Structure and Dynamics of the Acyl Chain of a Transmembrane Polypeptide

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Received October 21, 1993; Revised Manuscript Received December 17, 1993®

ABSTRACT: We have used acylated analogs of gramicidin as a model to study the interaction between a covalently coupled fatty acid and the hydrophobic part of a membrane-spanning protein in a bilayer environment. The acyl chain was covalently coupled to the C-terminal ethanolamine group of gramicidin which is located near the membrane interface, mimicking a situation found in acylated proteins. Either perdeuterated palmitic acid or palmitic acid deuterated at only C_2, C_3, C_{5-6}, C_{7-8}, C_9, or C_{13} was coupled to gramicidin and examined by ^{2}H-NMR in oriented bilayers of dimyristoylphosphatidylcholine. In this way, quadrupolar splittings of deuterons at specific carbons were assigned. The quadrupolar splittings and T_1 values were compared to those of free palmitic acid in oriented bilayers, with and without gramicidin. The results indicate that the covalently coupled fatty acid is highly immobilized near the carboxyl terminus because double quadrupolar splittings and very low T_1 values (4 ms) were found for the -CD_2- deuterons at carbon atoms C_2 and C_3. Control experiments with free fatty acid showed single quadrupolar splittings and higher T_1 values for this segment of the fatty acid. Molecular modeling of the carboxy-terminal segment of the covalently coupled acyl chain suggested that it has a defined structure with a bend near its attachment site. In contrast, the methyl end (C_{10}–C_{16}) of the covalently coupled fatty acid had quadrupolar splittings and T_1 values very similar to those found for free fatty acids. This indicates that the order and dynamics of the fatty acid near the methyl end is dominated by the surrounding lipids and is not significantly affected by the neighboring peptide.

Many proteins are post- or cotranslationally coupled to lipids. This covalent modification is believed to be important for protein localization and function. Examples of such lipid modifications include glypiation (Ferguson & Williams, 1988), isoprenylation (Marshall, 1993) and fatty acylation (Grand, 1989). Acylated proteins can roughly be divided in two groups (Schmidt, 1989). The first group contains proteins (mainly soluble) which are modified by covalent coupling of myristic acid to the N-terminal glycine. The second group contains proteins (mainly membrane-associated) that are palmitoylated at the amino acids cysteine or threonine.

Remarkably, in this latter class the fatty acid is usually coupled to amino acids that are part of or are close to an already very hydrophobic and probably membrane-spanning segment of the protein. For example, the extremely hydrophobic surfactant protein SP-C is palmitoylated at two cysteines (Beers & Fisher, 1992). Also, several transmembrane viral proteins are palmitoylated in the membrane-spanning region (Crise & Rose, 1992). The function of this type of acylation is unknown but could be diverse, such as regulation of protein function (Mouilloc et al., 1992), signal transduction (James & Olson, 1990), membrane fusion (Glick & Rothman, 1987), and protein localization (Strittmatter et al., 1992). Despite extensive insight into the structure and dynamics of lipids in biological membranes, virtually nothing is known about the orientation, structure, and dynamics of fatty acids covalently coupled to polypeptides.

We have initiated a line of research to get insight into the structural and functional consequences of acylation of transmembrane peptides using gramicidin A as a model peptide. The choice is based on the extensive knowledge of the different well-characterized functions (channel formation, regulation of lipid structure [see Killian (1992) for review]) of this transmembrane helical pentadecapeptide, the existence of a natural acylated analogue (Koeppe et al., 1985), and the possibility to synthesize acylgramicidins by covalent coupling of fatty acids to the C-terminal hydroxyl of the ethanolamine group which is located at the membrane–water interface (Vogt et al., 1991).

Our studies so far have described the consequences of acylation of gramicidin for its conformation (Vogt et al., 1991), its orientation at the air–water interface (Vogt et al., 1991), and its channel activity (Vogt et al., 1992). The results showed that the conformation and the single channel conductance of gramicidin are not influenced by acylation. The lifetime of the gramicidin channels increased 5 times upon acylation.

Now we report on the structure and dynamics of the covalently attached acyl chain in lipid bilayers. For that we synthesized specifically deuterated and perdeuterated palmitoylgramicidins and studied by ^{2}H-NMR[1] the chain order and dynamics in oriented bilayers of dimyristoylphosphatidylcholine using the analogous ^{2}H-labeled free fatty acid as a control system.

The results show that the order and dynamics of the methyl end of the covalently coupled fatty acid are hardly influenced by the nearby helical peptide and are dominated by the surrounding lipids. In contrast, the carboxyl end of the covalently coupled fatty acid is highly immobilized and takes up a defined orientation.

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® Abstract published in *Advance ACS Abstracts,* February 1, 1994.

[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; FID, free induction decay; ed, echo delay; SDS, sodium dodecyl sulfate; CD, circular dichroism.

0006-2960/94/0433-2063$04.50/0

MATERIALS AND METHODS

Materials

Gramicidin A was purified from the naturally occurring mixture of gramicidins A, B, and C as described before (Vogt et al., 1991). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Palmitic acid specifically deuterated at carbon atoms C_2, C_3, C_5 and C_6, C_7 and C_8, C_9, or C_{13} (numbered from the carboxyl end) was purchased from MSD Isotopes (Montreal, Canada). Perdeuterated palmitic acid-d_{31} was from Cambridge Isotope Laboratories (Woburn, MA). Gramicidin A was palmitoylated at the C-terminal ethanolamine group using the procedure of Vogt et al. (1991). The specifically deuterated [2,2-$^2H_2$]-, [3,3-$^2H_2$]-, [5,5,6,6-$^2H_4$]-, [7,7,8,8-$^2H_4$]-, [9,9-$^2H_2$]-, and [13,13-$^2H_2$]palmitoylgramicidin and the perdeuterated palmitoylgramicidin-d_{31} were characterized using ^{1}H-NMR, absorption spectroscopy, and analytical HPLC.

Methods

Preparation of Oriented Samples. Chloroform (400 μL) was added to a dried film prepared from a mixture of DMPC (30 μmol, in chloroform/methanol = 1:1) and either acylgramicidin (2 μmol, from ethanolic solution) or gramicidin and free fatty acid (2 μmol each). This solution was transferred to ±28 microscope cover slips (4 × 24 mm). A stream of nitrogen was used to evaporate the chloroform solution and thereby enhance the spreading of the lipid film onto the cover slips. The film was further dried under high vacuum for at least 16 h. The cover slips were then placed on top of each other in a sample holder (external dimensions 6 × 6 × 26 mm). Deuterium-depleted water (18 μL) was allowed to diffuse in between the glass plates before the sample holder was sealed. The samples were incubated at 40 °C to facilitate alignment of the lipid and gramicidin molecules. Complete alignment, as determined by ^{31}P-NMR, was usually reached in 3–4 days. The composition of the samples was checked after the NMR measurements using HPTLC and no degradation of the samples could be detected.

NMR. NMR measurements were performed on a Bruker MSL-300 spectrometer. The NMR samples were placed at a 0° or 90° orientation of the glass plates normal with respect to the magnetic field in a probe equipped with a solenoidal coil (internal diameter = 7.5 mm) at 40 °C.

Proton-decoupled ^{31}P-NMR spectra were recorded with a high-power probe at 121 MHz using a 2-μs (42°) pulse and an interpulse time of 1 s. A total of 1500 free induction decays (FID) were accumulated. Prior to Fourier transformation an exponential multiplication was performed resulting in a 10-Hz line broading. The chemical shift was determined relative to the isotropic peak of a lyso-PC suspension.

^{2}H-NMR spectra were recorded with a high-power probe at 46 MHz using the quadrupolar echo technique (Davis et al., 1976). A 3.4-μs 90° pulse, a spectral width of 500 kHz, an echo delay time of 35 μs, and a 150-ms interpulse time were used to record and accumulate 300 000 FIDs. Prior to Fourier transformation an exponential multiplication was performed resulting in a 250-Hz line broadening. Spectra were symmetrized to increase the signal-to-noise ratio.

T_1 measurements on deuterium-labeled components were carried out at 40 °C and at a 90° sample orientation using the inversion recovery sequence with quadrupolar echo detection (180°–τ–90°–ed–90°–ed–acquire), in which the echo delay time (ed) was 35 μs, τ was a variable delay time, and the interpulse time was at least $5T_1$.

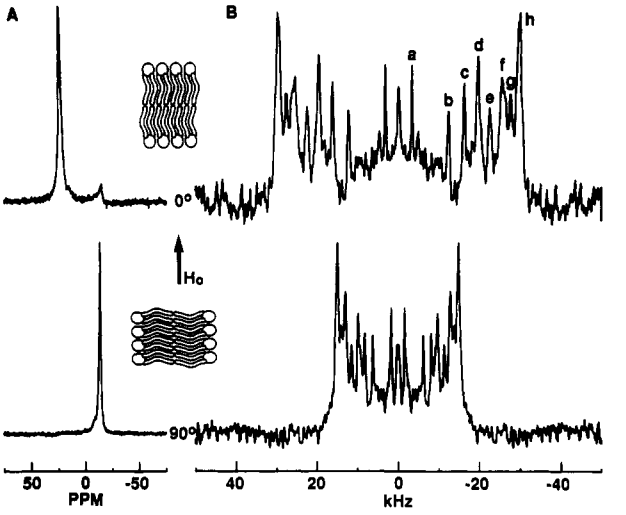

FIGURE 1: ^{31}P-NMR (A) and ^{2}H-NMR (B) spectra of oriented DMPC bilayers with 16:0-gramidicin-d_{31} (15:1 molar ratio) recorded at a 0° (top) or 90° (bottom) orientation of the normal to the glass plates with respect to the magnetic field. Peaks from 16:0-gramicidin-d_{31} are marked a–h.

Molecular Modeling. To visualize the structural constraints imposed upon a fatty acid covalently coupled to the C-terminal ethanolamine of gramicidin, computer graphics was employed using the computer simulation programs Insight II and Discover (both from Biosym Technologies, San Diego, CA). The three-dimensional structure of gramicidin was based on 2D ^{1}H-NMR of the SDS micelle-associated molecule (Arseniev et al., 1986) followed by energy minimization of the backbone as described (Killian et al., 1992). The gramicidin coordinates were a generous gift from R. E. Koeppe II.

RESULTS

We incorporated synthetic [^{2}H]palmitoylated gramicidins in oriented bilayers in order to analyze by ^{2}H-NMR the order and dynamics of a fatty acid covalently coupled to a transmembrane polypeptide. Palmitic acid was chosen because this fatty acid is the most abundant in membrane-associated acylated proteins. In such an approach, oriented bilayers offer advantages over randomly dispersed bilayers. The most important advantages are that information on the orientation of the chain in the bilayer can be obtained and that a more precise determination of the quadrupolar splitting is possible. A uniform orientation of both lipids and peptides is required to fully exploit these advantages. For the gramicidin/DMPC system a uniform orientation has been proven to occur (Nicholson et al., 1987; Chiu et al., 1991; Hing et al., 1990; Teng et al., 1991). Figure 1 demonstrates that this also holds for the palmitoylgramicidin/DMPC system. The sharp peaks at 29 and −14.5 ppm in the typical ^{31}P-NMR spectrum (Figure 1A) of the 16:0-gramicidin-d_3/DMPC system oriented at angles of 0° and 90°, respectively, between the glass plates' normal and the direction of the magnetic field prove that more than 95% of the DMPC molecules are oriented parallel to this normal and undergo fast axial rotation as expected for liquid crystalline bilayers (Seelig & Seelig, 1974a,b; Stockton et al., 1974). ^{31}P-NMR spectra of all other samples showed a similar orientation (data not shown).

The sharp peaks in the ^{2}H-NMR spectrum (Figure 1B) demonstrate that 16:0-gramicidin-d_{31} is also uniformly oriented in the sample. From the 2-fold larger values of the residual quadrupolar splitting ($\Delta\nu_q$) of all resonances in the 0° orientation spectrum, as compared to the 90° orientation

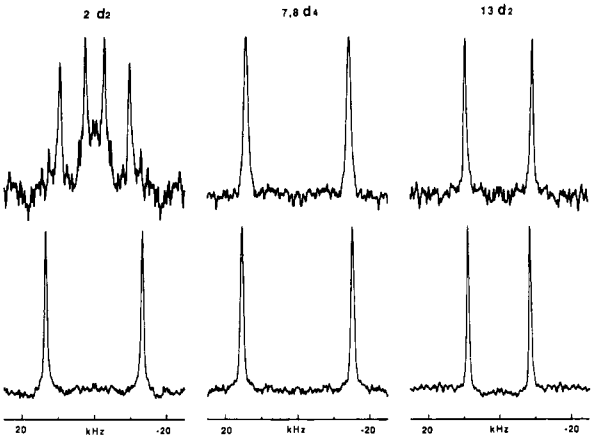

FIGURE 2: ^{2}H-NMR spectra of palmitic acid covalently coupled to gramicidin (top, DMPC:^{2}H-16:0-gramicidin = 15:1 molar ratio) or free in the presence of gramicidin (bottom, DMPC:gramicidin:^{2}H-16:0 = 15:1:1), specifically labeled at carbon atoms 2 (left), 7 and 8 (middle), or 13 (right), recorded at a 90° orientation of the glass plate normal with respect to the magnetic field.

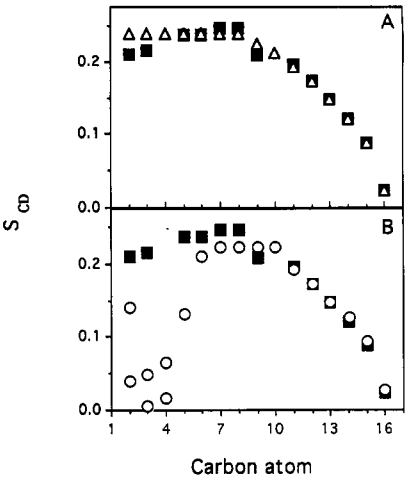

FIGURE 3: Order parameter (S_{CD}) vs carbon atom position of 6.7 mol % palmitic acid incorporated in oriented DMPC bilayers in the absence (A, △) or presence (A and B, ■) of equimolar amounts of gramicidin or covalently coupled to gramicidin (B, ○).

spectrum, it can be concluded that the acylgramicidin molecules also undergo fast axial rotation, presumably like gramicidin around the helix axis (Smith & Cornell, 1986; Datema et al., 1986), which is aligned parallel to the glass plates' normal. The ^{2}H-NMR spectrum of the 16:0-gramicidin-d_{31}/DMPC system showed similarities with a spectrum obtained from a 16:0-d_{31}/DMPC control system [Oldfield et al. (1978) and data not shown]. These include the total width of the spectrum and the characteristic pattern of the resonances. However, several differences were noticed such as changes in the shape of composed peaks suggesting changes in chain order between the two systems. To facilitate the spectral assignment, we studied in addition samples containing palmitoyl chains which were specifically deuterated. Figure 2 shows as an example ^{2}H-NMR spectra recorded at a 90° sample orientation of oriented DMPC bilayers containing [2,2-$^2H_2$]-, [7,7,8,8-$^2H_4$]-, and [13,13-$^2H_2$]-16:0-gramicidin (top) and as a control the equivalent system containing free ^{2}H-labeled fatty acid and gramicidin (bottom). As expected, sharp peaks are observed in all cases, with $\Delta\nu_q$ values which are half those observed for the 0° sample orientation (not shown). For [13,13-$^2H_2$]-16:0-gramicidin a spectrum with a single quadrupolar splitting of 18.9 kHz is observed (corresponding to peak d in the spectrum of the perdeuterated sample in Figure 1B), demonstrating that both deuterons are structurally and motionally equivalent. The spectrum of the control sample has a $\Delta\nu_q$ value of 18.8 kHz, which immediately demonstrates that at this position the order of the acyl chains is very similar for the two systems. Also for [7,7,8,8-$^2H_4$]-16:0-gramicidin a single quadrupolar splitting is observed of 28.5 kHz (corresponding to peak h in the spectrum in Figure 1B), similar to the situation in the control sample, which has a $\Delta\nu_q$ of 31.3 kHz. These data furthermore show that in both experimental systems the chain order increases from the C_{13} to the $C_{7,8}$ position. The spectrum of the [2,2-$^2H_2$]-16:0-gramicidin/DMPC system exhibits some remarkable features. First, two doublets are observed with quadrupolar splittings of 5.0 and 18.0 kHz, demonstrating that the deuterons at this position are not equivalent. In contrast, the control system shows the expected doublet (Stockton & Smith, 1976), with a quadrupolar splitting of 26.6 kHz. The behavior of the covalently attached acyl chain strongly resembles that observed at the C_2 position of acyl chains esterified to the *sn*-2 position of phosphatidylcholines where two quadrupolar splittings arise from the bend in the acyl chain at that position (Seelig & Seelig, 1974a,b). A second remarkable feature of the spectrum of [2,2-$^2H_2$]-16:0-gramicidin is the strong decrease in signal-to-noise ratio as compared to the control system. For the [3,3-$^2H_2$]-16:0-gramicidin/DMPC system also, two doublets with a low-signal-to-noise ratio were observed with $\Delta\nu_q$ values of 0.8 and 6.2 kHz (spectra not shown). From the measured values of $\Delta\nu_q$ obtained from specifically deuterated and perdeuterated 16:0 and by assuming a decrease in $\Delta\nu_q$ from C_{10} toward the methyl end (Seelig & Seelig, 1974a, 1975), values for $\Delta\nu_q$ were assigned to specific carbon atoms of the palmitoyl chain. A value of the order parameter (S_{CD}) was calculated from the measured values of $\Delta\nu_q$ (Seelig & Niederberger, 1974). Two order parameters were assigned to C_4 using arguments described in the discussion.

Figure 3A illustrates as a reference system the order parameter profiles of 6.7 mol % palmitic acid in oriented DMPC bilayers in the absence or presence of 6.7 mol % gramicidin. As generally observed for lipids in bilayers (Seelig & Seelig, 1975), two regions can be distinguished: a plateau region from C_2 to C_8 with a maximal value of $S_{CD} = 0.24$ and a region in which S_{CD} gradually decreases from a value of 0.23 for C_9 to 0.05 for C_{16}. The presence of 6.7 mol % gramicidin had only minor effects on the order parameter profile, in agreement with previous studies on the effect of gramicidin on the chain order in phospholipid systems (Rice & Oldfield, 1979).

A strikingly different picture emerges for the covalently coupled acyl chain in 16:0-gramicidin (Figure 3B). Comparison with the free fatty acid in the control system immediately reveals the changes in the order parameter profile of C_2–C_6. The values for S_{CD} at C_2–C_6 are always lower for the covalently coupled fatty acid as compared to the control situations. Furthermore, two values for S_{CD} are calculated for the deuterons attached to C_2, C_3, and C_4. A gradual increase in S_{CD} is observed from C_5 (0.13) to a maximum value for C_7–C_{10} of 0.23. For C_{11}–C_{16} a gradual decrease in S_{CD} (from 0.20 to 0.05) is observed for the covalently coupled fatty acid, similar to that found for the control situations. This indicates that the order of the covalently coupled fatty acid is greatly influenced near the carboxyl terminus, while toward the methyl end the order is not influenced by the neighboring peptide.

The dynamics of the covalently coupled fatty acid were investigated using deuterium T_1 relaxation measurements. Typical inversion recovery T_1 relaxation measurements are

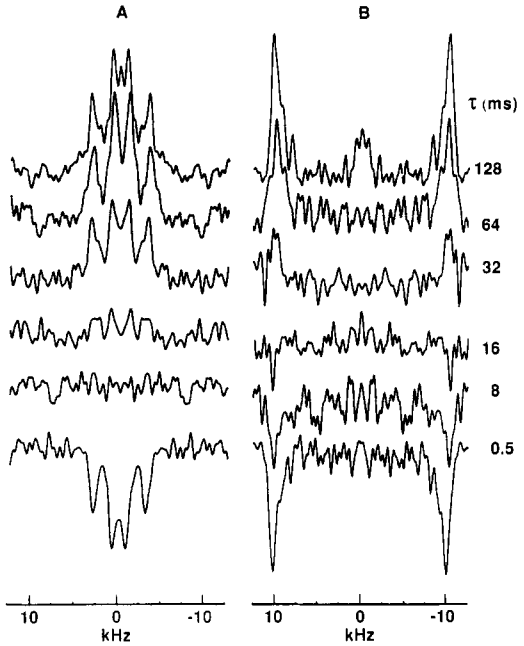

FIGURE 4: Inversion recovery T_1 measurement of [3,3-$^2H_2$]- (A) and [13,13-$^2H_2$]palmitoylgramicidin (B) incorporated in an oriented DMPC bilayer (DMPC:16:0-gramicidin = 15:1 molar ratio) at a 90° orientation using variable delays (τ) as indicated.

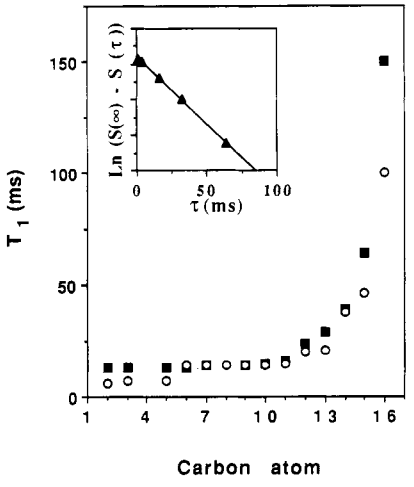

FIGURE 5: T_1 values for palmitic acid covalently coupled to gramicidin (○) and free in the presence of gramicidin (■). The inset shows the T_1 fit of [13,13-$^2H_2$]-16:0-gramicidin from Figure 4B.

shown in Figure 4 for [3,3-$^2H_2$]-16:0-gramicidin (A) and [13,-13-$^2H_2$]-16:0-gramicidin (B). Clearly visible are the differences in T_1 relaxation behavior for the deuterons at different positions of the covalently coupled fatty acid. Two doublets are observed for [3,3-$^2H_2$]-16:0-gramicidin with a very similar T_1 relaxation behavior. Similar results were observed for [2,2-$^2H_2$]-16:0-gramicidin (not shown). Values for T_1 were determined from the measured intensities at different delay times (τ), as shown in the insert of Figure 5 for [13,13-$^2H_2$]-16:0-gramicidin. The curve of ln τ vs intensity is best fitted with a straight line, indicating a single-exponential decay. A smaller value for T_1 indicates a lower mobility for the deuterons if the correlation time is in the fast correlation time regime (Fyfe, 1983). That this is the case was argued from results obtained from temperature-dependent T_1 measurements (data not shown). Figure 5 shows that the T_1 relaxation profile of 16:0-gramicidin and the control situation are similar with a gradual increase in the values for T_1 from C_{10} to C_{16}. However, T_1 values for 16:0-gramicidin are slightly lower near the methyl

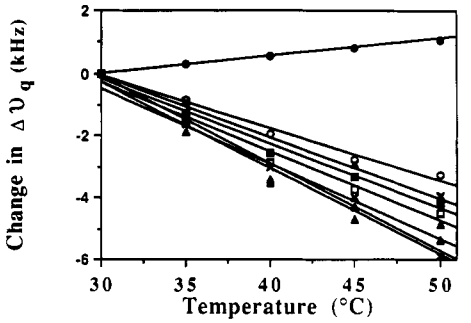

FIGURE 6: Temperature dependence of $\Delta\nu_q$ of 16:0-gramicidin specifically labeled at the 2 (×), 3 (▲), 5 (●), 6 (○), 7, 8, and 9 (■), and 13 (□) positions of the acyl chain. Samples were prepared and measured as described in the legend of Figure 2. The $\Delta\nu_q$ values were related to the values observed at 30 °C.

end of the chain and are reduced by about 30%. Interestingly, in the region of C_{2-5} the T_1 profile shows much larger differences as compared to the control situation. In this region T_1 values were found to be reduced by 54%. From these results it can be concluded that not only the order but also the dynamics of the carboxy-terminal part of the fatty acid are strongly influenced by covalent coupling to gramicidin. In contrast, the dynamics of the methyl end appear to be more or less adapted from the surrounding lipids rather then from the neighboring gramicidin.

For fatty acids in a membrane environment an increase in temperature results in an increase in motion and a subsequent decrease in $\Delta\nu_q$ (Bienvenue et al., 1982). Since covalent coupling to gramicidin strongly influences the order and dynamics of the carboxyl end (C_{2-5}) of palmitic acid, we thought it of interest to investigate the temperature dependence of $\Delta\nu_q$ in this part of the fatty acid. Figure 6 shows that most of the deuterons of the covalently coupled fatty acid indeed show the expected decrease in $\Delta\nu_q$ with increasing temperature. This was also found for deuterons at other positions from C6 down the chain and for all deuterons in the free fatty acid in the control situation (data not shown). However, a strikingly different temperature dependence of $\Delta\nu_q$ is observed at position C_5, which shows an increase with increasing temperature. Since all increases in motion will lead to a decrease of $\Delta\nu_q$, it can be concluded that the temperature-dependent changes in $\Delta\nu_q$ near the carboxy-terminal part must at least be partially attributed to changes in orientation of the fatty acyl chain.

DISCUSSION

The ^2H NMR data obtained on [^2H]palmitoylgramicidin in oriented bilayers allow us to describe for the first time the order parameter profile and dynamics of a fatty acid residue covalently coupled to a transmembrane helical peptide. The nonperturbing character of ^2H as the reporter group means that the obtained results will closely mimic the situation expected for the natural fatty acid.

Striking differences were observed in the NMR parameters obtained near the site of covalent attachment (C_{1-6}) and the remainder of the palmitoyl chain (C_7 till the methyl end). The order parameter profile of this latter and largest part of the acyl chain follows closely the order parameter profile of acyl chains in bilayers of lipid model systems and biological membranes (Seelig & Seelig, 1974a, 1975; Marcelja, 1974; Stockton et al., 1977) in that from C_7 to C_{10} the order is constant and is gradually decreasing toward the end of the acyl chain. Such behavior was also observed in our control experiments where the free deuterated palmitic acid in both the gramicidin-free and gramicidin-containing DMPC bilayer

showed a very comparable acyl chain order profile in this region. The gradual decrease in S_{CD} is the result of an increased probability of *trans–gauche* isomerizations and internal motion toward the methyl end of the acyl chain.

The results lead to the conclusion that this part of the acyl chain has a chain order identical to that observed in the bulk of a liquid crystalline bilayer. Except for some small effects toward the methyl end, the same holds for the motional properties of this segment of the acyl chain as revealed by the deuterium T_1 values. The sharp ^2H resonances observed for ^2H-16:0-gramicidin and the 2-fold increase in $\Delta\nu_q$ upon rotation of the sample from a 90° to 0° orientation of the bilayer normal with respect to the magnetic field demonstrate that the acyl chain undergoes fast axial reorientation and that its motional long axis is oriented parallel to the bilayer normal. Because it is known that the motional axis of gramicidin is also aligned parallel to the helical axis of gramicidin (Prosser et al., 1991; Separovic et al., 1993), this means that the acyl chain runs in immediate proximity to the gramicidin molecule and that, despite this close proximity, the majority of the acyl chain is not influenced in its motional characteristics by the polypeptide. At this point it should be mentioned that membrane proteins in general have been found to induce no large effects on chain order of lipids in (model) membranes, as measured by ^2H-NMR (Tamm & Seelig, 1983; Bienvenue et al., 1982; Seelig et al., 1981). Our results show that this is even true for a covalently attached acyl chain, at least near the interior of the bilayer. This conclusion is valid for the motions that determine the residual quadrupolar splitting ($\tau_c < \approx 10^{-5}$ s) and does not exclude the possibility that other motions are influenced by the neighboring polypeptide.

The present findings are consistent with previous results on the molecular dimensions of acylgramicidins as inferred from their behavior in monomolecular layers at the air–water interface (Vogt et al., 1992). These results showed that the limiting molecular area of acylgramicidins is independent of the length of the covalently coupled fatty acid, which led to the suggestion that the acyl chain must be organized with its long axis parallel to the gramicidin helix.

From the $\Delta\nu_q$ values the average length (L) of an acyl chain in a liquid crystalline bilayer can be calculated (Seelig & Seelig, 1974a; Sankaram & Thompson, 1990):

$$L = \sum_{i=1}^{n} L_i = l_{C-C} \sum_{i=1}^{n} [1 - 0.5(1 - S_{mol,i})]/1.125 \qquad (1)$$

in which l_{C-C} is the effective length of a C–C bond (1.25 Å), $S_{mol,i}$ is the order parameter at C_i ($-2S_{CD}$; Smith et al., 1977), and n is the number of carbon atoms. The C_{6-16} segment of the palmitoyl chain attached to gramicidin can be considered to be in a liquid-crystalline state and will then have a length of 8.8 Å (C to C). An additional 1 Å has to be added to the calculated length of this segment to correct for the contribution of the CD_3 group (Sankaram & Thompson, 1990). The length of the gramicidin monomer in the channel conformation is approximately 12.5 Å (Arseniev et al. 1986; Koeppe & Kimura, 1984), suggesting that the difference in length (2.7 Å) is provided by the remaining carboxyl-terminal 6-carbon-atom segment of the acyl chain. However, the length of this segment of free palmitic acid in a DMPC bilayer is much larger (4.8 Å), as can be calculated accordingly from the data presented in Figure 3. This difference of 2.1 Å indicates that the structure and dynamics of the carboxyl end of the covalently coupled fatty acid differs significantly from that of the free fatty acid. The simplest view is that this part is more rigid and makes a bend, thereby reducing its effective length.

Indeed, the ^2H-NMR experiments showed remarkable changes in properties of the C_{1-6} region of the palmitoyl chain upon covalent attachment to gramicidin. The double quadrupolar splittings of approximately identical intensity of the $-CD_2-$ groups at C_2 and C_3 with their strikingly different $\Delta\nu_q$ values, the low signal-to-noise ratio of the spectra [probably due to fast T_2 relaxation (Rice & Oldfield, 1979)], and the very short T_1 values are all indicative of an ordered structure of this part of the covalently bound acyl chain. This is also supported by the unusual temperature dependence of $\Delta\nu_q$ at the carboxy-terminal part of the chain, which suggests that at least up till the C_5 position it is a defined orientation of the chain, rather than its motional properties, which determines the value of $\Delta\nu_q$. At this point it is of interest to note that the order parameter profile for this part of the covalently coupled palmitic acid shows some similarities with the order parameter profile reported for cholesteryl esters (Valic et al., 1979; Gorrissen et al., 1981). These authors explained the effects by the presence of a bend in the acyl chain.

The double quadrupolar splittings in the various spectra must arise from the individual deuterons of the $-CD_2-$ group in which the C–D bonds take up defined average positions with respect to the rotational long axis of the gramicidin molecule. This axis closely parallels the bilayer normal (Separovic et al., 1993). Such conditions allow for an analysis of possible conformations of this part of the acyl chain because the $\Delta\nu_q$ values directly relate to the angle of the C–D bond vector with respect to the applied magnetic field (for details see Appendix).

In the following paragraph we will try to get some insight into the average orientation of the carboxyl end of the covalently coupled fatty acid. For simplicity we assume that this part of the fatty acid is rigid. Without this simplification, however, it is virtually impossible to analyze the conformation of the acyl chain.

By fixing C_2 in space, sets of coordinates can be calculated for the carbon atoms C_1–C_4 which are compatible with the $\Delta\nu_q$ values experimentally obtained for C_2 and C_3. To calculate coordinates for C_5 requires knowledge of the $\Delta\nu_q$ values of C_4 for which no specifically labeled palmitic acid chain was available. However, in the spectrum of the perdeuterated palmitoylgramicidin two unidentified resonances with $\Delta\nu_q$ values of 2.3 and 8.7 kHz were observed, which both had short T_1 values and which we assigned to the deuterons of C_4. Using these $\Delta\nu_q$ values, coordinates for C_4 and C_5 could be calculated (for details of the calculation procedure, see Appendix). The deuterons attached to C_5 and C_6 both showed one $\Delta\nu_q$ value, strongly suggesting that they form a motional transition region connecting the ordered part of the acyl chain with the more flexible part which at C_7 already is motionally equivalent to the liquid crystalline bilayer. The calculations revealed some 30 possible conformational solutions for the C_1–C_7 segment which are compatible with the NMR data.

Most probable solutions were then selected on the basis of the following constraints: (1) The difference in depth between C_1 and C_6 should be close to 2.7 Å, (2) the acyl chain should not run into the gramicidin molecule, and (3) torsion angles of 180° and ±60° are preferred over other angles. Interestingly, without imposing any constraints, most torsion angles were already found to be close to either 180° or ±60°, supporting the validity of our calculations.

The six solutions which matched these criteria are shown in Figure 7. The open circles indicate the localization of the carbonyl oxygen and the ethanolamine oxygen, which overlap but for clarity are drawn separately. From the top view in

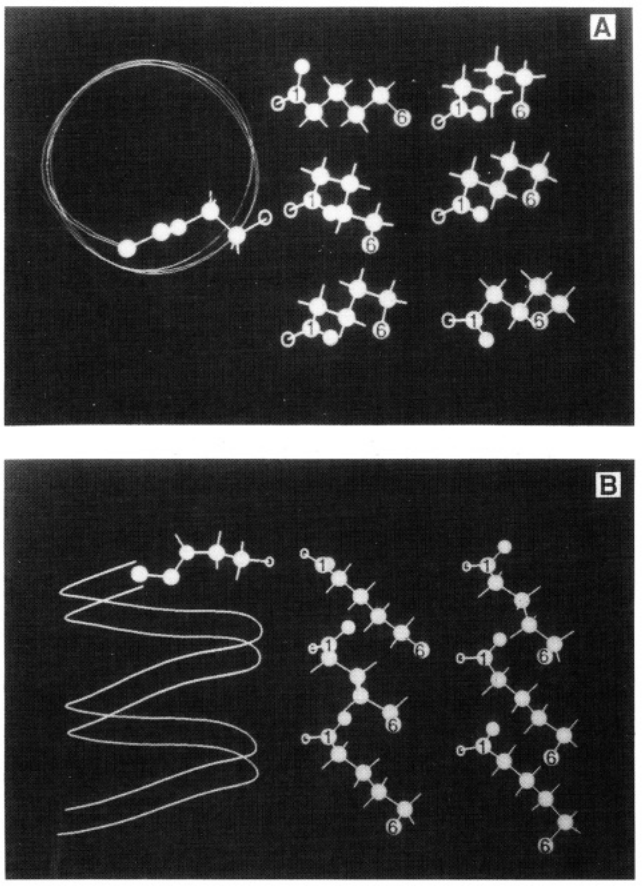

FIGURE 7: Top view (A) and side view (B) of six possible conformations of the carboxyl terminus of the covalently coupled palmitic acid residue. The point of connection between the C-terminal ethanolamine of gramicidin and the carboxyl end (C_1) of the fatty acid is marked by a circle. In the side view the point of connection and C_6 are aligned in the plane of the drawing.

Figure 7A it can be calculated that in all cases the chain extends outward by 7–9 Å from the center of the channel. For comparison, according to the energy-minimized model, the indole group of Trp-11 extends by about 7 Å. The localization in the plane of the drawing is not known and rotation about an axis perpendicular to this plane (parallel to the helix axis) is possible, with the constraint that the chain should not run into the gramicidin molecule. Figure 7B shows a side view of the six possible solutions. For all solutions the line connecting C1 and C6 makes an angle of 39–49° with respect to the helix axis. Around C5 or C6 the chain makes a bend and from there till the methyl end it runs parallel to the helix axis.

In the interpretations presented above it was assumed that the backbone structure of gramicidin does not significantly change upon acylation. Support for this was obtained from single channel experiments, which showed no change in channel conductance upon acylation (Vogt et al., 1992), and from CD measurements, which indicated that gramicidin and acylgramicidin both prefer the $\beta^{6,3}$ helical conformation (Vogt et al., 1991).

The model presented in Figure 8 summarizes the results of the present study and depicts to scale the localization of palmitoylgramicidin in a DMPC bilayer. The chain runs in between the side chains of Trp-9 and Trp-11, as one might expect for an acyl chain running down from the ethanolamine end with gramicidin being in the $\beta^{6,3}$ helical conformation. This is supported by 2D ^{1}H-NMR studies, which show that the fatty acid is in close proximity to the side chain of Leu-10

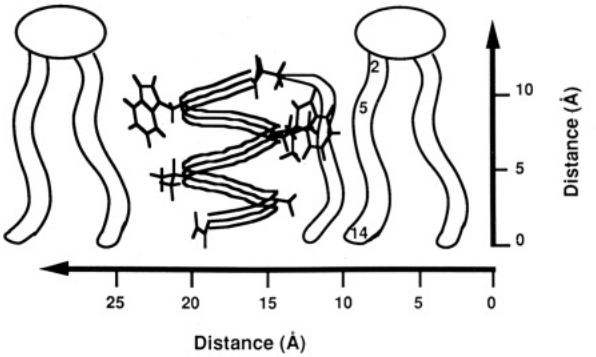

FIGURE 8: Schematic representation to scale of a 16:0-gramicidin molecule incorporated in half a DMPC bilayer. The acyl chain makes a well-defined bend near the carboxyl terminus and its methyl end runs into the bilayer, parallel to the helix axis and the lipid acyl chains. The chain furthermore runs in between Trp-9 and Trp-11 of the gramicidin molecule. To illustrate the dimensions of the gramicidin molecule only the side chains are depicted that form the outer contour.

(Taylor et al., 1991). From C_7 down to C_{16}, the methyl end of the chain is at about the same depth as the methyl ends of the fatty acid chains of the lipids, i.e., from C_5 down to C_{14}.

It is tempting to speculate about a possible function of the covalently coupled fatty acid, such as found in gramicidin K (Koeppe et al., 1985). We propose that the acyl chain stabilizes a local curvature of the interface of the bilayer around the polypeptide. This would be consistent with observations from single channel measurements (Vogt et al., 1992) that acylation increases the lifetime of the gramicidin channel, for which a model was proposed along these lines.

Finally, our results on the structure of acylgramicidin are helpful to suggest general possible functions of acylation of hydrophobic membrane proteins. Induction of stabilization of local curvature, as described above, is one candidate for a more general function, particularly since many membrane processes involve (temporarily) areas of high surface curvature.

Another, more speculative possibility derives from the observed bend in the acyl chain, which like the bend in the acyl chain at the *sn*-2 position of a PC molecule (Seelig & Seelig, 1974a,b) allows for an alignment of the chain parallel to the bilayer normal. Such a bend could serve as a recognition site by specific proteins. Finally, it is possible that acylation induces subtle changes in side-chain conformation, which may affect protein conformation and/or protein–protein interactions. It is presently under investigation to what extent the conformation of some of the amino acid side chains of gramicidin in close proximity to the covalently coupled fatty acid are influenced by the nearby fatty acyl chain.

ACKNOWLEDGMENT

The authors would like to thank Professor R. E. Koeppe II for the gramicidin coordinates and many discussions and Professor J. Kroon for the use of the Silicon Graphics computer.

APPENDIX

Deuterium (^{2}H) NMR spectra are dominated by the quadrupolar interaction. In ^{2}H-NMR a doublet is observed for each deuteron. This doublet is symmetrically distributed around the origin. The separation between the two resonances is called the quadrupolar splitting ($\Delta\nu_q$). In the absence of motion $\Delta\nu_q$ is dependent on the angle (α) between the magnetic field and the field gradient tensor (to a good approximation

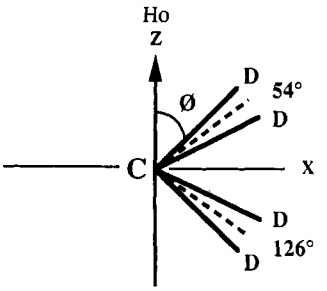

FIGURE 9: Four possible angles (ϕ) are calculated for a C–D bond using eq A3. These possibilities arise from the facts that the C–D bond can point either up or down and that the sign of the quadrupolar splitting is unknown and could be either positive or negative. The z-axis is chosen to be parallel to the magnetic field (H_0) and $y = 0$.

the C–D bond vector) according to

$$\Delta\nu_q = {}^3/_4(e^2qQ/h)\,(3\cos^2\alpha - 1) \qquad \text{(A1)}$$

in which the quadrupolar coupling constant (e^2qQ/h) has a value of 168 kHz (Burnett & Muller, 1971). In the presence of motion $\Delta\nu_q$ decreases due to motional averaging. In our system, which contains deuterium-labeled 16:0-gramicidin molecules incorporated in hydrated oriented DMPC bilayers, three different factors determine the total extent of averaging: (1) fast axial rotation around the helix axis of gramicidin, (2) internal motion of the deuterium-labeled palmitoyl chain (e.g., *trans–gauche* rotations or local motions), and (3) wobbling of 16:0-gramicidin about the bilayer normal. The quadrupolar splitting is then given by

$$\Delta\nu_q = {}^3/_8(e^2qQ/h)\,(3\cos^2\beta - 1)\,\langle(3\cos^2\phi - 1)\rangle\,\omega \qquad \text{(A2)}$$

in which β is the angle between the direction of the magnetic field and the axis of motional averaging, $\langle(3\cos^2\phi - 1)\rangle$ is the time-averaged order parameter (S_{CD}), ϕ is the instantaneous angle between the C–D bond and the helix axis, and ω is a factor to correct for wobbling of the helix axis relative to the bilayer normal.

For gramicidin it was shown that the axis of motional averaging is parallel to the bilayer normal (Prosser et al., 1991). An order parameter analysis of available NMR data on the Gly_2–Ala_3 peptide plane of gramicidin (Separovic et al., 1993) showed that the helix axis has an order parameter of 0.93 relative to the bilayer normal. This order parameter probably results from wobbling around the helix axis and leads to an averaging of all values of $\Delta\nu_q$. In the following calculation we assume that the $\Delta\nu_q$ values obtained for the carboxyl end of the covalently coupled palmitic acid are not averaged by internal motion but only by wobbling of the gramicidin molecule ($\omega = 0.93$). This assumption allows the calculation of an average conformation. The angle ϕ can now be calculated directly from the experimentally obtained $\Delta\nu_q$ values obtained at $\beta = 0°$ according to

$$\Delta\nu_q = {}^3/_4(e^2qQ/h)\,(3\cos^2\phi - 1)\,\omega \qquad \text{(A3)}$$

Calculation of the orientation of the carboxyl end of the acyl chain was then done as follows. A tetrahedral system C_{i-1}, C_i, C_{i+1}, D_{i1}, D_{i2} was defined, in which i stands for the ith carbon atom from the carboxyl end and D_{i1} and D_{i2} represent the two deuterons attached to C_i. This tetrahedral system is placed in an orthogonal coordinate system with C_i in the origin and with the z-axis of this coordinate system parallel to the axis of motional averaging and the direction of the magnetic field. The coordinates (x_1,y_1,z_1) of D_{i1} relative

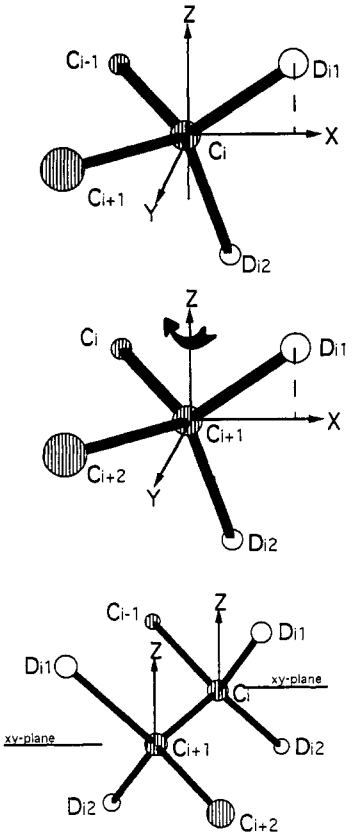

FIGURE 10: From the values of $\Delta\nu_q$, angles of the CD bonds with respect to the motional axis (z-axis) can be calculated. From these values sets of coordinates can be calculated for each tetrahedral system, as shown for C_i and C_{i+1}. As a result of the assumptions made in the calculations (see text), the coordinate system of C_{i+1} is rotated relative to C_i around the z-axis. Identification of possible solutions (and chain elongation) is therefore possible by matching the coordinates of C_{i+1} with those calculated for C_{i+1} from the adjacent carbon atom C_i and vice versa.

to C_i are now calculated from the angle ϕ in eq A3 as follows:

$$x_1 = R_{CD}\sin\phi_1\cos\theta$$

$$y_1 = R_{CD}\sin\phi_1\sin\theta \qquad \text{(A4)}$$

$$z_1 = R_{CD}\cos\phi_1$$

in which R, ϕ, and θ are the polar coordinates and R_{CD} represents the length of a C–D bond (1.08 Å). For convenience we have chosen to place D_{i1} in the xz plane such that $\theta = 0$ and $y = 0$. Because the sign (+ or –) of $\Delta\nu_q$ is not known, a maximum of four solutions can be found for D_{i1} (Figure 9). In the calculation of D_{i2} it was further assumed that the tetrahedral angle (Ψ) is 109.5°. Eight possible sets of coordinates (x_2,y_2,z_2) for D_{i2} were calculated for each set of coordinates of D_{i1} according to

$$x_2 = R_{CD}\{[\cos\Psi - (\cos\phi_1\cos\phi_2)]/\sin\phi_1\}$$

$$y_2 = \pm(R_{CD}{}^2 - x_2{}^2 - z_2{}^2)^{1/2} \qquad \text{(A5)}$$

$$z_2 = R_{CD}\cos\phi_2$$

Due to geometrical constraints the number of possible solutions is reduced from a possible 32 to 24. The coordinates of the carbon atoms C_{i-1} and C_{i+1} are then similarly calculated from the positions of the two deuterons. The coordinates for C_{i-1}

and C_{i+1} are interchangeable. The solutions are, in sets of two, mirror images in the *xy* and *zy* plane.

Chain elongation is possible by combining the results of individual calculations on two adjacent carbon atoms. A result of the assumption that $\theta = 0$ is that the coordinate system for the tetrahedral system of C_{i+1} is different from that of C_i. Effectively a rotation around the *z*-axis is performed for C_{i+1} relative to C_i. The coordinates of C_{i+1} as calculated from the tetrahedral system of the adjacent carbon atom C_i can be used to link the different chain segments (see Figure 10).

Torsion angles χ_1, χ_2, and χ_3 are calculated from the coordinates of the carbon atoms C_1–C_6. A number of solutions were found, most of which had combinations of torsion angles with values of $\pm 60° \pm 5°$ and $180° \pm 5°$.

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