Structural Restraints and Heterogeneous Orientation of the Gramicidin A Channel Closed State in Lipid Bilayers

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ABSTRACT Although there have been several decades of literature illustrating the opening and closing of the monovalent cation selective gramicidin A channel through single channel conductance, the closed conformation has remained poorly characterized. In sharp contrast, the open-state dimer is one of the highest resolution structures yet characterized in a lipid environment. To shift the open/closed equilibrium dramatically toward the closed state, a lower peptide/lipid molar ratio and, most importantly, long-chain lipids have been used. For the first time, structural evidence for a monomeric state has been observed for the native gramicidin A peptide. Solid-state NMR spectroscopy of single-site ^{15}N-labeled gramicidin in uniformly aligned bilayers in the L_α phase have been observed. The results suggest a kinked structure with considerable orientational heterogeneity. The C-terminal domain is well structured, has a well-defined orientation in the bilayer, and appears to be in the bilayer interfacial region. On the other hand, the N-terminal domain, although appearing to be well structured and in the hydrophobic core of the bilayer, has a broad range of orientations relative to the bilayer normal. The structure is not just half of the open-state dimer, and neither is the structure restricted to the surface of the bilayer. Consequently, the monomeric or closed state appears to be a hybrid of these two models from the literature.

INTRODUCTION

Single channel conductance of gramicidin A (gA) in membranes clearly shows monovalent selective ion channels that open and close with high fidelity, but, although the conducting state represents one of the highest resolution membrane bound structures (Ketchem et al., 1993, 1997; Townsley et al., 2001), the nonconducting state in the membrane has not been extensively characterized (He et al., 1994). For three decades it has been known that the conducting state is dimeric (Hladky and Haydon, 1970, 1972; Veatch et al., 1975), and through electrophysiological measurements the closing of the channel appears to be first order with an activation energy of ~75 kJ/mol (Hladky and Haydon, 1972, 1984; Bamberg and Lauger, 1974). It is now generally accepted that the closed state is monomeric. Like many other protein systems, the uncomplexed state can be complicated by dynamics or structural heterogeneity to such an extent that a unique structural model may neither be appropriate nor definable. When combined with a bilayer environment and the potential for not only an incompletely defined structure but also heterogeneous orientation of the peptide with respect to its environment, the system becomes even more complex. Such complexity in the system equates to considerable challenges for characterizing the system. The closed state of the gramicidin channel is such a complex, heterogeneous system as will be described in this report.

Gramicidin A is a hydrophobic polypeptide of 15 residues with alternating L and D stereochemistry and blocked N- and C-terminal residues: formyl-Val^1-Gly^2-Ala^3-D-Leu^4-Ala^5-D-Val^6-Val^7-D-Val^8-Trp^9-D-Leu^{10}-Trp^{11}-D-Leu^{12}-Trp^{13}-D-Leu^{14}-Trp^{15}-ethanolamine. As a single-stranded dimer this peptide forms a monovalent cation selective channel that displays single channel currents (for reviews, see Hladky and Haydon, 1984; Andersen and Koeppe, 1992; Busath, 1993) with rapid gating between closed and open states. The conducting or open state is a β-strand conformation in which all side chains are on one side of the strand, thereby inducing a helix with intrastrand hydrogen bonding and 6.5 residues per turn. The dimer is formed by a set of six intermolecular hydrogen bonds at the bilayer center forming a pore that is ~4 Å in diameter and 26 Å long (Ketchem et al., 1997; Fu et al., 2000). This conformation places all of the indole N-H groups in a position so that they can hydrogen bond with the bilayer headgroup region (Hu et al., 1995; Meulendijks, et al., 1989; O'Connell et al., 1990; Scarlata, 1991; Maruyama and Takeuchi, 1997). Without these indole interactions, an intertwined double helix is the most stable structure in a bilayer environment, a nonconducting state (Cotten et al., 1997; Salom et al., 1995).

The conformation of gA is very sensitive to its environment. In addition to the conducting single stranded dimer that has been characterized in detergent micelles (Lomize et al., 1992: Townsley et al., 2001) as well as lipid bilayers in the L_α phase (Ketchem et al., 1993, 1997), there are a number of intertwined double helical conformations. These latter structures can be left- or right-handed, they can be parallel or antiparallel, and they can have a differing number of residues per turn. Some of these structures have been characterized by x-ray crystallography (Langs, 1988; Wallace and Ravikumar, 1988; Langs et al., 1991; Burkhart

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et al., 1998), solution NMR (Arseniev et al., 1984; Barsukov et al., 1987; Pascal and Cross, 1992, 1993; Doyle and Wallace, 1997; Townsley et al., 2001), and solid-state NMR (Cotten et al., 1997). This structural diversity induced by various environments suggests that the closed state could have a unique conformation. The suggestion that this state is an intertwined dimeric structure (Salemme, 1988) can be eliminated because of the lack of stability of such a structure in the membrane environment (Cotten et al., 1999), as well as the numerous hydrogen bonds that would have to be broken and reformed in an environment that is not conducive to such bond rearrangements (Arumugam et al., 1996; Xu and Cross, 1999). Alternatively, it has been suggested that the closed state could be virtually identical to the monomer of the conducting dimer or a different conformation altogether on the surface of the lipid bilayer (He et al., 1994).

Huang and co-workers (He et al., 1994) made the first substantial effort to characterize the closed state by modeling it as Boc-gA (tert-butoxycarbonyl-gA) using circular dichroism (CD), x-ray in-plane scattering, and lamellar diffraction. The samples were determined to be 70% monomer and Tl^+ was still found to bind to the peptide, albeit somewhat further from the bilayer center. These samples were prepared using a very short-chain lipid, dilaurylphosphatidylcholine (12:0). It was noted that the effective interaction radius in the plane of the bilayer increased and that the pitch of the helix decreased. Overall, the authors concluded that the monomeric state was a half-dimer state in the bilayer leaflet.

Studies of the conducting state have shown that the mean lifetime of the channel strongly increases with decreasing bilayer thickness (Hladky and Haydon, 1970, 1972), and therefore the dimeric conducting state is stabilized in the dimyristoylphosphatidylcholine (14:0) bilayers used for structural characterization (Nicholson et al., 1987; Smith and Cornell, 1986). Consequently, if it had not been for the Boc group on the N-terminus of gA in place of the native formyl group, the samples used by He et al. (1994) would be dimeric. Veatch et al. (1975) characterized the open/closed state equilibrium constant as a function of hydrophobic membrane thickness demonstrating that the equilibrium constant could be shifted by several orders of magnitude. Mobashery et al. (1997) showed that the concentration of gA had to be increased by nearly an order of magnitude when trying to prepare conducting channels in dieicosenoylphosphatidylcholine (20:1) lipids versus dioleoylphosphatidylcholine (18:1). Unfortunately, these measurements were obtained on hydrocarbon swollen bilayers that are known to be easily deformed compared to the pure diacylphosphatidylcholine lipids used here that are much more rigid. Consequently, the influence of thickening diacyl lipid bilayers on the equilibrium constant is underestimated by these studies. Indeed, Martinac and Hamill (2001) have been unable to form the conducting state of gA in 1,2-dierucoyl-*sn*-glycero-3-phosphatidylcholine (DEruPC, 22:1) without stretch activation of the samples that is known to thin the bilayers. Here, to characterize the nonconducting state, we studied gA in DEruPC and 1,2-dinervonoyl-*sn*-glycero-3-phosphatidylcholine (DNPC, 24:1).

Before considering various structural possibilities in a lipid environment it is important to recognize constraints that this environment places on the structural space. Any peptide exposed to the interstices of a lipid bilayer can be expected to have its amide backbone hydrogen bonding sites satisfied. In fact, it has been shown that it is difficult even to bury an indole side chain in the middle of the bilayer without a hydrogen bond acceptor for the $N_{\varepsilon 1}$-H site (Cotten et al., 1999). In forming a monomer from the conducting dimer, there will be three amide carbonyls and N-H groups exposed at the bilayer center on each monomer. Potentially, such a situation could be accommodated by a structural rearrangement or by distributing the charge through a cap of water molecules. Most likely some combination of these two mechanisms will be used. A surface location for the peptide, although avoiding exposure of the amides to the low dielectric environment, may be unfavorable because it would expose many of the hydrophobic side chains to the polar headgroup region. In addition, each monomer must disturb the bilayer significantly so that a monomer in the adjacent leaflet can be detected and a conducting dimer formed when there is such an opportunity; otherwise dimers would rarely form.

Solid-state NMR is rapidly developing as a structural and dynamics characterization tool for membrane bound peptides and proteins (Cross and Opella, 1994; Fu and Cross, 1999). A decade ago the first solid-state NMR-derived structure was solved (Ketchem et al., 1993), and today several structures characterized by solid-state NMR are deposited in the Protein Data Bank. Here, an effort has been made to prepare uniformly aligned lipid bilayers containing the gA monomer for the purpose of obtaining orientational restraints. To fully interpret these restraints it is necessary to know the spin interaction tensors for the site of interest in the environment of interest (Hu et al., 1995; Mai et al., 1993; Teng and Cross, 1989), i.e., not just from a crystalline model compound. Recording wideline spectra of unoriented lipid bilayer samples not only characterizes the chemical shift anisotropy (CSA characterized by $\sigma_{11} < \sigma_{22} < \sigma_{33}$) but also characterizes large amplitude molecular motions. For instance, in the gA conducting dimer there is rotational motion about the channel axis and bilayer normal that reduces the axial asymmetry to an axially symmetric tensor characterized by just two elements, $\sigma_{//}$ and $\sigma_{\perp}$. Depending on the orientation of the chemical shift tensor to this motional axis, the residual anisotropy can be nearly the static limit or averaged to zero, such that only the isotropic average is observed. The CSA can be viewed as an ellipsoid having a fixed orientation with respect to the molecular frame, where the Principal Axis Frame (PAF) of this tensor is coincident with the axes of the ellipsoid. For a given orientation of the nuclear site relative to the magnetic field, only the com-

ponent of the CSA tensor in the direction of the field is detected. This results in a single observed chemical shift frequency given by $\sigma_{obs} = \sigma_{11}\cos^2\theta_{11} + \sigma_{22}\cos^2\theta_{22} + \sigma_{33}\cos^2\theta_{33}$, where θ_{ii} represents the angle between a principal axis and the direction of the applied magnetic field. For a well-aligned dimer sample, the observed resonance when the bilayer normal is aligned parallel to the field is precisely the $\sigma_{//}$ component of the motionally averaged tensor. Here, we present the results of a solid-state NMR study of gA in long-chain phospholipids. Previously, α-helical peptides have been characterized in long-chain phospholipids by ^{15}N solid-state NMR (Harzer and Bechinger, 2000).

MATERIALS AND METHODS

Samples of gA ^{15}N-labeled separately in the Ala^5 and in the D-Leu^{10} residues were synthesized by solid-phase synthesis using 9-flouroenylmethoxycarbonyl (Fmoc) chemistry (Fields et al., 1989). The lipids used in this study, DEruPC (*cis*-13, 22:1) and DNPC (*cis*-15, 24:1) were obtained from Avanti Polar Lipids (Alabaster, AL). Unoriented samples of gA in DEruPC and of gA in DNPC were prepared (1:40 molar ratio) by codissolving 17 mg gA with 312 mg DEruPC or 331 mg DNPC, respectively, in 5% (v/v) ethanol in benzene. The solutions were frozen in liquid nitrogen and placed in a vacuum to dry overnight. Both the DEruPC samples and the DNPC samples were hydrated to equilibrium in a 93% relative humidity environment by incubating the DEruPC samples in an oven maintained at 308 K for 1 week and the DNPC samples at 315 K for 2 weeks.

Oriented bilayer samples were prepared by cosolubilizing 3.0 mg isotopically labeled gA with 57.7 mg DEruPC, or 3.0 mg peptide with 70 mg DNPC (1:40 molar ratio) in benzene/ethanol (95:5 v/v). Such a sample solution was spread in equal aliquots onto 52 clean glass coverslips and dried under vacuum overnight. These glass coverslips were then stacked into a 13- × 8- × 8-mm tube with HPLC-grade water added to each glass coverslip to fully hydrate the DEruPC and DNPC lipids. Subsequent to sealing both ends of the tube, the samples were incubated at 308 K for 1 week (DEruPC) and at 315 K for 2 weeks (DNPC) to obtain optimal alignment of the gramicidin-containing samples. Such temperatures are well above the phase transition temperatures of 284 K and 297 K, respectively (Caffrey and Feigenson, 1981; Lewis and Engelman, 1983).

The NMR spectra were obtained at 298 K and 313 K for the DEruPC and DNPC samples, respectively. The degree of bilayer alignment was monitored by ^{31}P NMR on a Bruker (Billerica, MA) DMX-300 NMR spectrometer using a homebuilt solenoid coil probe. All the ^{31}P data were obtained at a resonant frequency of 121.52 MHz using a single pulse experiment with a 90° pulse of 7 μs and a recycle delay of 4 s. ^1H decoupling was applied during the acquisition time. ^{15}N spectra of oriented and unoriented samples were obtained on a spectrometer built around a Chemagnetics data-acquisition system (Varian, Palo Alto, CA) and an Oxford Instruments (Concord, MA) 400/89 magnet, and some spectra were obtained on a new Bruker DMX console using the same Oxford magnet. The $^{15}N/^1H$ NMR probe was homebuilt using a square coil design. The ^{15}N resonant frequency was 40.56 MHz. Typically, spectra were recorded using cross-polarization (5-μs 90° pulse, 1-ms contact time), followed by a Hahn echo (70-μs echo delay) and a recycle delay of 4 s. All chemical shifts are reported relative to the frequency of a saturated solution of $^{15}NH_4NO_3$.

Data processing was performed on a Silicon Graphics workstation using FELIX (Molecular Simulations, San Diego, CA). An exponential window function with a line broadening of 200 Hz (5 ppm) and 140 Hz (1 ppm) was used in processing the ^{15}N and the ^{31}P experimental data, respectively. The principal components of the chemical shift anisotropy for axially asymmetric powder spectra, σ_{11}, σ_{22}, and σ_{33} were obtained by simulating the experimental powder pattern spectra of single-site-labeled samples.

RESULTS

The static principal values of the ^{15}N tensor for ^{15}N-Ala^5-gA in DEruPC were characterized from a hydrated powder sample of this peptide in a DEruPC bilayer that has been dried to achieve a static sample. This powder pattern spectrum (Fig. 1) is characterized by tensor elements: $\sigma_{11} = 40$, $\sigma_{22} = 65$, and $\sigma_{33} = 204 \pm 2.5$ ppm, where σ_{33} represents the tensor element closest to the N-H bond vector. The corresponding chemical shift tensor elements for ^{15}N-labeled Ala^5 gA in DMPC (1:8 molar ratio) using a similar preparation protocol were previously characterized, $\sigma_{11} = 38$, $\sigma_{22} = 67$, and $\sigma_{33} = 207$ ppm (Mai et al., 1993), and do not differ substantially from those reported here in DEruPC.

Initial samples of gA ^{15}N-labeled Ala^5 and D-Leu^{10} in the DEruPC and DNPC lipids were made in different peptide/lipid molar ratios ranging from 1:8 to 1:50. ^{31}P spectra of these samples oriented between glass plates showed the presence of a substantial isotropic lipid phase at a peptide/lipid molar ratio of 1:30 and at lower lipid ratios (data not shown). Samples for further analysis were prepared at a peptide/lipid molar ratio of 1:40 so as to minimize this

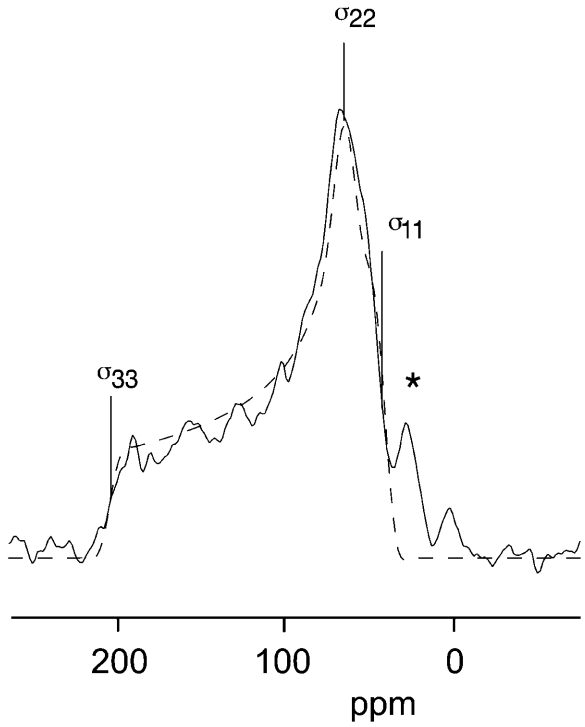

FIGURE 1 ^{15}N powder pattern spectrum obtained from dry powder sample of ^{15}N-Ala^5-gA/DEruPC using a peptide/lipid ratio of 1:40. A total of 24,000 free induction decays were acquired, and 5 ppm line broadening was applied. This spectrum provides a measurement of the principal elements of the chemical shift shielding tensor $\sigma_{11} = 40$, $\sigma_{22} = 65$, and $\sigma_{33} = 204 \pm 2.5$ ppm. The simulated axially asymmetric spectrum is shown by a dashed line. The asterisk indicates a natural abundance ^{15}N signal from the choline headgroup of DEruPC.

isotropic phase and at the same time ensure that a reasonable ^{15}N-gA signal could be obtained.

^{31}P spectra of hydrated unoriented sample of ^{15}N-D-Leu^{10}-gA in DEruPC and DNPC are shown in the inserts in Fig. 2, *B* and *D*, respectively. These spectra show nearly ideal L_α phase powder patterns illustrating the uniaxial rotation of the lipids about the bilayer normal. The ^{15}N spectra of these samples in the L_α phase are also shown in Fig. 2, *B* and *D*, and the characteristics identified from these spectra are presented in Table 1. Both tensors appear to be best fit by an axially asymmetric tensor with only a small degree of librational averaging based on a static DMPC sample (Table 1). A small amount of isotropic intensity (<10%) is observed in each of these ^{15}N spectra. Such small signals presumably arise from small liposomes and are difficult to avoid in the hydration of the peptide/lipid samples depending on sample rheology. However, the ^{31}P spectra do not show evidence of small liposomes, and therefore these small signals may arise via another mechanism.

The spectra of ^{15}N-D-Leu^{10}-gA in DEruPC (Fig. 2 *A*) and DNPC (Fig. 2 *C*) are from samples aligned between glass plates such that the bilayer normal is parallel with the magnetic field direction of the NMR spectrum. These spectra show resonances that are constrained to a small region of the spectral frequency range, for DEruPC ~29 (81–52) ppm and for DNPC ~26 (88–62) ppm at half-height of the observed

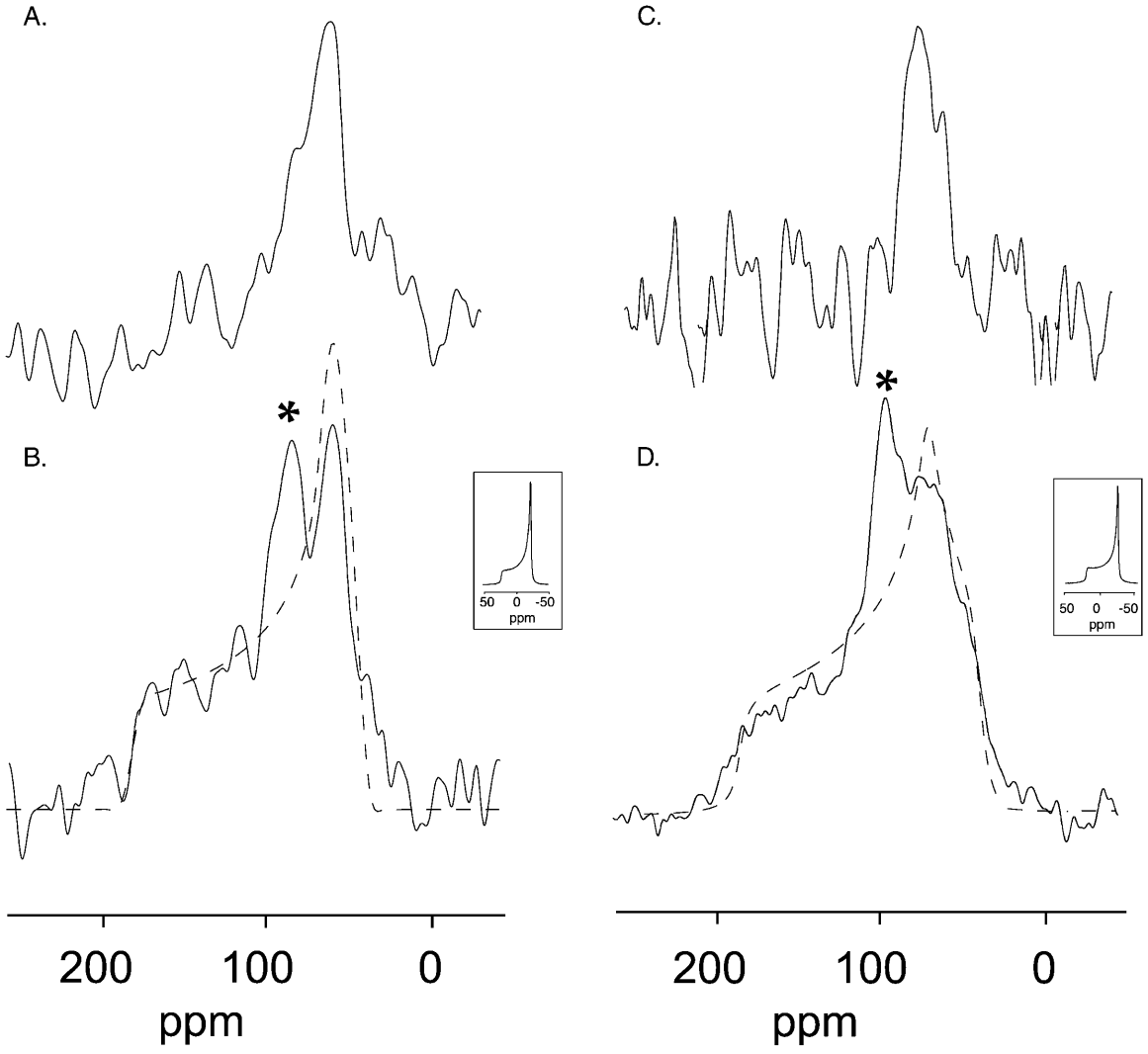

FIGURE 2 ^{15}N spectra of bilayer samples with ^{15}N-D-Leu^{10}-gA in a molar ratio of 1:40 (peptide/lipid) hydrated at ~40% by weight. Spectra were recorded with 30,000 and 42,000 acquisitions for oriented (*A* and *C*) and hydrated powder samples (*B* and *D*), respectively, and processed with 10-ppm line broadening. (*A*) ^{15}N spectrum of an aligned sample using DEruPC bilayers. (*B*) ^{15}N spectrum of an unoriented sample using DEruPC bilayers. The simulated axially asymmetric powder spectrum is shown by a dashed line having chemical shift-shielding tensor elements of $\sigma_{11} = 48$, $\sigma_{22} = 64$, and $\sigma_{33} = 182$ ppm. (*C*) ^{15}N spectrum of an aligned sample using DNPC bilayers. (*D*) ^{15}N spectrum of an unoriented sample using DNPC bilayers. The simulated spectrum has chemical shift-shielding tensor elements of $\sigma_{11} = 40$, $\sigma_{22} = 72$, and $\sigma_{33} = 190$ ppm. The insets are the ^{31}P spectra of the corresponding samples. The resonance intensities marked with an asterisk are isotropic contributions to the spectrum (intensity <10% from simulation).

TABLE 1 ^{15}N anisotropic chemical shift data (in ppm)

Lipid	Labeled gA site	σ_{11}	σ_{22}	σ_{33}	CSA
Anhydrous DEruPC	Ala^5	40	65	204	164
Hydrated DEruPC	Ala^5	55	69	193	138
Hydrated DNPC	Ala^5	48	62	202	154
Anhydrous DMPC*	D-Leu^{10}	38	68	204	166
Hydrated DEruPC	D-Leu^{10}	48	64	182	134
Hydrated DNPC	D-Leu^{10}	40	72	190	150

All tensor element magnitudes are reported here with an error of ±2.5 ppm, and all chemical shifts are reported relative to the frequency of a saturated solution of $^{15}NH_4NO_3$.
*Mai et al. (1993).

resonances. Thus, these spectra of ^{15}N-Leu^{10} demonstrate a relatively small set of ^{15}N chemical shift tensor orientations relative to the bilayer normal. Because these resonances from aligned samples do not conform to the σ_{33} frequency of the unoriented sample spectra, it confirms our assumption that the powder pattern spectra are not axially symmetric and that rotational motions about the bilayer normal are not occurring at a rate that is rapid on the timescale of the ^{15}N chemical shift tensor. The ^{15}N spectral data from both aligned and unoriented samples from these two long-chain lipids are consistent with each other demonstrating a level of reproducibility. The ^{15}N frequencies from the aligned samples show that the σ_{33} tensor element is approximately in the plane of bilayer.

^{31}P spectra of hydrated unoriented samples of ^{15}N-Ala^5-gA in DEruPC and DNPC are shown in the inserts in Fig. 3, *B* and *D*, respectively. These spectra show that >96% of the lipids are uniformly prepared in the L_α phase and have rotational freedom about the bilayer normal. A small amount of isotropic phase (<4%) is observed, but is not substantial, which potentially comes from a small amount of cubic phase (de Planque et al., 2002; Killian et al., 1996). The ^{15}N spectra of these samples show broad powder patterns with sharp spectral discontinuities (Table 1). The well-defined tensor elements mean that the librational averaging for all of the labeled Ala^5 sites are identical. The powder pattern spectra show no evidence of being axially symmetric as they are in the spectra of the gA channel form. This suggests that the structural complex with associated lipids is too large to rotate freely about the bilayer normal on the 10-kHz timescale. The general features of the powder pattern spectra from DEruPC bilayers and DNPC bilayers are similar, although the amplitude of librational averaging appears to be somewhat greater in the presence of DEruPC that has the shorter chain length and the lower phase transition temperature.

The ^{31}P spectrum of aligned ^{15}N-Ala^5-gA in hydrated DEruPC bilayers is shown in the insert in Fig. 3 *A* and that for DNPC bilayers is shown in the insert in Fig. 3 *C*. Both spectra show a predominantly aligned sample with no evidence of isotropic phase. The only other component in the spectra is some powder pattern intensity reflecting a portion of the sample having a random distribution of orientations. These results are in sharp contrast to the ^{15}N spectra of these samples (Fig. 3, *A* and *C*) where there is no predominant chemical shift frequency for an aligned component, but neither does the broad distribution of frequencies reflect a random distribution of orientations. Therefore, it is not possible to say that the broad distribution of resonances arises simply because the peptides are associated with the portion of unoriented lipid domains. Consequently, it appears that the ^{15}N-Ala sites have a broad but nonrandom distribution of orientations in the aligned bilayers of both DEruPC and DNPC. This is a surprising observation, since the ^{15}N-D-Leu^{10}-gA spectra of the aligned samples (Fig. 2, *A* and *C*) show relatively uniform alignment in the vicinity of the residue 10.

DISCUSSION

There are many reasons to believe that the observations reported here are of the monomeric nonconducting state in a lipid bilayer. Clearly, it is not the conducting dimer that has been so well characterized (Ketchem et al., 1997) or even double helical dimers that have been observed in lipid bilayers (Cotten et al., 1997; 1999; Arumugam et al., 1996). Moreover, the data is not consistent with any of the other well-defined structures characterized by x-ray crystallography or solution NMR in bilayer mimetic environments. Based on the results of Veatch et al. (1975), Mobashery et al. (1997), and Martinac and Hamill (2001), the dimerization constant should be $<10^{10}$ mol^{-1} cm^2 in the bilayers used in this article, and therefore >90% of the gA will be monomeric. Such a monomeric state will be nonconducting.

Unlike the conducting dimeric state, where a global rotational motion has been well characterized (Nicholson et al., 1987; Lee et al., 1993) leading to axially symmetric powder patterns, here the powder patterns are axially asymmetry. This is confirmed by the spectra of aligned samples that would show a resonance consistent with the unique tensor element, $\sigma_{//}$ of an axially symmetric powder pattern if axial motion about the bilayer normal occurred. Alternatively, axial motion could occur about an axis in the molecular structure, such as the helical axis, but this is also clearly not the case for the Leu^{10} site and it would be very difficult to imagine axial rotation for one region of the polypeptide and not for the other. Therefore, we conclude that axial rotation is not occurring on the ^{15}N chemical shift anisotropy timescale (10 kHz).

There are many reasons to believe that the monomeric structure of gA will be a significantly structured state. Clearly, from single channel conductance studies there is a rapid and efficient equilibrium process between conducting and nonconducting states (Andersen and Koeppe, 1992; Busath, 1993). This is generally accepted to be a monomer-dimer equilibrium. It is also known that it is difficult to break and form hydrogen bonds in the low dielectric environment

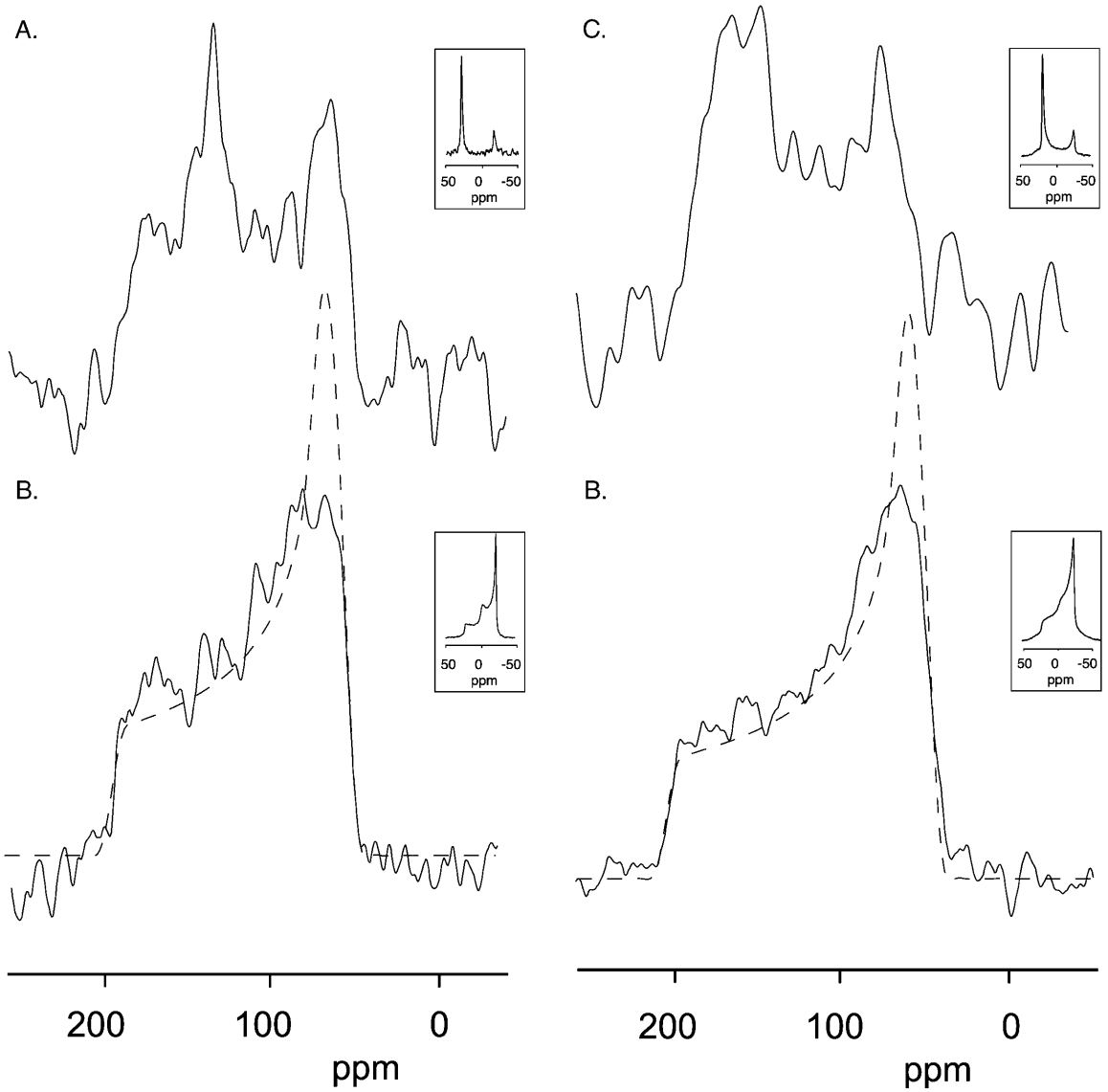

FIGURE 3 ^{15}N spectra of bilayer samples with ^{15}N-Ala^5-gA in a molar ratio of 1:40 (peptide/lipid) hydrated at ~40% by weight. Acquisition numbers are 30,000 and 42,000 for oriented (*A* and *C*) and hydrated powder samples (*B* and *D*), respectively. A line broadening of 10 ppm was applied. (*A*) ^{15}N spectrum of an aligned sample using DEruPC bilayers. (*B*) ^{15}N spectrum of an unoriented sample using DEruPC bilayers. The simulated axially asymmetric powder spectrum is shown by a dashed line having chemical shift-shielding tensor elements of $\sigma_{11} = 55$, $\sigma_{22} = 69$, and $\sigma_{33} = 193$ ppm. (*C*) ^{15}N spectrum of an aligned sample using DNPC bilayers. (*D*) ^{15}N spectrum of an unoriented sample using DNPC bilayers. The simulated spectrum has chemical shift-shielding tensor elements of $\sigma_{11} = 48$, $\sigma_{22} = 62$, and $\sigma_{33} = 202$ ppm. The insets are the ^{31}P spectra of the corresponding samples.

of a lipid bilayer (Xu et al., 1996; Xu and Cross, 1999; Popot and Engelman, 2000). Therefore, it is likely that the number of hydrogen bonds that need to be broken and reformed in the monomer-dimer equilibrium will be minimized; hence the gA monomer should be a relatively well structured state. This concept is supported by the scattering results from Boc-gA (He et al., 1994) where they demonstrated Tl^+ binding to the monomer. The sharp discontinuities in almost all of the ^{15}N powder patterns for ^{15}N-Ala^5 and ^{15}N-Leu^{10}-gA in these two lipid environments show that all of the molecules in a given sample have the same motional freedom. Such homogeneous dynamics suggest homogeneous structure (Lazo et al., 1993), since these librational motions typically reflect local motions of the polypeptide backbone that are, in turn, characteristic of different molecular configurations. This uniformity in dynamics and structure within a sample further argues against any heterogeneous aggregation as a possible explanation for the broad lineshapes from the aligned samples. In addition, the Leu^{10} data from aligned samples show a relatively uniform orientation for this amide site relative to the bilayer normal, indicating a well structured site.

The data from the aligned samples of Ala^5 and Leu^{10} are decidedly different, both from each other and from the conducting dimer. The observation of a relatively narrow

resonance for the ^{15}N-Leu^{10}-gA aligned samples and the broad spectral patterns from the unoriented samples suggests that the C-terminal domain of this monomeric conformation is both uniformly structured and relatively well aligned. The resonances from the Leu^{10} gA site centered at 67 ppm (DEruPC) and 75 ppm (DNPC) suggest that the σ_{33} tensor element is roughly in the plane of the bilayer, in sharp contrast to the conformation of the conducting dimer defined by a single sharp ($\Delta\nu_{1/2}$ = 4 ppm) resonance at 144 ppm (Mai et al., 1993). Huang and co-workers (He et al., 1994) noted that the peptide moved away from the bilayer center in going from the dimeric to monomeric state and that it had a larger effective radius in the bilayer. The tilting of the Leu^{10} peptide plane noted above and its hydrogen bond partners for this amide site is consistent with these scattering results from Boc-gA. This is also consistent with our result that the peptide no longer has axial rotation about the bilayer normal. Because of the hydrophobic nature of this peptide, it is important not to think of this peptide as being on the surface of the lipid bilayer but rather buried at the boundary of the hydrophobic region and headgroup regions formed by phosphatidylcholine lipids (Wiener and White, 1992).

On the other hand, the observed resonant frequency for the Ala^5 site is distributed over a wide range of frequencies, and hence the σ_{33} tensor element appears to have a broad range of orientations from parallel to perpendicular with respect to the bilayer normal. This disorder is either static or dynamic on a timescale that is slow compared to the chemical shift anisotropy (10 kHz). Structural heterogeneity is unlikely based on the sharp discontinuities of the ^{15}N chemical shift anisotropy that strongly suggests a uniform structure. In addition, sample heterogeneity is also unlikely based on respectable ^{31}P spectra for these samples and the ^{15}N spectra of aligned Leu^{10} labeled gramicidin A. Furthermore, if this heterogeneity represented local disorder of the Ala^5 peptide plane, we would expect much greater librational amplitudes and frequencies than those observed. Therefore, we speculate that the Ala^5 site reflects a different structural domain from that of the D-Leu^{10} site, one in which the N-terminal domain possesses a range of orientations that may be as great or greater than 90° with respect to the bilayer normal. Because the N-terminal domain is tethered to the C-terminal domain and because the N-terminal domain is very hydrophobic, this range of orientations would be in the hydrophobic region of the bilayer to provide a uniform electronic environment for the Ala^5 site in these different orientations. It is well known that gramicidin A has very limited stability in water, and hence the speculation here is that the N-terminal domain is in the hydrophobic region of the bilayer environment. To be stable in the hydrophobic region of the bilayer, amide carbonyls and N-H groups must be hydrogen bonded and not exposed to the hydrophobic interstices of the lipid bilayer, and therefore this domain is likely to have a well defined conformation. This is consistent with He et al. (1994) who have shown that Boc-gA binds Tl^+.

Various orientations would then be achieved by pivoting about a kink site between the C- and N-terminal regions. Interestingly, there is a significant break in the pattern of observed chemical shift frequencies in the conducting dimer in the vicinity of D-Leu^{10}. Residues Val^1, Ala^3, Ala^5, Val^7, and Trp^9 have resonance frequencies in aligned dimer samples between 196 and 198 ppm, whereas residues Trp^{11,13,15} have resonance frequencies between 181 and 185 ppm, suggesting two inherent domains in the conducting dimer. Indeed, these two domains represent the cation binding site and the pore region of the conducting dimer.

Nature does not always provide structural biologists with well defined molecular systems to characterize as we have seen with the numerous purified proteins that are unstructured in isolation from their binding partners (Dunker et al., 2002). Although the open state of gA represents a well-oriented and structured system in biological membranes, the closed state of gA is different. From a biological and thermodynamic perspective, the closed state must be able to find its partner in the opposing bilayer leaflet with high fidelity, and it must be able to structurally rearrange to form the open state with a minimal energy barrier. At the same time, the closed state must be energetically stable in this environment. Whereas none of the previously described models for the closed state satisfy these constraints, the model suggested here fits these constraints quite well. The multiple orientations of the N-terminal domain may act as a sensor for a monomer in the adjacent bilayer leaflet. Once the intermolecular hydrogen bonds start to form, facilitated by water molecules (Arumugam et al., 1996; Xu et al., 1996; Xu and Cross, 1999), the C-terminal domains will zipper onto the nascent structure. Although much of this mechanism is conjecture, it fits with many of the requirements discussed earlier in this article. It is hoped that with such a model for the gating of this well-characterized channel that additional experiments will be forthcoming to test these many assumptions.

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