Orientation of the Valine-1 Side Chain of the Gramicidin Transmembrane Channel and Implications for Channel Functioning. A ^{2}H NMR Study†

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ABSTRACT: The orientation of the valine-1 side chain of gramicidin was determined by solid-state ^{2}H NMR using valine-1-deuterated (d_8) gramicidin. The peptide was incorporated into DMPC bilayers that were oriented between glass plates. When the plates were oriented with their normal perpendicular to the magnetic field, four quadrupolar splittings were observed of 106, 68, 9.7, and 2.0 kHz. These resonances were assigned to $C_\alpha D$, $C_\beta D$, and the deuterons of each of the $C_\gamma D_3$ methyl groups, respectively. The average orientation of the various C–D bonds was calculated with respect to the helix axis. The angle obtained for the C_α–D resonance was consistent with a single-stranded $\beta^{6.3}$-helical model for the backbone but not with double-helical models. The angles of the side chain were then fitted to a model for the right-handed $\beta^{6.3}$-helix. Rotation of the valine-1 side chain yielded a set of torsion angles that matched the angles as determined from the ^{2}H NMR measurements. The corresponding orientation of the valine-1 side chain ($\chi_1 = -5°$) was found to be quite unusual, but it explains well the importance of a branched side chain at position 1 for channel formation and stability. A van der Waals interaction between valine-1 of one monomer and alanine-5 of the other helps to stabilize the gramicidin dimer.

Detailed knowledge of the structure and dynamics of membrane-associated proteins and peptides is essential for an understanding of their functioning and their interaction with membrane lipids. This may be illustrated by the mode of action of the channel-forming peptide–antibiotic gramicidin A. The primary structure of this hydrophobic polypeptide is HOC-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NH-CH_2CH_2-OH (Sarges & Witkop, 1965a). Due to its alternating sequence of L and D amino acids gramicidin can form various types of β-helices (Urry, 1971; Veatch et al., 1974; Wallace et al., 1981), of which the single-stranded $\beta^{6.3}$-helical conformation is the channel conformation (Urry, 1985). Transmembrane channels are formed by end-to-end association via hydrogen bonding of the formyl–NH termini of two monomers present in this $\beta^{6.3}$-helical conformation (Urry, 1985; Andersen, 1984). The peptide backbone then lines a hydrophilic pore with a diameter of about 4 Å, and the hydrophobic amino acid side chains are buried in the lipid acyl-chain region of the bilayer. The $\beta^{6.3}$-helical conformation also appears to be the thermodynamically preferred conformation of gramicidin in a lipid bilayer (Killian et al., 1988a,b; Baño et al., 1989) as well as the conformation that is responsible for the strong lipid structure modulating activity of the peptide (Killian et al., 1990; Tournois et al., 1987).

The channel properties of gramicidin A have been extensively investigated. Black lipid membrane studies on gramicidin analogs show that a single amino acid substitution can significantly affect channel conductance and lifetime (Morrow et al., 1979; Bradley et al., 1981; Mazet et al., 1984; Russell et al., 1986). Moreover, studies on N-terminal valine-substituted analogs indicate that even though the channel properties are dramatically changed, in most cases the peptide backbone appears structurally unaltered by the substitution (Durkin et al., 1990). Therefore, structural information not only on the peptide backbone but also on the various side chains is essential for an understanding of channel functioning.

A useful technique to investigate the structure and dynamics of membrane-associated peptides and proteins is solid-state NMR[1] [for a recent review, see Opella (1990)]. This technique has been applied successfully to study the gramicidin channel and has yielded much information on the structure and dynamics of the backbone (Cornell et al., 1988a; Datema et al., 1986; Davis, 1988; Hing et al., 1990a,b; Nicholson et al., 1987, 1991; Nicholson & Cross, 1989; Lograsso et al., 1989; Prosser et al., 1991; Smith et al., 1989). Most of these studies were performed on samples that were macroscopically aligned in the magnetic field. The advantage is that for each labeled site in the molecule, sharp resonance lines can be obtained, the positions of which are determined by the average orientation of this site with respect to the magnetic field. Therefore, by making use of oriented gramicidin molecules, it is possible to obtain detailed information on the configuration and the dynamics of the side chains.

In this study we used solid-state ^{2}H NMR to gain insight into the structure and dynamics of the backbone and valine-1 side chain of gramicidin upon incorporation in oriented bilayers of dimyristoylphosphatidylcholine (DMPC). We will demonstrate that this side chain has a preferred and well-defined orientation with respect to the helix axis. As shown by molecular modeling, this orientation permits a maximal van der Waals interaction of the valine-1 side chain with alanine-5 of the other monomer, which helps to stabilize the $\beta^{6.3}$-dimer. The results explain well the effects on channel function of different side-chain substitutions at position 1.

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[1] Abbreviations: NMR, nuclear magnetic resonance spectroscopy; DMPC, dimyristoylphosphatidylcholine; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate.

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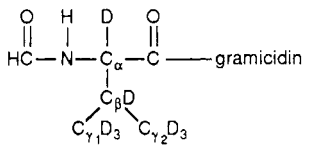
MATERIALS AND METHODS

Materials. Valine-1-deuterated (d_8) gramicidin was prepared as follows: L-Valine-d_8 from MSD Isotopes (St. Louis, MO) was treated with D-amino acid oxidase (Sigma Chemical Co., St. Louis, MO) to remove possible residual traces of the D-isomer and was recrystallized from H_2O–ethanol (Greenstein & Winitz, 1961). Formyl-L-valine-d_8 was then prepared, recrystallized, and coupled to des(formylvalyl)gramicidin A by the procedures of Weiss and Koeppe (1985). The desired product was purified by chromatography over Sephadex LH-20 and Bio-Rad AGMP-50 (Weiss & Koeppe, 1985) and finally by preparative reversed-phase HPLC on phenyl-silica (Koeppe & Weiss, 1981; Koeppe et al., 1985). A single component, with R_f identical to gramicidin A, was then observed on an analytical octyl-silica HPLC column [e.g., see Koeppe et al. (1992)]. For the valine-1 deuterons the following nomenclature is used:



1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was prepared and purified as described (Van Deenen & De Haas, 1964; Geurts van Kessel et al., 1981).

Sample Preparation. Oriented bilayers of gramicidin and DMPC were prepared essentially as described by Moll and Cross (1990). Gramicidin (3 μmol) and DMPC (30 μmol) were codissolved in 300 μL of a benzene–ethanol (97/3, by volume) mixture, and the solution was divided over approximately 30 glass plates (0.15 × 4.8 × 24 mm) by repeatedly spreading aliquots of 5 μL on each plate with intervals of several minutes to allow air-drying of the samples. The plates were stored under high vacuum overnight and then stacked in a small cuvette (outer dimensions: 6.6 × 6.6 × 25 mm). Then 15 μL of ^{2}H-depleted water was added, corresponding to 36% of the total sample weight, and the cuvette was sealed. The sample was allowed to equilibrate for 3 days at 40 °C prior to NMR measurements.

NMR Measurements. ^{2}H NMR spectra of valine-1-deuterated (d_8) gramicidin were recorded on a Bruker MSL 300 spectrometer at 46.0 MHz using the quadrupolar echo sequence (Davis et al., 1976) with full-phase cycling. Dry powder spectra were obtained from 40 mg of gramicidin in 5-mm outer diameter glass tubes using a high-power probe with a 5-mm-diameter solenoid coil. A total of 5000–10 000 scans were accumulated with a 1-MHz spectral width, a 2.4-μs 90° pulse, a 35-μs echo delay time, a 1-s interpulse time, a 2K time domain, and a dwell time of 0.5 μs. ^{2}H NMR spectra of the samples, oriented between glass plates were recorded using a 7.5-mm-diameter solenoid coil. A total of 200 000–800 000 scans were accumulated, employing a 3.4-μs 90° pulse, a 200-ms interpulse time, and a 60–100-μs echo delay time. The signal-to-noise ratio in the spectra of the hydrated samples and the dry powder was increased by applying a 200–300-Hz line broadening and a 2-kHz line broadening, respectively. Quadrupolar splittings in the dry powder were measured from spectra to which no line broadening was applied. Unless otherwise stated, all spectra shown are normalized to the same height.

^{31}P NMR spectra of the oriented sample were recorded at 121.47 MHz with gated proton decoupling, using a high-power dual observe probe with a 7.5-mm-diameter solenoid coil. A total of 200–1000 free induction decays were accumulated with a 4.5-μs 90° pulse, a 2-s interpulse time, 4K data points, and a 38.5-kHz spectral width. Prior to Fourier transformation an exponential multiplication was applied, resulting in a 100-Hz line broadening.

Molecular Modeling. The structure of Arseniev et al. (1986) for the gramicidin dimer in SDS micelles was used as a starting point. Energy minimization was performed in a manner similar to that of Struthers et al. (1991) using the molecular simulation program DISCOVER (Biosym Technologies, San Diego, CA), version 2.7.0, on a Silicon Graphics Personal Iris Model 4-D computer. The nonbonded and valence force field parameters for the amino acid residues were obtained from the consistent valence force field library provided by Biosym. The peptide torsion angle ω was constrained to 180° for all residues using a force potential of 100 kcal/rad^2. A dielectric of 2 was chosen to roughly approximate the dielectric of the interior of a membrane. The energy was minimized using the steepest descent method (Dauber-Osguthorpe et al., 1988) until the maximum Cartesian derivative was less than 0.8 kcal mol^{-1} Å^{-1}. The model was further minimized using the conjugate gradient methods (Dauber-Osguthorpe et al., 1988) until the maximum derivative was less than 0.08 kcal mol^{-1} Å^{-1}. The root-mean-square (rms) deviation of the initial Arseniev structure and the final minimized structure was 0.29 Å for all atoms and 0.17 Å for backbone atoms.

RESULTS AND DISCUSSION

Solid-state ^{2}H NMR measurements were performed on valine-1-deuterated (d_8) gramicidin to obtain insight into the structure and dynamics of the peptide backbone and the valine-1 side chain upon incorporation in a lipid bilayer. To characterize the different resonances and the extent to which averaging of these resonances can occur due to various types of motion, we first measured ^{2}H NMR spectra of valine-1-deuterated (d_8) gramicidin as a dry powder in the absence of lipids, and next the peptide was incorporated into bilayers that were oriented with respect to the magnetic field.

Gramicidin as a Dry Powder. In the absence of motion the peak separation between the resonance lines from a deuteron in a particular C–D bond is a function of the orientation of that bond with respect to the magnetic field (H_0) and is given by (Seelig, 1977; Smith & Oldfield, 1984)

$$\Delta\nu_q = {}^3/_2(e^2qQ/h)({}^1/_2[3\cos^2\theta - 1]) \quad (1)$$

in which e^2qQ/h is the quadrupolar coupling constant, which is approximately 168 kHz for C–D bonds (Burnett & Muller, 1971), and θ is the angle between the C–D bond and the direction of the magnetic field (H_0). The powder pattern obtained in a randomly oriented sample is a superposition of resonances, each representing a particular orientation of the labeled site with respect to the magnetic field. $\Delta\nu_q$ in such a spectrum is defined as the distance between the dominant peaks, which represent all orientations perpendicular to the magnetic field (at $\theta = 90°$). Since any motional averaging will lead to a reduction of $\Delta\nu_q$, the maximum value of $\Delta\nu_q$ for C–D bonds in a powder pattern is about 126 kHz.

Figure 1 shows ^{2}H NMR spectra of gramicidin at 0 and at 40 °C. In both cases two spectral components are observed. One is characterized by a quadrupolar splitting ($\Delta\nu_q$) of 125 kHz, which was found to be independent of temperature. The second component has a much higher intensity and a reduced $\Delta\nu_q$ of 36 kHz at 0 °C and about 34 kHz at 40 °C. As illustrated by comparison of spectra A and B in Figure 1,

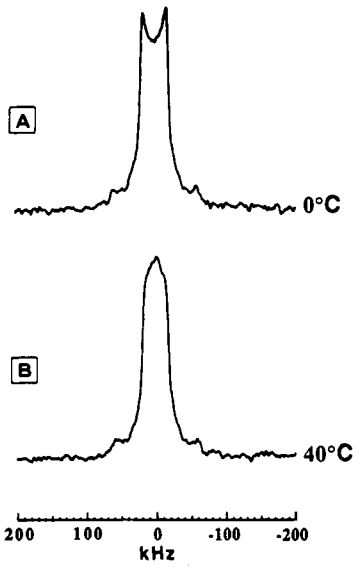


FIGURE 1: 46.0-MHz ^{2}H NMR spectra of valine-1-deuterated (d_8) gramicidin as a dry powder at 0 and at 40 °C.

when the temperature is raised, a change in line shape occurs, characterized by a gradually increasing intensity near the isotropic position. The component with $\Delta\nu_q$ of 125 kHz, which is close to the maximum peak separation for C–D bonds in a powder pattern, is assigned to the $C_\alpha D$ and $C_\beta D$ deuterons, which are expected to be nearly immobile on the ^{2}H NMR time scale. The component with the small value of $\Delta\nu_q$ and the relatively large intensity is assigned to the six deuterons of the valine methyl groups, in which fast rotation [$\tau_c \sim 10^{-10}$ s (Keniry et al., 1984)] about the C_β–C_γ bonds results in an averaging of the quadrupolar interaction, according to

$$\Delta\nu_q = {}^3/_2(e^2qQ/h)({}^1/_2[3\cos^2\phi - 1])({}^1/_2[3\cos^2\psi - 1]) \quad (2)$$

in which ϕ is the angle between the C–D bonds and the axis of motional averaging (along the C_β–C_γ bond) and ψ is the angle between the axis of motional averaging and H_0. In a randomly oriented sample again a powder pattern will be obtained but now with a quadrupolar splitting corresponding to the peak separation at $\psi = 90°$. Thus, in case of tetrahedral geometry ($\phi \approx 109.5°$) fast methyl reorientation wil result in a 3-fold reduction of the quadrupolar splitting, leading to $\Delta\nu_q$ of ~42 kHz. As for the valine-1 methyl groups in gramicidin, generally a slightly smaller value of $\Delta\nu_q$ is observed for methyl groups in protein side chains (Kinsey et al., 1981; Keniry et al., 1984; Batchelder et al., 1983), due to a small deviation from tetrahedral geometry and/or additional small amplitude motions about the C_β–C_γ bonds (Batchelder et al., 1983). The differences in line shape of the spectra at 0 and 40 °C in Figure 1 indicate that when the temperature is raised, additional motions occur, the origin of which is not known.

Gramicidin in Oriented Bilayers. Next the structure and dynamics of the Val-1 side chain were investigated after incorporation of gramicidin into oriented bilayers of DMPC. ^{31}P NMR measurements demonstrate that these bilayers are well oriented (Figure 2). In the liquid-crystalline phase lipids undergo fast axial rotation, and the resonance frequency ν_α depends on the angle of this motional axis of the lipid with respect to the magnetic field, according to (Seelig, 1978; Cullis & De Kruijff, 1978)

$$\nu_\alpha\ (\text{ppm}) = -{}^2/_3\Delta\sigma({}^1/_2[3\cos^2\alpha - 1]) \quad (3)$$

in which $\Delta\sigma$ is the residual chemical shift anisotropy, which under these conditions is about –36 ppm (Moll & Cross, 1990; Cornell et al., 1988b); 0 ppm corresponds to the resonance

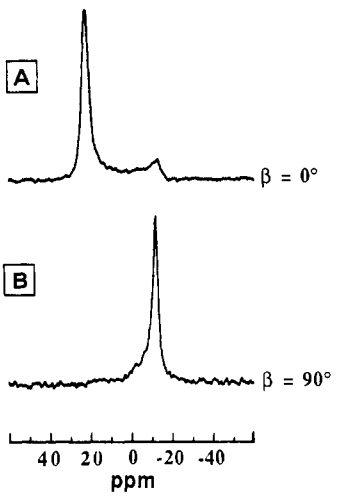


FIGURE 2: 121.5-MHz ^{31}P NMR spectra of valine-1-deuterated (d_8) gramicidin/DMPC (1/10 molar ratio) oriented bilayers at 40 °C. Samples were aligned with the glass plates normal parallel ($\beta = 0°$) and perpendicular ($\beta = 90°$) to the magnetic field.

position of lipids that undergo fast isotropic motion. When the glass plates are oriented with their normal parallel to the magnetic field ($\beta = 0°$), a sharp resonance occurs at 24 ppm (Figure 2A), demonstrating that the majority of the lipids are oriented with their rotational axis parallel to the magnetic field. Upon 90° reorientation of the sample ($\beta = 90°$), one peak is observed at –12 ppm (Figure 2B), corresponding to a perpendicular orientation of the lipids.

When incorporated in a lipid bilayer in the liquid-crystalline phase, the gramicidin molecules will also undergo rapid reorientation about their long axis (Hing et al., 1990a,b; Cornell et al., 1988a; Nicholson et al., 1987), leading to additional motional averaging of the quadrupolar interaction. Assuming that the axis of motional averaging coincides with the helix axis and is parallel to the bilayer normal, then for CD groups, in the absence of other motions

$$\Delta\nu_q = {}^3/_2(e^2qQ/h)({}^1/_2[3\cos^2\chi - 1])({}^1/_2[3\cos^2\beta - 1]) \quad (4)$$

in which χ is the angle between the C–D bond and the helix axis and β is the angle between the glass plate normal and H_0. For CD_3 groups undergoing fast reorientation of the methyl groups, long axis rotation will lead to further averaging according to

$$\Delta\nu_q = {}^3/_2(e^2qQ/h)({}^1/_2[3\cos^2\phi - 1]) \times ({}^1/_2[3\cos^2\zeta - 1])({}^1/_2[3\cos^2\beta - 1]) \quad (5)$$

in which ϕ is again the angle between the C–D bond and the C_β–C_γ bond and ζ is the angle between the axis of motional averaging for methyl rotation (along the C_β–C_γ bond) and the helix axis. In case of tetrahedral geometry ($\phi \approx 109.5°$) rapid methyl reorientation thus also in this case leads to a 3-fold reduction of $\Delta\nu_q$.

Figure 3 shows ^{2}H NMR spectra at 40 °C of the same sample as in Figure 2. At $\beta = 0°$ two spectral components are observed (Figure 3A), with quadrupolar splittings of 4.1 and 19.5 kHz. Upon 90° reorientation of the sample (Figure 3B) these splittings are reduced by a factor of 2 to 2.0 and 9.7 kHz, respectively. Upon vertical expansion and symmetrization of a spectrum of the sample at $\beta = 90°$ (Figure 3C), additional resonance lines become visible with low and about equal intensity and with $\Delta\nu_q$ of 68 and 106 kHz.

The maximum peak separation for deuterons of methyl groups at $\beta = 90°$ is about 42 kHz. Therefore, the resonance

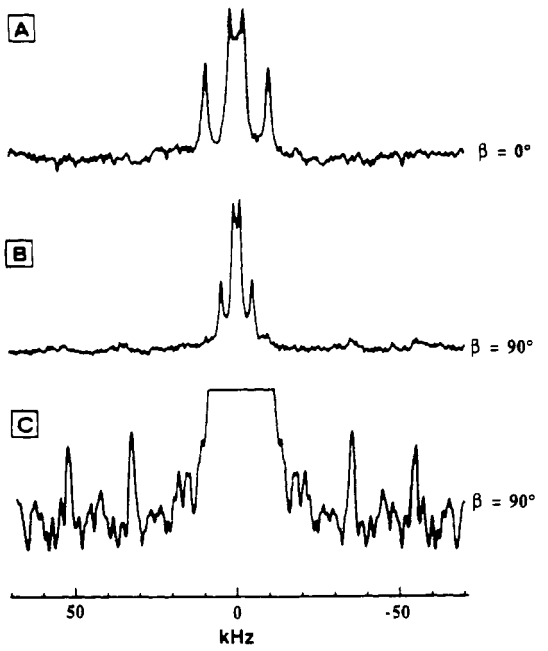


FIGURE 3: 46.0-MHz ^{2}H NMR spectra of valine-1-deuterated (d_8) gramicidin/DMPC (1/10 molar ratio) oriented bilayers at 40 °C at $\beta = 0°$ (A), at $\beta = 90°$ (B), and at $\beta = 90°$ after vertical expansion and symmetrization (C).

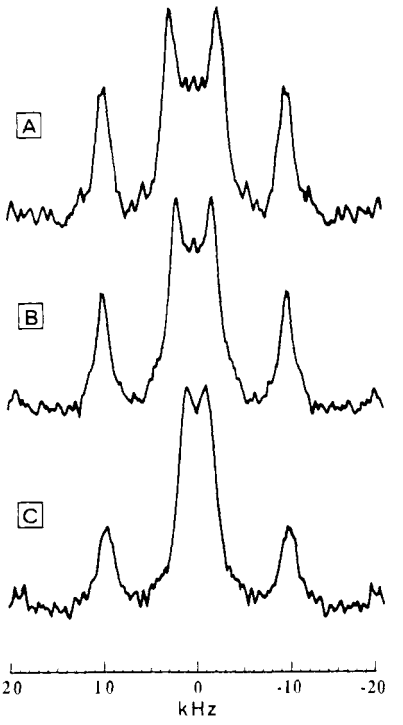


FIGURE 4: 46.0-MHz ^{2}H NMR spectra of valine-1-deuterated (d_8) gramicidin/DMPC (1/10 molar ratio) oriented bilayers at $\beta = 0°$ at 30 °C (A), at 40 °C (B), and at 55 °C (C). Spectra were symmetrized to increase the signal-to-noise ratio.

lines in Figure 3C can originate only from the C_α and C_β deuterons. The orientation of the C_α–D bond of Val-1, and hence the value of $\Delta\nu_q$, is expected to be rather similar to that of the Ala-3 C_α–D bond (Prosser et al., 1991), for which under comparable experimental conditions a single quadrupolar splitting at $\beta = 90°$ was observed with $\Delta\nu_q$ of about 103 kHz (Hing et al., 1990a). Therefore, the component with $\Delta\nu_q$ of 106 kHz in Figure 3C is assigned to the C_α deuteron and the other one to the C_β deuteron. Upon 90° reorientation of the sample these resonance lines disappeared, but no distinct resonance lines could be observed at twice the value of $\Delta\nu_q$ (not shown). This is consistent with a loss of intensity, as reported for the Ala-3 C_α–D upon rotating from $\beta = 90°$ to $\beta = 0°$ (Hing et al., 1990a). The low intensity is most likely due to a short T_2 of these relatively rigid deuterons.

The spectral components in panels A and B of Figure 3 are then assigned to the deuterons of each of the methyl groups, for which NMR signals with a relatively small value of $\Delta\nu_q$ and with a high intensity are expected. The 2-fold reduction in $\Delta\nu_q$ and the observation that also at $\beta = 90°$ sharp resonance lines are observed indicate that gramicidin is undergoing rapid reorientation ($\tau_c < 10^{-5}$ s) about its long axis and that this axis indeed is oriented parallel to the bilayer normal, in agreement with previous results (Hing et al., 1990a,b; Cornell et al., 1988a; Nicholson et al., 1987).

The two components show a difference in intensity that is not due to differences in either T_1 or T_2 relaxation time, since increasing the interpulse time or varying the echo delay time did not significantly change their relative intensity. Most likely it is an artifact resulting from an underlying broad component that contributes to the intensity near the isotropic position and that could arise from residual HOD, from natural-abundance deuterons in the lipids and in the peptide, and/or from valine-1 methyl deuterons of a small fraction of gramicidin molecules that are randomly oriented, present in another conformation, or aggregated. This latter possibility seems likely, since the spectra of gramicidin as a dry powder, in which it probably is present in an aggregated form and in a nonchannel configuration, also showed a broad isotropic component at 40 °C (Figure 1B).

Figure 4 shows spectra at $\beta = 0°$ of the methyl deuterons at different temperatures above the gel to liquid-crystalline phase transition of the lipid. For one methyl group $\Delta\nu_q$ remains constant at about 19.5 kHz, while the value of $\Delta\nu_q$ for the deuterons of the other methyl group is clearly temperature sensitive, ranging from 5.0 kHz at 30 °C to 2.2 kHz at 55 °C. At all temperatures, 90° rotation of the sample resulted in a 2-fold reduction in $\Delta\nu_q$ (not shown). That a temperature dependence of $\Delta\nu_q$ is observed for only one of the methyl groups is puzzling. The result could be due to a combination of a small change in geometry, coupled with a slight increase in motional averaging. Since a change of only 1° in average orientation of the corresponding C_β–C_γ bonds at $\beta = 0°$ can lead to a change in $\Delta\nu_q$ of about 2 kHz, a minor change in geometry or orientation of the side chain, possibly related to a decrease of the bilayer thickness with increasing temperature, is easily detectable.

The relative intensity of the two components appears to change with temperature. Again, the presence of an underlying broad isotropic component could provide an explanation if its line width were temperature dependent.

Calculation of Torsion Angles. If no motions other than fast axial rotation and reorientation of the methyl groups occur that affect the quadrupolar splittings of the various deuterons, then the angles of the various C–D and C_β–C_γ bonds with respect to the axis of motional averaging can be calculated according to eqs 4 and 5. These angles are presented in Table I (column 3). For calculation of the bond angles the tetrahedral angle of the methyl deuterons was assumed to be 111.5°, which is the experimentally determined angle in methyl groups attached to another C atom (Kuchitsu, 1968). The value also corresponds well with the angles obtained by molecular modeling after energy minimization according to the Biosym program (see Materials and Methods). For both methyl groups the quadrupolar splittings lie in a region in

Table I: Quadrupolar Splittings of the Valine-1 Deuterons of Gramicidin (Column 2) and Calculated Bond Angles with Respect to the Helix Axis (Column 3) upon Incorporation of the Peptide in Oriented Bilayers of DMPC (1/10 Molar Ratio) at 40 °C and at $\beta = 90°$[a]

bond	$\Delta\nu_q$	angle (deg)[b]	angle (deg) with motional averaging to 92%	angle (deg) of minimized model with $\chi_1 = -5°$
C_α–D	106	18.6	13.6	13.6
C_β–D	68	33.5	31.5	30.6
C_β–$C_{\gamma_1}D_3$	2.0	52.6 or 56.9[c]	52.4 or 57.1	51.2
C_β–$C_{\gamma_2}D_3$	9.7	44.6 or 66.3[c]	43.9 or 67.4	66.2

[a] If all values of $\Delta\nu_q$ are motionally averaged to 92% of their true value, this will result in a small change in angles (column 4). The last column (column 5) shows the angles calculated for a minimized model with $\chi_1 = -5°$. [b] For calculation of the C_α–D and C_β–D angles, the values for the quadrupolar coupling constant were derived from the experimentally observed $\Delta\nu_q$ of 125 kHz. For the methyl groups, a coupling constant of 168 kHz (Burnett & Muller, 1971) and a tetrahedral angle of 111.5° (see text) were used. [c] Depending on the sign of the interaction.

which the sign of the interaction can be either positive or negative, and therefore two possible orientations can be calculated. The C_α and C_β deuterons are both outside this region, resulting in only one angle.

The experimentally observed value of 106 kHz for $\Delta\nu_q$ for the valine-1 C_αD predicts an upper limit of 18.6° for the angle of the C_α–D bond with respect to the helix axis; the angle would be less if $\Delta\nu_q$ were partly averaged by additional motions, such as rapid fluctuations in the orientation of the motional axis of the helix or small-amplitude, high-frequency motions of the C_α–D bond. Similarly, in case of additional motional averaging of the valine-1 side-chain resonances, it follows from eqs 4 and 5 that the angles smaller than 54.7° can be considered as upper limits and the larger ones as lower limits. It is unlikely, however, that extensive motional averaging occurs in the side chain, since such motions are expected to be temperature sensitive and since for the C_β–$C_{\gamma_2}D_3$ deuterons no temperature dependence of $\Delta\nu_q$ was observed.

In principle, the experimental values of $\Delta\nu_q$ of the side chain could have been affected by tetrahedral jump motions about the C_α–C_β axis. Such hopping motions have been observed for the longer and more flexible leucine side chain (Batchelder et al., 1982; Leo et al., 1987; Colnago et al., 1987), resulting in typical line shapes with asymmetry parameter $\eta = 1$, which could be simulated using hopping rates of about 10^6 rad·s^{-1} (Batchelder et al., 1982). For the valine side chain, however, less consistent results were reported. Studies on deuterated valine, biosynthetically incorporated into bacteriorhodopsin (Keniry et al., 1984; Kinsey et al., 1981), indicated that the side chain is relatively rigid and motion about the C_β–C_γ axis was reported to be slow ($<10^5$ s^{-1}), while studies on the fd coat protein (Leo et al., 1987; Colnago et al., 1987) suggested that side-chain jump motions can occur for valine. However, for two reasons we consider it unlikely that the valine-1 side chain in the gramicidin channel would undergo such hopping motions. First, it can be argued that the time that each site is occupied then must be long on the time scale of the long axis rotation of gramicidin [$\sim 5 \times 10^6$ s^{-1} for gramicidin in DMPC bilayers (MacDonald & Seelig, 1988)], or otherwise at $\beta = 90°$ no sharp resonances with a 2-fold reduction in $\Delta\nu_q$ would have been obtained. The jump rate would then be near the "intermediate exchange" range, in which characteristic changes in line shape and spectral intensity are expected when the echo-delay time is varied (Beshah et al., 1987; Griffin et al., 1988). No such changes were observed (not shown).

Table II: Predicted Angles (θ) between the C_α–H Bonds and the Helix Axis for the Model of Arseniev et al. (1986) before and after Minimization

bond label	original Arseniev θ (deg)	minimized θ (deg)	bond label	original Arseniev θ (deg)	minimized θ (deg)
H–formyl	54.6	48.5	H–C_α Val-8	−27.4	−27.9
H–C_α Val-1	10.5	13.6	H–C_α Trp-9	21.5	17.9
H–C_α Gly-2	−22.1	−20.3	H–C_α Leu-10	−19.3	−20.4
H–C_α Ala-3	15.7	15.1	H–C_α Trp-11	14.3	14.9
H–C_α Leu-4	−20.3	−22.1	H–C_α Leu-12	−14.4	−14.7
H–C_α Ala-5	14.4	12.4	H–C_α Trp-13	9.0	5.0
H–C_α Val-6	−11.5	−12.3	H–C_α Leu-14	−18.6	−21.6
H–C_α Val-7	7.7	7.4	H–C_α Trp-15	16.1	14.1

Second, a 2-fold tetrahedral jump motion between two equally populated sites reduces the line width of the powder pattern by a factor of 2 (Colnago et al., 1987). If such motions would occur for valine-1 in the gramicidin channel, then $\Delta\nu_q$ of the C_β deuteron should be smaller than the predicted maximum value of 62.5 kHz at $\beta = 90°$. The next step is to compare the calculated angles with molecular models of the gramicidin helix, starting with the angle of the C_α–D bond of the peptide backbone with respect to the helix axis.

Backbone Folding Motif and the C_α–D Bond. Assuming that gramicidin is present as a right-handed $\beta^{6.3}$-helix (Nicholson & Cross, 1989; Koeppe et al., 1992), the C_α–D angle was first calculated from the coordinates of the model given by Arseniev et al. (1986), based on 2-D NMR analysis of gramicidin in SDS micelles. In this model, the errors in the more preliminary Arseniev (1985) model based on backbone torsion angles have been corrected. The two monomers are equivalent and are aligned head to head with respect to the same helix axis (there is no "kink"). Table II (middle column) shows the predicted angles for each of the C_α–H bonds with respect to the helix axis for the starting model.

Upon further minimization, the angle of the formyl C–H bonds with respect to the helix axis decreased from 54.6° to 48.5°. This value is in good agreement with the experimental ^{2}H NMR data of Hing et al. (1990b). The angles for several of the C_α–H bonds with respect to the helix axis also changed somewhat (Table II, last column). In particular, the angle θ for the valine-1 C_α–H bond changed from 10.5° to 13.6°.

In the absence of motions other than fast axial reorientation, an angle of 13.6° for the C_α–D bond would correspond to an ideal $\Delta\nu_q$ of 115 kHz at $\beta = 90°$. Since the experimentally observed value of $\Delta\nu_q$ is 106 kHz (Table I, column 2), this suggests that $\Delta\nu_q$ for C_αD is motionally averaged to about 92% of its true value. This is in agreement with previous observations for other backbone C_α deuterium-labeled gramicidin molecules in DMPC bilayers (Hing et al., 1990a; Prosser et al., 1991). If this averaging were due to motion of the entire molecule, then the same or even greater averaging would occur in the side chain. While consistent with a $\beta^{6.3}$-helix, $\Delta\nu_q$ for the valine-1 C_αD *precludes* double-helical models for the gramicidin channel. A $\beta^{7.2}$ double helix constructed by the algorithm of Koeppe and Kimura (1984) gives values of 32° for the angle between the L-residue C_α–D bonds and the helix axis; the $\beta^{5.6}$ double helix of Langs (1988) gives values of 32–47°. In general, other double-helical models give values in the range of 30–40° (Prosser et al., 1991).

Valine-1 Side-Chain Orientation. In Arseniev's model a valine-1 side-chain orientation with a torsion angle χ_1 near 180° was proposed mainly on the basis of energy minimization (Arseniev et al., 1986). However, the angles for the side chain in Arseniev's model did not fit our experimental data.

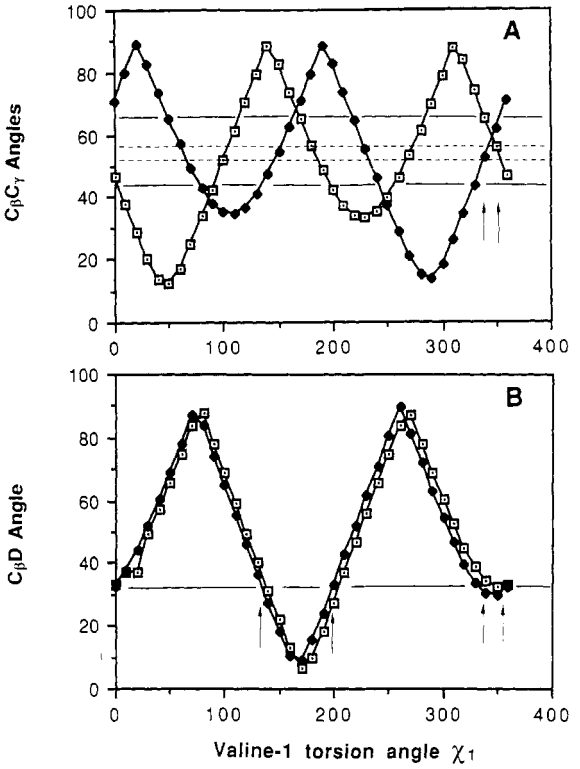


FIGURE 5: Plots of the angles of selected bonds with respect to the helix axis of the right-handed $\beta^{6.3}$ gramicidin dimer as a function of the torsion angle χ_1 of the valine-1 side chain. (A) Angles for the C_β–C_{γ_1} (□) or C_β–C_{γ_2} (◆) bond based on the minimized right-handed model (see Materials and Methods). The experimental ^2H quadrupolar splittings indicate that one of the angles should be at one of the closely dotted lines while the other should be at either one of the widely spaced dotted lines. (B) Angle for the C_β–D bond based on either the 1986 Arseniev model (□) or the minimized model (◆). Possible solutions are marked with arrows in each graph. Only the solutions at 340° and 355° will satisfy both graphs.

Therefore, to find the side-chain orientation, the valine-1 C_α–C_β torsion angle of the minimized model was next rotated through 360° at 5-deg intervals, and the angles of the C_β–D, C_β–C_{γ_1}, and C_β–C_{γ_2} bonds with respect to the helix axis were compared to the experimental values. The results of the search of torsional space are shown in Figure 5. For calculation of the experimental values of the C_β–D and C_β–C_γ angles with respect to the helix axis, it was assumed as a first approximation that the values of $\Delta\nu_q$ were motionally averaged to 92% of their true value by a concerted motion of the entire molecule, as suggested above for the C_αD. As shown in Table I (column 4), a 92% correction factor would only slightly affect the side-chain angles. Figure 5A shows that under these conditions only two values of χ_1 (340° and 355°) are found to simultaneously fit the ζ angles for both $C_{\gamma_1}D_3$ and $C_{\gamma_2}D_3$. In view of the many experimental variables this solution is surprisingly unequivocal. Even when a 5° error was allowed for in the experimentally determined angles, the only solutions are near these two angles. For comparison, if the tetrahedral angle ϕ were 109.5° instead of 111.5°, this would result in an error of less than 2°. Also, an error in the extent of motional averaging is unlikely to change this result, since even when additional motional averaging to 70% is taken into account, again the only solutions found are near these two angles of 340° and 355°. Finally, for the original C_α–D angle of 10.5° in Arseniev's model, nearly identical solutions were obtained. A search of the C_β–D orientation with respect to χ_1 (Figure 5B) shows four possible solutions, two of which agree with those for the C_{γ_1}–D_3 and C_{γ_2}–D_3 orientations (Figure 5A). The original and minimized Arseniev models gave very similar results also for the C_β–D orientation, illustrating that small adjustments to the backbone will not alter the conclusion concerning the side-chain torsion angle. The two solutions shown in Figure 5 correspond to χ_1 values near −5° and near −20° (for χ_1 of approximately −13°, the angles for C_β–C_{γ_1} and C_β–C_{γ_2} are equal). Since only these two values of χ_1 would fit, even when the range of possible angles of each bond was extended to include a correction for a relatively large extent of motional averaging of $\Delta\nu_q$ to 70% of its true value, this suggests that the side chain is nearly rigid. Both torsion angles are in good agreement with the experimental value for *J*-coupling of the valine-1 side chain as observed by Arseniev et al. (1985). From the two possible torsion angles, the −20° orientation can be excluded because of a close contact between the Val-1 C_{γ_2} and the Ala-5 C_β.

The angles corresponding to a model with χ_1 −5° are presented in Table I (column 5). A torsion angle of $\chi_1 = -5°$ is highly unusual, since normally found are the energetically more favorable staggered side-chain rotational isomers (χ_1 = −60°, +60°, or 180°). Interestingly, a recent molecular dynamics study on the side chains in the gramicidin channel suggested a rapid flip of the valine-1 side chain between torsion angles of $\chi_1 = -60°$ and $\chi_1 = +60°$ (Chiu et al., 1992; E. Jakobsson, personal communication). Although our NMR data exclude such a flip, it is noteworthy that (1) also these studies do not support the Arseniev model, since a $\chi_1 = 180°$ torsion angle was not observed, and (2) the average of the two orientations would correspond exactly to the eclipsed orientation that we propose here.

For proteins of known crystal structure, valine side chains are observed to be about 65–70% "trans" (χ_1 near 180°) and about 25–35% "gauche" (χ_1 near ± 60°), with other values represented occasionally (Ponder & Richards, 1987; McGregor et al., 1987). However, eclipsed orientations can occur. Examination of the valines of the photosynthetic reaction center, a crystalline membrane–protein complex, 1PRC in the Protein Data Bank (Deisenhofer & Michel, 1989), shows that, of the 86 valine orientations, 4 are eclipsed. The χ_1 values derived from the reported coordinates of valines H-44 and H-69 are −16° and −6°, respectively, both being near the orientation that we report here for valine-1 of gramicidin. Interestingly, unusual torsion angles are often found to be of functional importance. Indeed this appears to be the case for valine-1 of the gramicidin A channel.

Implications for Channel Function. At χ_1 of −5°, both methyl groups of the β-branched valine-1 are in van der Waals contact (4.1–4.4 Å carbon–carbon distance) with $C_\beta H_3$ of alanine-5 of the opposite monomer of the gramicidin dimer (Figure 6A,C). This side-chain interdigitation could play a role in "docking" of the monomers and provide net stabilization for the gramicidin dimer. When valine-1 is semisynthetically replaced with norvaline, the branching at the β-carbon of the position 1 side chain is lost, and the mean channel lifetime is reduced by a factor of 3.5, while the channel formation rate remains unchanged (Russell et al., 1986). This corresponds to a net stabilization of about 3.1 kJ/mol for the valine-1 gramicidin dimer relative to the norvaline-1 gramicidin dimer.

If indeed the channel is stabilized by side-chain stacking involving a branched β-carbon at position 1, then one would predict that this stabilization holds true for other isomeric pairs of branched vs straight side chains. This was in fact observed, e.g., with isoleucine vs norleucine (O. S. Andersen and R. E. Koeppe II, unpublished results). Moreover, upon substitution of alanine-5 by valine, this side-chain packing would not be possible due to steric hindrance, and one would

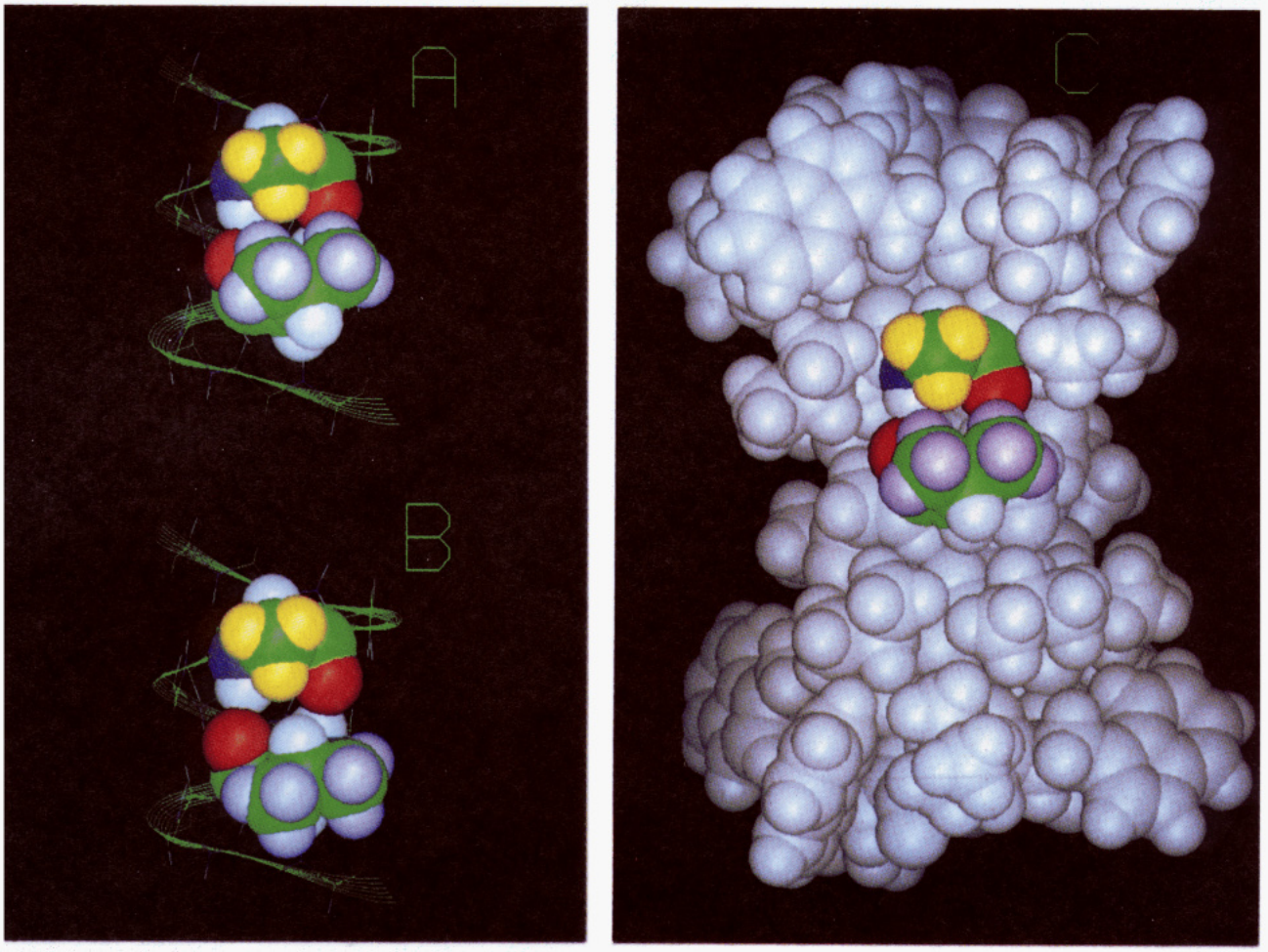


FIGURE 6: CPK representation of the valine-1a-to-alanine-5b interaction at the monomer–monomer interface of a gramicidin dimer in DMPC. The two residues are hydrogen bonded to each other with their respective C═O and N—H groups. The deuterium NMR data indicate a χ_1 angle near −5° for valine-1. In this orientation (panel A), there is a favorable van der Waals interaction between the valine (purple) and alanine (yellow) side chains that provides additional stabilization for the dimer. For comparison, the original valine-1 orientation of Arseniev and co-workers (1986) is shown in panel B. Panel C shows a CPK representation of the entire gramicidin dimer, using the minimized coordinates of Arseniev (1986), but with the new orientation for valine-1.

expect a similar decrease in channel lifetime. Preliminary experiments suggest that this is the case (Andersen et al., 1992).

An important implication of this channel stabilization by side-chain interdigitation is that the majority of the gramicidin molecules in a DMPC bilayer must be present as a $\beta^{6.3}$-helical *dimer*, since a torsion ange $\chi_1 = -5°$ would not be an energy minimum for valine in a gramicidin monomer. This is due to the absence of the favorable van der Waals interaction between valine-1a and alanine-5b, which in the gramicidin transmembrane channel will help to overcome the rotational energy barrier and to place the valine-1 side chain in a near-eclipsed orientation.

Finally, in view of the present results it is interesting to note that in about 10–20% of the naturally occurring species of gramicidin, valine-1 is replaced by isoleucine (Sarges & Witkop, 1965b). Since this is the only other naturally occurring hydrophobic amino acid that has a β-branched side chain, and since such branching at position 1 is important for channel stabilization, this supports the notion (Mandl & Paulus, 1985) that channel formation is important for the biological function of gramicidin in *Bacillus brevis*.

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Registry No. DMPC, 18194-24-6; Val, 72-18-4; Ala, 56-41-7; gramicidin A, 11029-61-1.