed. The measured quadrupole splittings decreased from 126 kHz at 265 K to 110 kHz at 325 K, and the resemblance between these spectra and that of the dry powder of ([$^2H_5$-Trp]$_4$)GA shown in Figure 2 is evident.

In contrast, the ^2H NMR spectra from the mixture DOPC–[$^2H_5$-Trp]$_4$)GA (10:1 M/M) showed a pronounced temperature dependence as illustrated in Figure 5A. At 265 K, the spectrum was distorted, showed a low signal-to-noise ratio, and appeared to consist of two components, a sharp central resonance plus a broad splitting of 96-kHz width at the base. With increasing temperature, the spectra gradually evolved to an apparently axially asymmetric spectral line shape which showed an improved signal-to-noise ratio and had a separation of 96 kHz between its outermost edges (at 325 K). Visible as well were two ill-resolved splittings of approximately 40 and 15 kHz.

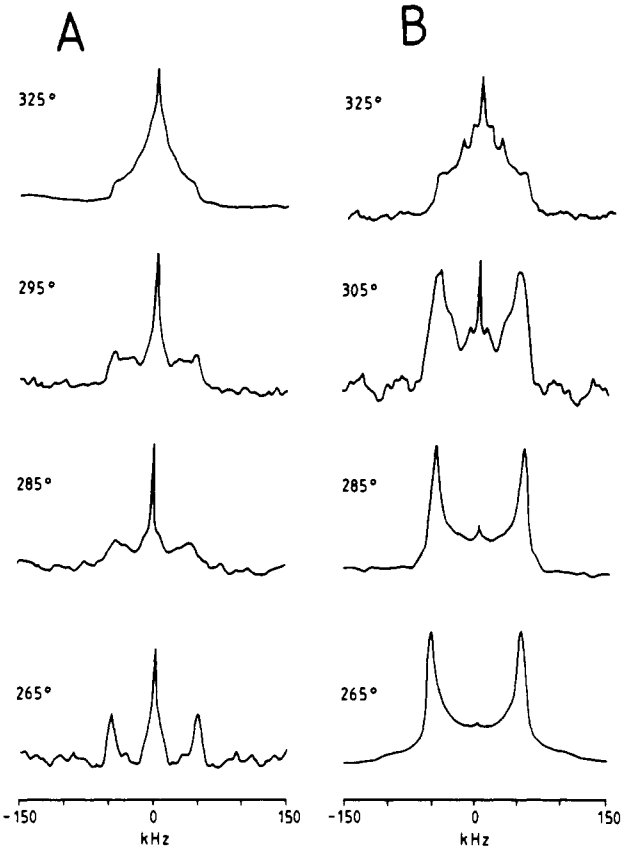

FIGURE 5: (A) ^2H NMR spectra of DOPC–GA (10:1 M/M) at various temperatures. The number of acquisitions at each temperature (degrees kelvin) was approximately 70 000. (B) ^2H NMR spectra of DMPC–GA (15:1 M/M) at various temperatures. The number of acquisitions at each temperature (degrees kelvin) was approximately 40 000.

The temperature dependence of the ^2H NMR spectra was different yet again with the mixture DMPC–[$^2H_5$-Trp]$_4$)GA (15:1 M/M) (Figure 5B). At temperatures below the DMPC phase transition of 23 °C, the spectra exhibited a good signal-to-noise ratio and were axially symmetric with a quadrupole splitting of 110 kHz. Increasing the temperature into the region of the DMPC phase transition caused a progressive narrowing of the spectra as well as a sharp decrease in the signal-to-noise ratio accompanied by a distortion in the line shape such that the spectra appeared to consist of two components, a sharp central resonance plus a broad splitting of 90-kHz separation. Upon further increasing the temperature, the signal-to-noise ratio improved, and the spectra evolved to a complex line shape with multiple quadrupole splittings and a total width of 90 kHz.

For two of these phospholipid–gramicidin mixtures, we measured the deuterium quadrupole echo decay time constant, T_{2e}, as a function of temperature. The results (Figure 6) show that T_{2e} was almost constant over a wide range of temperatures for the LPPC–[$^2H_5$-Trp]$_4$)GA, (4:1) mixture, varying between 140 and 170 μs. In contrast, a minimum in T_{2e} was measured at 295–305 K for DMPC–[$^2H_5$-Trp]$_4$)GA (15:1 M/M), i.e., at the gel to liquid-crystal phase transition of DMPC. At lower temperatures, T_{2e} was almost equal for the two mixtures while at higher temperatures T_{2e} for the DMPC-containing mixture was approximately half that of the LPPC-containing mixture. The poor spectral intensities observed with DOPC–([$^2H_5$-Trp]$_4$)GA (10:1 M/M) precluded meaningful T_{2e} measurements at all but the highest temperatures where T_{2e} was found to be approximately equal to that measured for the high-temperature end of the DMPC–GA mixture.

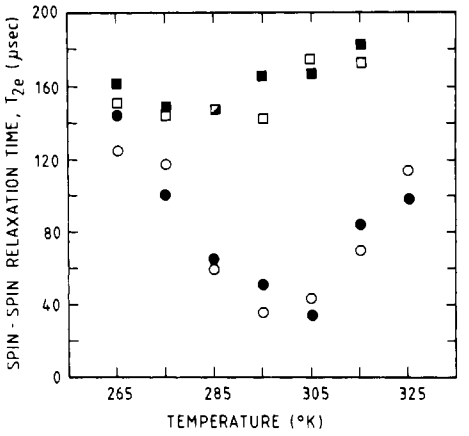

FIGURE 6: Quadrupole spin-echo relaxation time, T_{2e}, for the mixtures GA–LPPC (1:4 M/M) (squares) and GA–DMPC (1:15 M/M) (circles) as a function of temperature. Closed and open symbols represent the results of duplicate determinations.

We investigated as well the dependence of the deuterium NMR line shape in the LPPC-, DOPC-, and DMPC-containing mixtures on the two parameters τ_A and T_R, which refer to the interpulse delay and the total recycling time, respectively, in the quadrupole echo pulse sequence. As shown by the results in Figure 7, the ^2H NMR line shapes obtained with DMPC–[$^2H_5$-Trp]$_4$)GA (15:1 M/M) were sensitive to the value of τ_A, particularly at 325 K. With the LPPC–[$^2H_5$-Trp]$_4$GA (4:1 M/M) mixture, the dependence of the line shape on τ_A was less marked. Only at 325 K could some decrease in intensity in the central spectral region be observed upon increasing τ_A. At all temperatures for all mixtures, the shapes of the spectra were independent of T_R values between 1 and 10 s.

Discussion

In the present study, we have taken gramicidin A as a model of the membrane-spanning portion of an integral membrane protein and have reconstituted the peptide into bilayers of differing lipid compositions in order to investigate the hydrophobic aspects of lipid–protein interaction. When incorporated into lipid bilayers, the 15 alternating L- and D-amino acids of GA form a transmembrane ion channel consisting of an N-terminal to N-terminal helical dimer [for recent reviews, see Anderson (1984) and Urry (1985)]. The four tryptophan rings of GA, being situated in the carboxy-terminal half of the peptide, are consequently located toward the polar surfaces of the membrane rather than its interior (Boni et al., 1986). In GA crystals isolated from methanol solution, the Trp rings are directed outward from the central helical peptide backbone and are exposed to the solvent (Wallace, 1986). The same situation is expected to pertain in the bilayer GA structure where, although the helical form of GA is different from that in methanol, the Trp rings nevertheless will be exposed to the hydrophobic environment of the lipid fatty acyl chains.

The three different GA–phospholipid mixtures investigated here each emphasize different physical aspects. As demonstrated by the ^{31}P NMR results, all three mixtures form lamellar bilayers containing fluid lipids. In the case of DMPC–GA (15:1 M/M) at temperatures below 295 K, the lipids undergo a transition from a liquid-crystalline to a gel state. No such lipid thermotropic transition is observed for DOPC–GA (10:1 M/M), and the lipids remain liquid-crystalline due to the low thermotropic phase transition temperature of DOPC (Barton & Gunstone, 1974). In these latter two mixtures, stable bilayers may be formed over a wide range

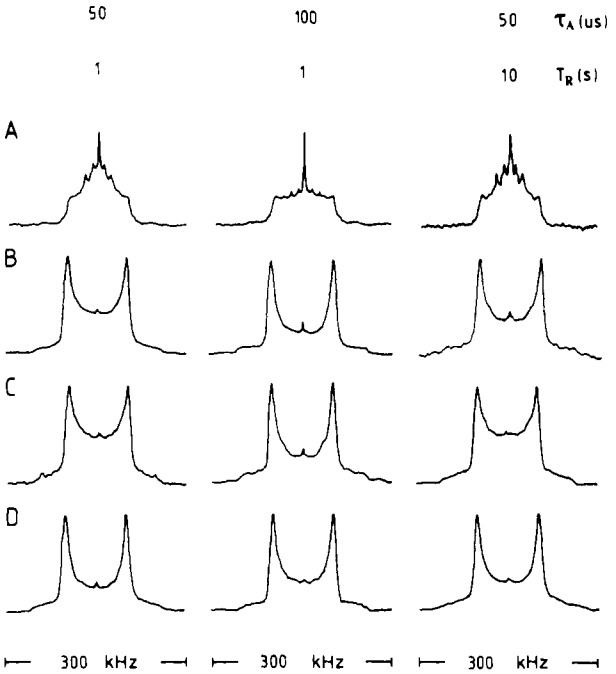

FIGURE 7: ^2H NMR spectra of GA–lipid mixtures for various values of the interpulse delay, τ_A, and the recycle delay, T_R, in the quadrupole echo sequence. All spectra are arranged left to right with τ_A = 50, 100, and 50 μs and T_R = 1, 1, and 10 s, respectively. (A and B) GA–DMPC (1:15 M/M) at 325 and 265 K, respectively. (C and D) GA–LPPC (1:4 M/M) at 325 and 265 K, respectively.

of GA:phospholipid ratios (Chapman et al., 1977; Killian et al., 1985). The third mixture, containing LPPC and GA, is the most unusual in that only at a fixed stoichiometry of about four LPPC molecules to one GA are homogeneous and stable bilayers formed when a mixed peptide–LPPC film is hydrated (Killian et al., 1983). However, heat incorporation of GA into LPPC micelles leads to bilayer aggregates with a different lipid:peptide stoichiometry of about 8:1 (Cavatorta et al., 1982; Spisni et al., 1983). In the latter system, the gramicidin A molecules are present as N-terminal-linked dimers (Urry et al., 1983), whereas in the former membrane they are thought to form C-terminal dimers. The structure of the 4 LPPC:1 GA membrane requires, however, further investigation.

Detailed characterizations of the molecular properties of the lipids in the three membrane systems indicate that both the lipid conformation and the lipid dynamics resemble closely those observed in pure lipid membranes (Rice & Oldfield, 1979; Killian et al., 1986; Chupin et al., 1987). Remarkably, despite the high protein content of the membranes, the lipids remain highly fluid over a wide temperature range with correlation times of 10^{-9}–10^{-10} s for the segmental fluctuations of the hydrocarbon chains. Typical values of the bilayer microviscosity, at least as far as the lipid rotational diffusion is concerned, therefore range from 1 to 10 P (Peters & Cherry, 1982).

Let us now compare the motional properties of gramicidin A in the three membrane systems. Application of eq 1 predicts that the rotational correlation time of GA will increase from 0.6×10^{-9} s in methanol to at least 10^{-7}–10^{-6} s in bilayer membranes, a rate which falls near the "intermediate exchange" range of ^2H NMR. In the presence of motions occurring at "intermediate" rates, characteristic changes of the ^2H NMR spectra are observed for (1) the line shape, (2) the total width of the spectrum, and (3) the spectral intensity and the quadrupole echo relaxation time (with variation of the pulse parameters) (Spiess & Sillescu, 1981; Griffin, 1981; Torchia, 1984; Spiess, 1980, 1985; Pauls et al., 1985).

In the present study, a further complication occurs because each GA molecule contains four tryptophans with altogether 4 × 5 deuterons. The experimentally observed ^2H NMR spectrum therefore arises from a superposition of 20 deuterons. Under these conditions, the quantitative analysis of the ^2H NMR spectra is facilitated by evaluating the so-called "second moment" M_2:

$$M_2 = \int_0^\infty (\omega - \omega_0)^2 f(\omega)\, d\omega / \int_0^\infty f(\omega)\, d\omega \qquad (2)$$

Here ω is the NMR resonance frequency (radians per second) and $f(\omega)$ the line shape function. For powder-type spectra with an asymmetry parameter $\eta^* = 0$, the second moment may be calculated from the separation, $\Delta\nu_Q$, of the 90° edges of the powder-type spectrum (i.e., the most intense peaks in the spectrum) according to [cf. Davis (1979)]

$$M_2 = (4\pi^2/5)\Delta\nu_Q^2 \qquad (3)$$

For other line shapes, M_2 can be determined by numerical integration.

(*A*) *GA–LPPC* (*1:4 M/M*). This membrane system exhibits just one type of ^2H NMR spectrum (cf. Figures 4 and 7); i.e., over the whole temperature range, the observed line shape corresponds to that of an almost rigid solid with an asymmetry parameter of $\eta^* \simeq 0$. Hence, the structural organization of the membrane molecules must be such that the gramicidin A motions are severely restricted and the deuterons experience no large-amplitude movements. In contrast, the lipids retain their fluidity and freedom of motion. One possible organizational model would envisage a network of GA molecules associating, perhaps, via intermolecular Trp–Trp interactions with the LPPC molecules occupying the interstitial spaces. Though the translational diffusion of the lipids would be hindered, the model would still allow free rotational diffusion of the lipids in the interstitial spaces. Fluorescence quenching (Cavatorta et al., 1982) and electron microscopy studies (Spisni et al., 1983) of GA–LPPC membranes support the concept of extensive intermolecular Trp–Trp interactions and GA–GA associations in such mixtures.

Even though the GA molecule as a whole is immobilized in this membrane, the ^2H NMR spectra nevertheless provide evidence for rather rapid deuteron fluctuations with small angular amplitudes. With increasing temperature, the quadrupole splitting $\Delta\nu_Q$ narrows continuously from 126 kHz at 265 K to 110 kHz at 325 K (Figure 4), reducing the second moment from 1.25×10^{11} s^{-2} to 9.55×10^{10} s^{-2}. Even this moderate averaging of the line shape requires angular fluctuations of 5–15° and a correlation time of the order of $\tau_c \ll (12.5 \times 10^{10} - 9.5 \times 10^{10})^{-1/2}$ s $= 6 \times 10^{-6}$ s. The existence of rather rapid oscillations is further supported by the observation that an increase in the recycle delay from 1 to 10 s (Figure 7) does not change the integrated spectral intensity. Thus, a recycle delay of 1 s is already sufficient to produce a fully relaxed spectrum. From ^2H NMR relaxation time theory, one therefore estimates $\tau_c < 5 \times 10^{-8}$ s. A more accurate value of τ_c could be derived by measuring the spin–lattice T_1 relaxation time. Unfortunately, such an experiment is not feasible at present because of extremely long measuring times.

(*B*) *GA–DMPC* (*1:15 M/M*). DMPC bilayers undergo a gel to liquid-crystal phase transition at 297 K [cf. Mabrey and Sturtevant (1976)] characterized by a conformational change of the hydrocarbon chains from a rigid all-trans conformation to a flexible, disordered chain structure. Addition of gramicidin A to DMPC bilayers at the molar ratio given above broadens the phase transition but does not shift the transition temperature. This can be demonstrated by measuring the phosphorus chemical shielding anisotropy which with and without GA exhibits a distinct decrease when the membrane is heated through the phase transition [cf. Rajan et al. (1981)]. This was also observed in the present experiments. The ^2H NMR spectra of deuteriated GA in DMPC membranes below the phase transition temperature appear to be virtually identical with those discussed above for the GA–LPPC membrane. The line shape corresponds to that of a rigid solid with a somewhat reduced quadrupole splitting of $\Delta\nu_Q = 110$ kHz (the total width at the base of the spectrum is 220 kHz) in the range of 265–295 K. This result is again indicative of immobilized GA molecules with rapidly fluctuating tryptophan side chains. The excursions of the deuterons are again limited to a very small angular interval.

Upon heating the membrane through the DMPC phase transition, a dramatic narrowing of the line shape takes place. The solidlike line shape with a maximum separation of 220 kHz between the outer wings is transformed into a complex powder pattern with multiple splittings and only 90-kHz total width. Such a reduction of the solidlike spectrum is possible only by the onset of large-amplitude fluctuations. We tentatively assign this process to the rotational diffusion of GA monomers or GA dimers in the liquid-crystalline DMPC membrane. The rate of this reorientational diffusion process can be estimated from the second moment and the quadrupole echo relaxation rate. Below the lipid phase transition, M_2 is 9.5×10^{10} s^{-2} (at 265 K); above T_c, M_2 is 3.5×10^{10} s^{-2}. In the same temperature interval, the quadrupole spin-echo relaxation time goes through a minimum which is centered approximately at the transition temperature T_c. For the fast correlation time regime, i.e., at temperatures above T_c, the reorientational correlation time τ_c may then be calculated by the approximate formula (Pauls et al., 1985)

$$T_{2e}^{-1} \simeq \Delta M_2 \tau_c \qquad (4)$$

For $T_{2e} = 100$ μs (at 325 K) and $\Delta M_2 = 6 \times 10^{10}$ s^{-2}, one finds $\tau_c \simeq 2 \times 10^{-7}$ s. If the rotating moiety is again the GA dimer, application of eq 1 leads a membrane viscosity of 1.7 P.

These results are in excellent agreement with studies on a hydrophobic model peptide consisting of 30 amino acids, i.e., identical in size with the GA dimer (Pauls et al., 1985). This synthetic molecule has a central piece of 24 leucine residues, spanning the membrane probably in an α-helix. By exchanging the backbone amide deuterons, it was possible to monitor the rotational diffusion of the molecule in the membrane. In bilayers composed of 1,2-dipalmitoylphosphatidylcholine, a correlation time of $\tau_c \simeq 2 \times 10^{-7}$ s and a bilayer viscosity of $\eta \simeq 1.1$ P were derived above the phase transition. Another estimate for the bilayer viscosity comes from the rotational and translational diffusion of bacteriorhodopsin reconstituted with DMPC. The evaluation of quite different measurements leads to a consistent result of $\eta \simeq 1.8$–4 P for temperatures above T_c (Hoffmann & Restall, 1984; Figure 5.11b).

(*C*) *GA–DOPC* (*1:10 M/M*). ^{31}P NMR spectra were recorded from 265 to 325 K and demonstrated a fluidlike bilayer phase for this mixture over the whole temperature range. In contrast, the simultaneous formation of a hexagonal H_{II} phase has been documented recently for the same mixture (Chupin et al., 1987; Killian et al., 1987). As yet, we have no explanation for this discrepancy. We note, however, that the method of sample preparation and the sample handling may also play a role in the formation of the H_{II} phase.

The ^2H NMR results for GA incorporated into DOPC bilayers indicate that the tryptophan deuterons experience a considerable motional freedom over the entire range of tem-

peratures investigated. Even at 265 K the quadrupole splitting is narrowed from a static limit of $\simeq$130 kHz for dry GA powder to at least 90 kHz for membrane-bound GA. Since DOPC bilayers remain liquid-crystalline over the whole temperature interval, any hindrance of motional freedom could only result from GA–GA interactions. The spectrum at 265 K also shows a second spectral component with a total width of only 40 kHz. Hence, at this temperature, two groups of deuterons with different extents of motional narrowing can be distinguished. Unfortunately, the spectra allow no decision to be made if the two signals arise from different deuterons on the same tryptophan, different tryptophans on the same GA, or different populations of GA molecules.

A further complication is the possibility that these spectra exhibit line shape irregularities arising from the "echo distortion" effect (Spiess & Sillescu, 1981) which becomes significant in the presence of motions occurring at rates near the "intermediate exchange" region. The poor signal-to-noise ratio and peculiar line shape of the ^{2}H NMR spectra at low temperatures with DOPC and at temperatures near the DMPC phase transition (cf. Figure 5A,B) are consistent with such an interpretation.

With increasing temperature, the spectral width is reduced even further, and at 325 K, the spectral line shape becomes similar to that of the GA–DMPC system at high temperature. A quadrupole echo relaxation time of $\simeq$100 μs was measured for the GA–DOPC membrane at 325 K. By the same reasoning as given above, we calculate a rotational correlation time of $\tau_c \simeq 2 \times 10^{-7}$ s which is again consistent with the rotational diffusion of GA monomers or dimers about the long helical axis within the plane of the bilayer. The present experiments do not exclude, however, the possibility of additional, large-amplitude fluctuations of tryptophan side chains with correlation times of $\tau_c \ll 10^{-7}$ s.

Concluding Remarks. The overall isotropic tumbling of GA in methanol solution which has a correlation time of $\tau_c \simeq 6 \times 10^{-10}$ s was restricted to an anisotropic rotation in the lipid bilayers of DMPC and DOPC with a correlation time of $\tau_c \simeq 2 \times 10^{-7}$ s corresponding to a bilayer viscosity of 1–2 P at the highest temperature. Within the same lipid bilayer, the protein rotational diffusion may be further influenced by the thermotropic phase transition. The gel-state lipids prevent large-amplitude motions but allow rapid fluctuations of the tryptophan side chains of small angular amplitude. Perhaps most importantly, the special complementarity between protein and lipid in the LPPC–GA bilayer leads to a supramolecular organization in which protein rotational diffusion is many orders of magnitude slower than the rotational diffusion of the lipid molecules. In spite of a rigid protein network, the lipids retain their flexing motions and their rotational mobility. The peptide surface appears to be rather rigid, but due to protruding side chains, the rather uneven contour of the peptide matches with inherent disorder of the hydrocarbon chains.

Registry No. GA, 11029-61-1; LPPC, 17364-16-8; DOPC, 4235-95-4; DMPC, 18194-24-6.

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Annular and Nonannular Binding Sites for Cholesterol Associated with the Nicotinic Acetylcholine Receptor†

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ABSTRACT: Interactions between lipids and the nicotinic acetylcholine receptor from *Torpedo californica* have been measured in reconstituted membranes containing purified receptor and defined lipids. The ability of brominated lipids to partially quench the intrinsic fluorescence of the acetylcholine receptor has been exploited to monitor contacts between the protein and the surrounding lipid. Relative binding constants for lipid binding to the protein have been quantitatively determined by measuring quenching observed in mixtures of brominated and nonbrominated lipids by use of equilibrium exchange equations developed by London and Feigenson [London, E., & Feigenson, G. W. (1981) *Biochemistry 20*, 1939–1948] and by Simmonds et al. [Simmonds, A. C., Rooney, E. K., & Lee, A. G. (1984) *Biochemistry 23*, 1432–1441]. Dioleoylphosphatidylcholine and its dibromo derivative are the two principal lipids used in the reconstituted membranes to establish the quenching parameters. Competition studies between cholesterol and phosphatidylcholine indicate that cholesterol does not compete effectively for the phospholipid sites presumed to surround the membrane-embedded portions of the receptor (annular lipids). However, dibromocholesterol partially quenches the receptor and leads to additional quenching of receptor in pure dibromophosphatidylcholine membranes. The results are consistent with the presence of additional binding sites for cholesterol that are not accessible to phospholipids (nonannular sites). Similar results are obtained by using cholesterol hemisuccinate and its dibromo analogue, both of which can be introduced into membranes more easily than cholesterol because of their greater solubility in water. Fatty acids appear to compete for both annular and nonannular sites, and analysis of the quenching data suggests that there are 5–10 nonannular sites associated with the receptor. Cholesterol has been shown to play a critical role in both acetylcholine receptor structural stabilization and ion channel activity, and the results presented here provide additional information about cholesterol–receptor interactions.

Our understanding of biological membranes is largely based on the now classical "fluid-mosaic" model of Singer and Nicolson (1972) where membrane proteins are embedded either wholly or partially in a fluid lipid bilayer. In recent years it has become apparent that the role of the lipids is not merely passive, since the lipids may directly regulate a wide variety of cellular functions. While such regulation is not completely understood, it is likely to be of physiological significance since the lipid composition of cell membranes is known to be affected by changes in diet, disease, or other physiological stresses (Sarzala et al., 1975; Ansell et al., 1973; Lee, 1985). Since effects of lipids are exerted ultimately on the membrane proteins, much interest has been focused on the nature of lipid–protein interactions (Devaux & Seigneuret, 1985). One of the most successful approaches has been to use model membrane systems consisting of proteins that can be readily purified and reconstituted into defined lipid environments (Jones et al., 1987). Among the most extensively characterized systems are $(Na^+ + K^+)$-ATPase (Esmann & Marsh, 1985), $(Ca^{2+} + Mg^{2+})$-ATPase (Froud et al., 1986), rhodopsin (Baldwin & Hubbell, 1985), D-glucose transporter (Tefft et al., 1986), cytochrome *c* oxidase (Griffith et al., 1986), and the nicotinic acetylcholine receptor of *Torpedo californica*

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