Protein Stability and Conformational Rearrangements in Lipid Bilayers: Linear Gramicidin, a Model System

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ABSTRACT The replacement of four tryptophans in gramicidin A by four phenylalanines (gramicidin M) causes no change in the molecular fold of this dimeric peptide in a low dielectric isotropic organic solvent, but the molecular folds are dramatically different in a lipid bilayer environment. The indoles of gramicidin A interact with the anisotropic bilayer environment to induce a change in the molecular fold. The double-helical fold of gramicidin M, as opposed to the single-stranded structure of gramicidin A, is not compatible with ion conductance. Gramicidin A/gramicidin M hybrid structures have also been prepared, and like gramicidin M homodimers, these dimeric hybrids appear to have a double-helical fold, suggesting that a couple of indoles are being buried in the bilayer interstices. To achieve this equilibrium structure (i.e., minimum energy conformation), incubation at 68°C for 2 days is required. Kinetically trapped metastable structures may be more common in lipid bilayers than in an aqueous isotropic environment. Structural characterizations in the bilayers were achieved with solid-state NMR-derived orientational constraints from uniformly aligned lipid bilayer samples, and characterizations in organic solvents were accomplished by solution NMR.

INTRODUCTION

Integral membrane protein folding is thought to occur primarily outside of the native environment for the protein; i.e., in the aqueous phase or, more likely, in the sizable membrane interfacial region. This generates a considerable problem in that protein conformation is dependent on solvent environment. Consequently, it is not clear how the minimum energy configuration of an integral membrane protein is achieved. Linear gramicidins span lipid bilayers, forming monovalent cation selective channels (Andersen, 1984; Andersen and Koeppe, 1992; Finkelstein and Andersen, 1981; Killian, 1992), but their structure has been shown to be solvent dependent (Veatch et al., 1974), and nonminimum energy conformations have been kinetically trapped in the lipid bilayer environment as metastable states (Arumugam et al., 1996). Furthermore, various polypeptide analogs have a lesser propensity for channel formation or lower conductance rate (Becker et al., 1991). The finding of nonminimum energy conformational states suggests that the folding path and hence the folding environment are critical for achieving a functional state. Moreover, because some amino acids are believed to mediate key peptide-lipid interactions, the study of peptide analogs will shed light on the driving force for conformational rearrangements along the folding pathway. Here we compare the structure of gramicidin A (gA, containing four tryptophan residues) to that of gramicidin M (gM, each tryptophan replaced with a phenylalanine) in both a lipid bilayer environment and in a mixed organic solvent system.

Conformational interconversion in nonpolar environments is difficult to achieve because of the increased stability of hydrogen bonds and electrostatic interactions (Zaks and Klibanov, 1988). The ubiquitous presence of water in studies of water-soluble proteins has concealed important roles that the solvent plays in protein structure, stability, and dynamics. In studies of membrane-bound peptides in which the presence of water is highly restricted, these solvent roles are becoming apparent. This has led to the observation of kinetically trapped states described above, which are rarely observed in water-soluble proteins (Baker and Agard, 1994).

Such difficulties for conformational interconversion suggest that protein folding in this bilayer environment is not a viable option because the search over conformational space would be very restricted. Similar conclusions have been achieved based on energetic arguments (Engelman and Steitz, 1981). These authors went on to propose the helical hairpin hypothesis for the insertion of a pair of helices into a membrane, and today it remains a viable model (Baumgartner, 1996). Popot and Engelman (1990) developed a two-stage model in which helices are inserted and associate with each other once in the bilayer to form the tertiary structure. This model has been further extended by Deber and Goto (1996), who describe the α-helical structure as developing in the interface region. White and co-workers (Weiner and White, 1992; White and Wimley, 1994) have described the detailed structure of the fluid phase of dioleoylphosphatidylcholine bilayers by joint refinement of x-ray and neutron diffraction data. The width of the two interface regions nearly equals the width of the hydrophobic core. Until recently the substantial thickness of the bilayer interface was not a significant feature of membrane protein insertion models, but Brown and Huestis (1993) have

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shown that a tripeptide that is unstructured in an aqueous environment becomes structured at the lipid-aqueous interface of phosphatidylcholine vesicles. Indeed, the interface may prove to be the scaffolding yard in which secondary structures are formed before insertion.

Gramicidin A is a linear polypeptide of 15 residues in which both of the terminal groups are blocked:

HCO-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

Consequently, there are no formal charges in this very hydrophobic polypeptide. In organic solvents, a variety of dimeric structures are known to exist, some of which have been crystallized (Langs, 1988; Langs et al., 1991; Wallace and Ravikumar, 1988) and some of which have been studied by solution NMR (Abdul-Manan and Hinton, 1994; Pascal and Cross, 1992, 1993). The interconversion rate between these conformers is known to be dependent on the solvent (Bañó, 1988, 1989, 1991; Pascal and Cross, 1992; Veatch et al., 1974). Recently it has been shown that protic solvents bind to the amide backbone sites, potentially to facilitate the hydrogen bond exchange that is necessary for conformational interconversion (Xu et al., 1996). In fact, it appears as if protic solvents catalyze these conformational interconversions. The structure of the single-stranded channel-forming dimer of gramicidin A has been solved in the hydrated lipid bilayer environment (Ketchem et al., 1993). The structure is a β-strand in which all of the side chains are on one side of the strand (because of the alternating stereochemistry of the amino acid residues), and therefore the strand wraps into a helix in which there are intrastrand hydrogen bonds between the turns of the helix. This helix is ~6.5 residues per turn, and it has a right-handed sense. To span the bilayer, the channel is a symmetrical dimer with the formylated amino termini at the bilayer center and the indole-rich carboxy termini at the bilayer surface. The indole N-H groups are all oriented toward the hydrophilic surface and presumably hydrogen bond to this surface, thus orienting the channel axis parallel to the bilayer normal, which is appropriate for channel function.

The orientation of the gramicidin channel in the anisotropic lipid environment is only one of several important roles for tryptophan in this molecule and potentially in other membrane proteins. Electrostatic interactions are effective over long distances in a low dielectric medium, and an excellent correlation between the dipole (indole)-monopole (cation) interaction energy and cation conductance has been established (Hu and Cross, 1995), by using the conductance data for a wide range of phenylalanine for tryptophan analogs of gramicidin (Becker et al., 1991). In the photosynthetic reaction center, a dielectric asymmetry has been described to distinguish between two nearly equivalent electron transfer pathways (Steffen et al., 1994). The role of long-range electrostatic interactions originating from indoles may have significant functional implications in many membrane proteins. Here the role of the indoles is shown to be fundamentally important as the driving force behind the conversion of double-stranded helices to single-stranded helices.

The gramicidin channel structure has been solved to high resolution by using solid-state NMR-derived orientational constraints from samples uniformly aligned with respect to the magnetic field. In anisotropic environments chemical shift, dipolar, and quadrupolar interactions are orientation dependent, and therefore these NMR observables can be used to constrain the orientation of specific spin interaction tensors with respect to the magnetic field direction. Because the tensors can be oriented with respect to the molecular frame (i.e., the covalent bonds of the atomic site), and because the bilayer normal and global molecular axes (in this case, the channel axis) are aligned parallel to the magnetic field direction, specific covalent bonds can be orientationally constrained with respect to the channel axis. The three-dimensional structure of gramicidin was assembled from a number of such constraints. Here additional orientational constraints are presented for gramicidin analogs that clearly demonstrate the presence of different molecular structures. In addition, standard high-resolution ^1H NMR techniques were used to characterize the chemical and conformational purity of samples in organic solvents and to establish the dominant conformation in these organic solvents. Matrix-assisted laser desorption ionization (MALDI) Fourier transform ion cyclotron resonance (FT-ICR) was used to show the chemical purity and molecular mass of the polypeptides (Hettich and Buchanan, 1991). MALDI FT-ICR mass spectrometers offer ultrahigh mass resolving power, precise mass measurement, and multistage MS^n in a single instrument (Solouki et al., 1995, 1996). Moreover, MALDI is a "soft ionization" technique, thereby minimizing fragmentation and dissociation processes.

MATERIALS AND METHODS

The gramicidins (A and M) were synthesized by solid-phase synthesis, using Fmoc (9-fluorenylmethoxycarbonyl) chemistry on an Applied Biosystems model 430A peptide synthesizer. Isotopically labeled amino acids were purchased from Cambridge Isotope Laboratories (Woburn, MA), and the blocking chemistry was performed in our laboratory. Details of the synthesis and blocking chemistry have been described previously (Fields et al., 1988, 1989).

Once cleaved from the solid-phase support, the peptides were characterized and the purity assessed. Both peptides were analyzed on a Beckman model 344 high-performance liquid chromatography (HPLC) system, using a Beckman Ultrasphere or YMC C-18 reverse-phase analytical column at 35°C. Twenty-microliter aliquots of a 1 mg/ml solution of gA were injected onto a column that was eluted isocratically at 0.7 ml/min with MeOH/H_2O in a 85/15 solvent ratio. The retention times monitored at 280 nm (indole wavelength maximum) was compared to a separation of natural gramicidin A′ products. Typical purity was in excess of 98%, and such peptides were used without further purification. When purification was needed, a semipreparative HPLC protocol was utilized as described by Fields et al. (1989).

Crude gramicidin M (gM) was purified by recrystallization at room temperature after the peptide was dissolved at 55°C in HPLC-grade MeOH. The characterization of gM differed from that of gA, because gM is more hydrophobic and much less soluble in MeOH and MeOH/H_2O mixtures. Second, gM has a wavelength absorption maximum at 254 nm, with a molar extinction coefficient that is almost a factor of 30 weaker. Therefore,

the conditions for HPLC analysis were altered by injecting 50-μl aliquots of a 0.2 mg/ml solution onto the column and by using a MeOH/H_2O mobile phase in the ratio of 95/5 (v/v). The column was monitored simultaneously at 214 and 254 nm, and the peaks were scanned for their wavelength maximum. Under these conditions aggregation was minimized, and the sensitivity was adequate to identify the gM peak.

In addition, because of the problems with HPLC, both mass and ^{1}H NMR spectroscopy were used to characterize the polypeptides. The MALDI FT-ICR mass spectra were acquired with an FTMS-2000 Fourier transform ion cyclotron resonance mass spectrometer (Finnigan Corp., Madison, WI) equipped with a 3 T superconducting magnet, automatic insertion probe, and Odyssey data system. Mass spectra were acquired in the source compartment of a dual cubic ion trap. Desorption/ionization was performed with a cartridge-type pulsed N_2 laser (model VSL-33ND; Laser Science, Newton, MA) operated at a wavelength of 337.1 nm with a pulse width of 3 ns (laser power density of 10^6 watts·cm^{-2}). The experimental details and instrument configuration have been published elsewhere (Solouki et al., 1995, 1996). A 1 M stock solution of 2,5-dihydroxybenzoic acid matrix was prepared daily in methanol acidified with 0.1% (v/v) trifluoroacetic acid. MALDI FT-ICR mass spectra were obtained at a typical matrix-to-gM molar ratio of ~1000:1. Approximately 10 μl of the solution mixture containing gM and the matrix was applied to the solid insertion tip and allowed to dry in air before insertion into the mass spectrometer.

^{1}H NMR samples were prepared by dissolving gM in 95/5 (v/v) benzene-d_6/ethanol-d_5 (Cambridge Isotope Laboratories) at a concentration of 8 mM. Samples were recorded on a Varian Unity Plus 720 MHz spectrometer at 30°C, equipped with three axis pulse-field gradients. 2D NOESY spectra were collected in a phase-sensitive mode, using the hypercomplex method (States et al., 1982). For GCOSY spectra, N-type data were collected and presented in absolute mode. The residual benzene peak was referenced to 7.15 ppm.

Oriented samples for solid-state NMR were prepared by codissolving gA or gM and dimyristoylphosphatidylcholine (DMPC) in a 1:8 molar ratio in a 95/5 (v/v) benzene/ethanol mixture. For the samples containing both gA and gM, the two peptides were first dissolved and then DMPC was added after allowing for sample equilibration overnight. After a freeze-thaw cycle, the solution, while still cool, was spread on glass coverslips. Partial evaporation of the solvents was allowed to occur at room temperature. The samples were then dried overnight under vacuum and stacked in a square glass tube before 50% HPLC-grade water (by weight of the peptide-lipid samples) was added. The tubes were then sealed and incubated at 45°C for a minimum of 2 weeks until the samples became transparent, uniformly hydrated, and oriented such that the bilayers were parallel to the glass slides. Some samples were further incubated at 68°C.

Deuterium exchange of amide protons was performed by opening a previously hydrated sample and exposing the sample to a saturated D_2O atmosphere at 45°C for 4 days in a closed container. If a significant weight loss occurred during the incubation, D_2O was added to compensate. The samples were then sealed and further incubated.

Solid-state NMR spectra were acquired using a spectrometer built around a Chemagnetics data acquisition system and an Oxford Instruments 400/89 magnet. The ^{15}N resonant frequency was 40.58 MHz, and the spectra were recorded by using cross-polarization (5 μs 90° pulse, 1 ms contact time, 7 s recycle delay) and ^{1}H dipolar decoupling. The ^{15}N spectra were referenced to a saturated solution of $^{15}NH_4NO_3$.

All data were processed with Varian VNMR software. A sine bell function of 0.047 s and 0.014 s was applied to the f2 and f1 dimensions, respectively, in processing the GCOSY data. For the NOESY spectra, a Gaussian function of 0.051 s and 0.012 s was applied to the f2 and f1 dimensions, respectively.

RESULTS

Fig. 1 shows the mass spectrum of a single-site ^{15}N-labeled synthetic gM (1727.2 Da). Molecular adduct ion peaks at 1750 $(gM + Na)^+$ and 1766 $(gM + K)^+$ were the dominant species. The low abundance peaks at lower values of m/z represent a small amount of fragmentation that occurred during the MALDI ion formation. This result confirms that the major HPLC peak, with an absorption maximum at 254 nm, is the gM peak (data not shown).

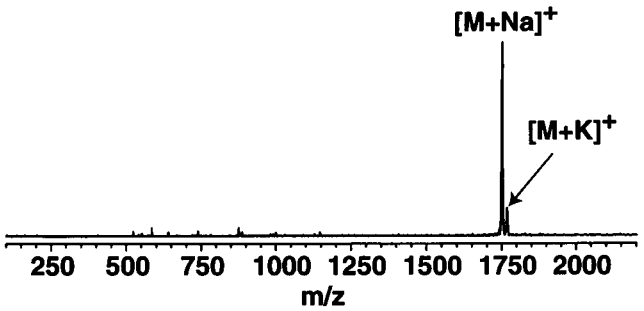

FIGURE 1 MALDI FT-ICR single laser shot mass spectrum obtained from a sample of ^{15}N Ala_5 gramicidin M (MW = 1727.2). Two molecular adduct ions, at m/z = 1750 and 1766, are observed.

The ^{1}H fingerprint region of the GCOSY spectrum for ^{15}N Ala_3, d_3 $(\alpha,\beta)Leu_4$ gM dissolved in deuterated benzene/ethanol is shown in Fig. 2. The structural and conformational purity is demonstrated by a single set of NH-C_αH cross-peaks. The resonance assignments were accomplished by standard procedures, using this spectrum in combination

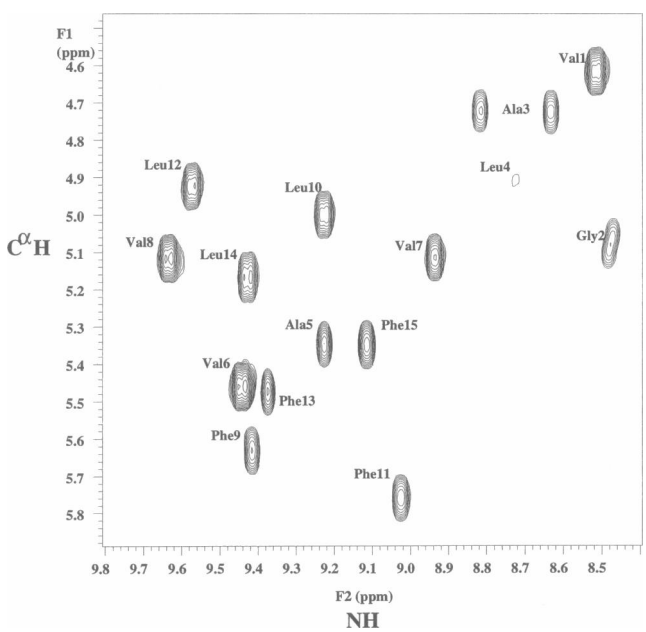

FIGURE 2 The fingerprint region of GCOSY for doubly labeled ^{15}N-Ala_3, d_3-Leu_4 gM (8 mM) in 5% d_5-EtOH in C_6D_6 solution. The assignment was made in conjunction with ^{1}H 2D TOCSY and NOESY experiments. ^{15}N-labeled Ala_3 has a split NH peak along the F2 dimension due to ^{15}N-^{1}H spin-spin coupling. The weak Leu_4 cross-peak results from the lack of a Leu_4 C_α proton. The C-terminal group, Et_{16}, is not shown in this region. The spectrum was recorded on a Varian UnityPlus 720 MHz spectrometer at 30°C, with two scans per time increment and a total experimental time of 8 min. A gradient level of 0.32 gauss/cm and a duration time of 1.9 ms were used for coherence selection.

with TOCSY and NOESY experiments. The amide proton for Ala_3 is split because of the spin-spin coupling between ^1H and ^{15}N, whereas the Leu_4 cross-peak is weak, because of deuteration of the Leu_4 C_α site. The NOESY spectrum shown in Fig. 3 shows interchain cross-peaks. Whereas NOE cross-peaks between residues 15–1, 13–3, and 11–5 are diagnostic for an antiparallel structure, those between 8–12 and 16–4 are characteristic for a left-handed helical sense. Therefore, the specific pattern of cross-peaks is entirely consistent with an antiparallel, left-handed, double-stranded conformation (known as species 3). This conformation has already been well characterized for gA in the same solvent (Zhang et al., 1992).

Solid-state ^{15}N NMR spectra of uniformly aligned samples of both gA and gM in DMPC bilayers are shown in Fig. 4. The samples are aligned such that the bilayer normal is parallel with the magnetic field direction. The anisotropic chemical shifts shown in Fig 4, *A* and *B*, reflect the orientation of the amide ^{15}N chemical shift tensors with respect to the magnetic field direction. If the tensor orientation is known with respect to the molecular frame (i.e., the covalent bond vectors), then the observed spin interaction magnitude in the oriented samples will constrain the molecular frame orientation with respect to the magnetic field direction. Because the various gramicidin structures are all versions of a β-strand conformation, the structural repeat unit is a dipeptide. Furthermore, the gramicidin structures are regular structures, and therefore there is a repeating pattern of the dipeptide spin interaction observables. By way of example, Fig. 5 illustrates the orientation dependence of the ^{15}N-^2H dipolar interaction for both D and L amino acid residues; the single-stranded, right-handed helical channel conformation; as well as the left-handed, double-stranded species 3 structure.

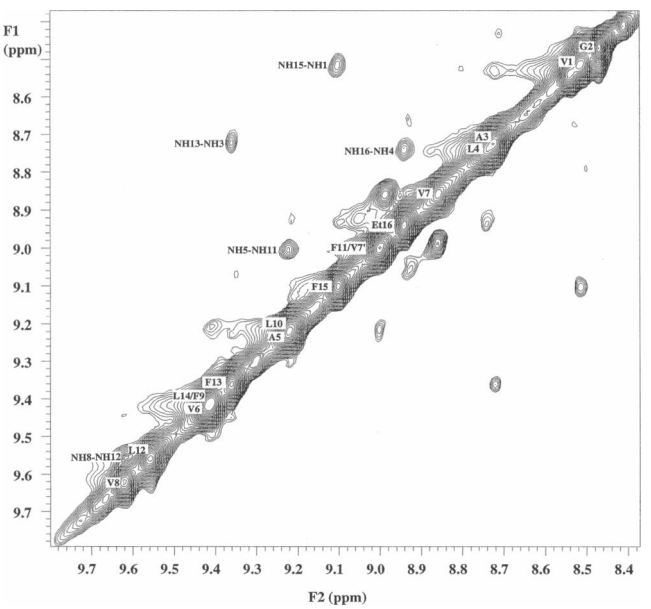

FIGURE 3 The backbone NH-NH region of a NOESY spectrum for ^{15}N-Val_7-labeled gM in 5% d_5-EtOH in C_6D_6 solution acquired as in Fig. 2, using a 300-ms mixing time and a 1.5-s recycle delay, with 16 acquisitions per time increment. The assignments relevant to the dimeric strand orientation (i.e., parallel versus antiparallel) and handedness (right versus left) characterization are given. ^{15}N-labeled Val_7 gM has a split NH peak on the diagonal, marked V7 and V7′ due to the ^{15}N-^1H spin spin coupling.

For gA, the pattern of ^{15}N chemical shift observables has the L sites near 190 ppm and the D sites near 140 ppm. A reversal of these values for the D and L residues would yield a change in handedness of the helix (Nicholson and Cross, 1989). gM shows such a change in the alternating pattern of the observed chemical shifts, and furthermore it shows a considerable change in frequency difference between the D and L residues of ~100 ppm (see Table 1). The doubling of this frequency difference correlates with a doubling of the helical pitch (Arumugam et al., 1996). Therefore these data for gM suggest that a double-helical structure with a left-handed helical sense is present in these planar lipid bilayers.

Moreover, the ^{15}N-^2H dipolar splitting between a spin 1/2 and a spin 1 nucleus shows a dramatic difference between D and L residues (Table 1). Such a dipolar interaction should yield a 1:1:1 triplet, and whereas the ^{15}N Leu_4 gM data crudely approximate this ideal, the ^{15}N Ala_5 gM data do not (Fig. 4 *F*). In this latter sample, the N_α-H appears to be more protected from hydrogen exchange than the Leu_4 site, and consequently the central peak of the triplet has a substantial contribution from the ^{15}N-^1H singlet (a result of ^1H decoupling). Data from several additional sites throughout the gM structure are consistent with the Leu_4 and Ala_5 data representing a dipeptide repeat unit.

Hybrid samples containing equal portions of gA and gM monomers were also prepared. Such preparations are a mixture of homo- and heterodimers. If a statistical distribution of gA and gM occurs, then a 1:2:1 ratio of gA_2:gA/gM:gM_2 would be expected, and for a label in gA, an equal intensity from the gA homodimer and the gA/gM heterodimer would be expected (which is similar to the pattern for a label in gM monomers). Fig. 6 *A* shows ^{15}N spectra of uniformly aligned ^{15}N Leu_{12} gA. The 135 ppm chemical shift value is consistent with the gA channel conformation. Fig. 6 *D* shows the same spectrum of ^{15}N Leu_4 gM in hydrated and oriented lipid bilayers as in Fig. 4 *B*. When ^{15}N Leu_{12} gA is observed in a hybrid mixture, there is no change in chemical shift compared to pure gA in oriented bilayers. Nor is there a change in chemical shift when ^{15}N Leu_4 gM is observed in hybrid mixtures compared with pure gM in oriented bilayers. However, when both samples are incubated at 68°C for 2 days, there is a very significant change in the gA spectrum in these hybrid samples compared to the gM spectrum, which remains unaffected. Whereas the initial structure (before 68°C incubation) in the hybrid preparations assessed by labels in gA is the channel state, the initial structure assessed by gM labels is a left-handed double-helical structure. It is anticipated that the homodimers of gA and gM will indeed be two different conformations, but the structure of a heterodimer cannot be a right-handed single-stranded helix and a left-handed double helix simultaneously. After incubation at 68°C, under

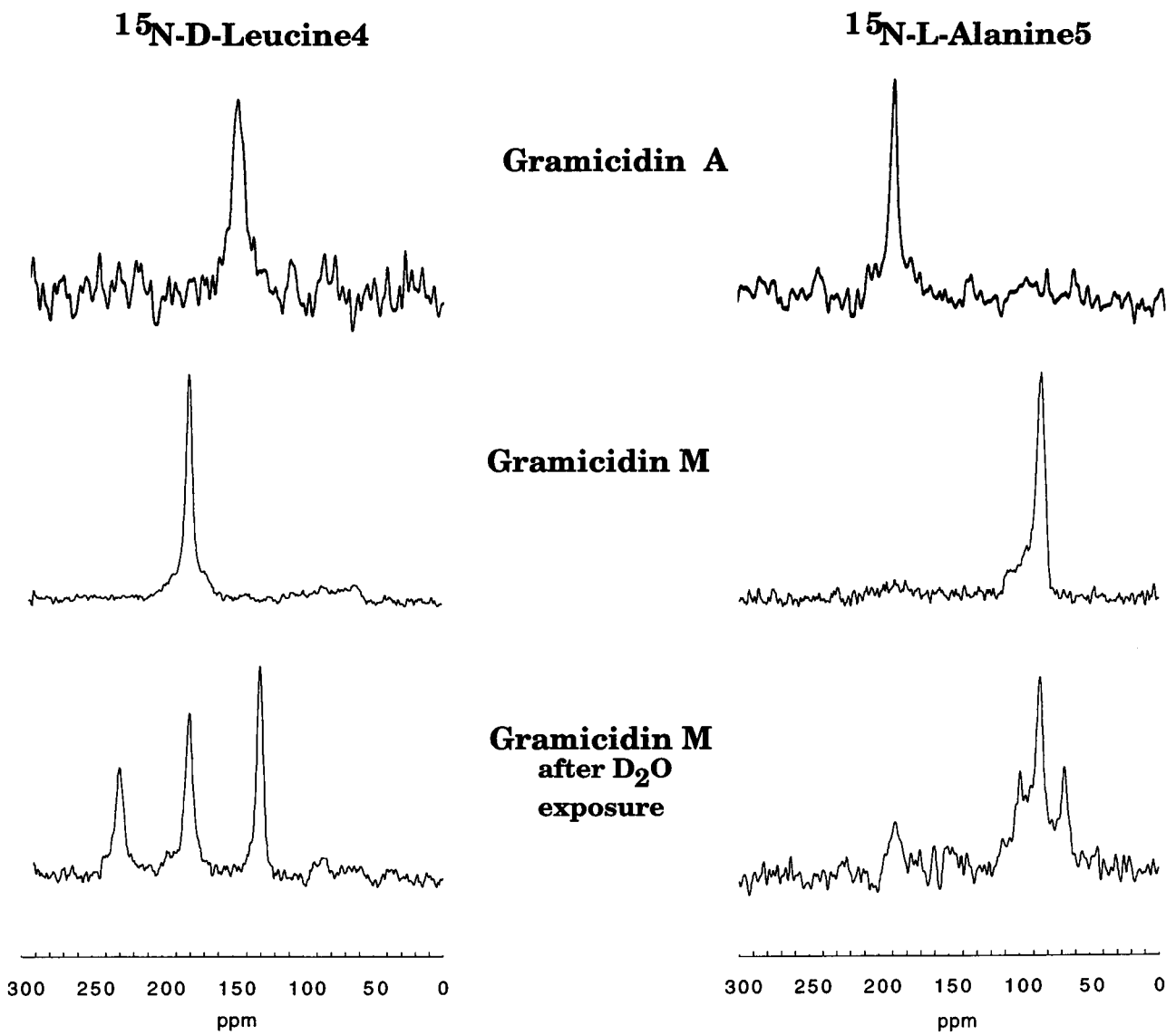

FIGURE 4 ^{15}N chemical shifts and ^{15}N-^{2}H dipolar interactions are observed for uniformly aligned samples of ^{15}N D-Leu_4- and ^{15}N-L-Ala_5-labeled gramicidins in DMPC bilayers prepared at a molar ratio of one peptide to eight lipid molecules. Spectra were obtained with cross-polarization and ^{1}H dipolar decoupling. The gramicidin A spectra were obtained at 20.1 MHz, and the gramicidin M spectra were obtained at 40.6 MHz.

conditions known to induce conformational transitions for kinetically trapped gramicidin conformers in lipid bilayers (Arumugam et al., 1996), the conformation assessed by the gA appears to be approximately a 1:1 mixture of the channel and double-helical states, and this is consistent with the data from gM labels.

DISCUSSION

Gramicidin M has been synthesized in high yield and purified by recrystallization from methanol. The peptide was characterized extensively by solution NMR spectroscopy and by FT-ICR and MALDI. The results have shown a peptide with the correct molecular weight, the correct sequence, and more than 95% purity. Although gM was previously synthesized and purified (Heitz et al., 1982), we found the more traditional approaches to purification and characterization by column chromatography and HPLC chromatography incompatible with the very low extinction coefficient and the high degree of hydrophobicity that led to extensive aggregation.

In benzene/ethanol (95:5 by volume), the conformations of gA and gM are shown to be the same. This conformation is known as species 3, a left-handed intertwined double helix. Similar conclusions were also achieved by using other low-polarity solvents, dimethyl sulfoxide and $CHCl_3$ (Heitz et al., 1986), in that the structures of gA and gM were shown to be the same. The presence of tryptophans or phenylalanines appears to have little effect on the polypeptide structure in this solvent. From studies of gA in a variety

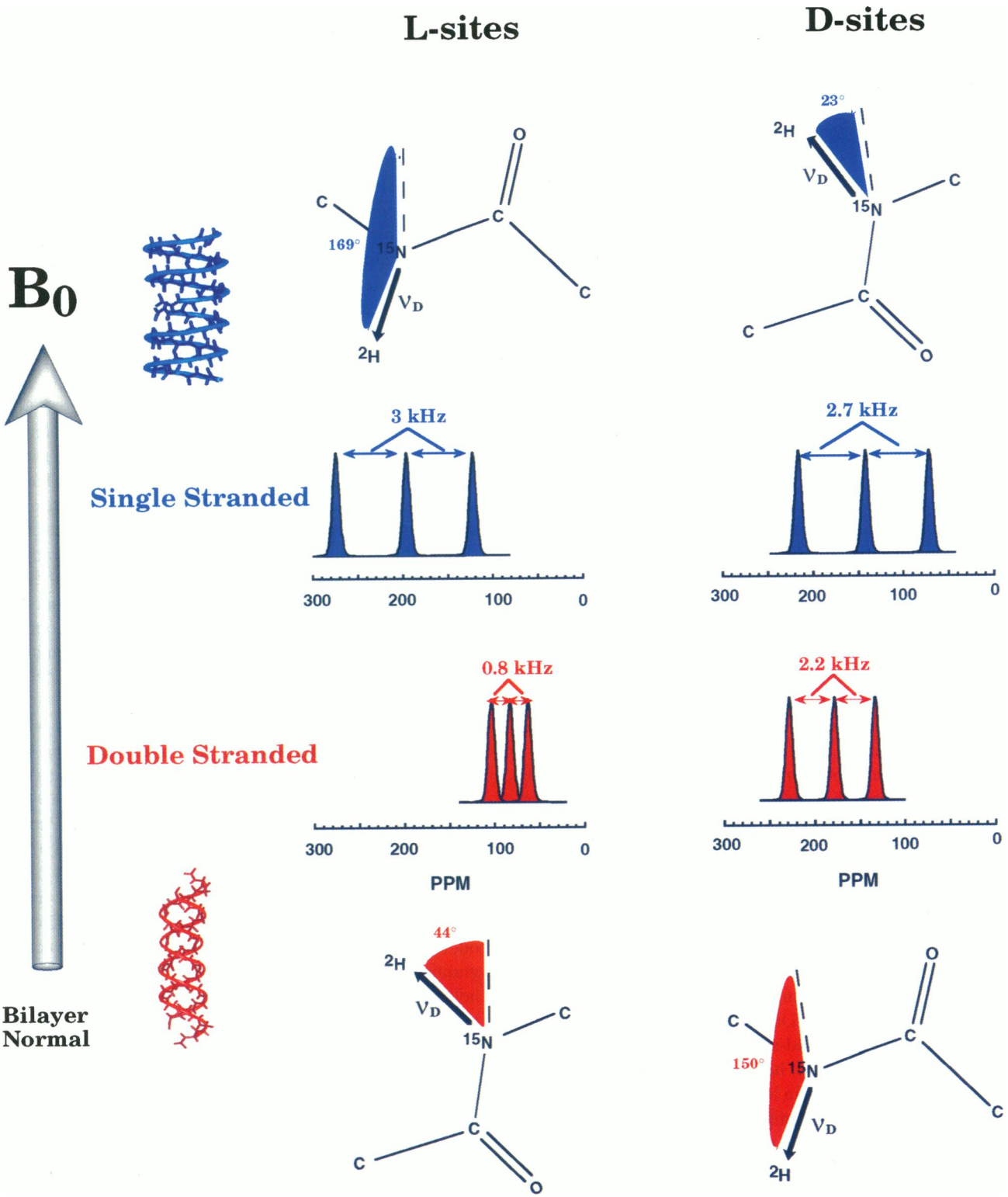

FIGURE 5 Gramicidin can adopt different solvent-dependent conformations that can be characterized by the observation of orientation dependent nuclear spin interactions. Because these various structures are variants on a β-strand secondary structure, the repeat unit is a dipeptide containing a D and an L residue. The right-handed single-stranded channel conformation (*blue*) shows ^{15}N-^{2}H dipolar splittings that are substantially different from the left-handed double-helical structure (*red*), which gM adopts in nonpolar organic solvents and which it is shown to adopt here in a lipid environment.

TABLE 1 Summary of the chemical shifts and N-H/N-D bond orientations with respect to the magnetic field of GA and GM

	CS (ppm)		N-H/N-D bond orientation (°)		Structural information
	Leu_4	Ala_5	Leu_4	Ala_5	
GA[§]	145	198	23.5*	169*	Single-stranded; right-handed
GM[¶]	182	86	30/150#	44/136# 67/113#	Double-stranded; left-handed

*N-H bond orientation.
#N-D bond orientation.
[§]Published results, references.
[¶]Experiments presented here.

of solvents, the antiparallel double helix is dominant in low dielectric solvents, presumably because of the low dipole moment of the antiparallel dimer versus the parallel dimer (Xu and Cross, unpublished results). Apparently, the dipole moment of the parallel structure is dominated by the β-strand-type backbone structure rather than the indole contributions, because parallel structures are not observed for gM in benzene/ethanol. β-Strands, unlike α-helices, have alternating orientations for the peptide planes, resulting in a dipeptide repeat unit. It is somewhat surprising that the dipole moment associated with the backbone is substantial; however, subtleties in the pattern of peptide plane orientations could generate a considerable dipole moment. Here it is emphasized that in this low dielectric isotropic solvent, the presence of the indoles has no significant impact on the peptide conformation.

In the lipid environment, gM assumes a conformation very different from that of gA. First, the gM conformation is unique and well aligned in this bilayer environment, as shown by the single sharp chemical shift resonances and the discrete triplets of the ^{15}N-^{2}H dipolar interactions. Second, these chemical shift and dipolar observables are very different from the gA right-handed, single-stranded channel conformational values. The gM data are consistent, however, with a left-handed intertwined double helix. Based on their clever HPLC experiments, Salom et al. (1995) have already shown that gM in DMPC vesicles is predominantly double-stranded. That solid-state NMR shows it to be left-handed suggests that gM has the same conformation in DMPC bilayers as it does in low dielectric organic solvents. Such a structure is not compatible with ion conductance, because the pore diameter is very narrow (Pascal and Cross, 1993; Smart et al., 1993; Zhang et al., 1992). Neither Langs (1988) by x-ray crystallography nor Xu et al. (1996) by NMR has observed solvent in this double-helical structure. Wallace and Ravikumar (1988) have shown atoms in the pore of a double-helical structure; but it had 6.4 residues per turn instead of 5.6 residues per turn as in species 3. The CsCl ion pairs substantially distorted the structure. Furthermore, no conductance of gM homodimers consistent with double-stranded structures (i.e., very long channel lifetimes) has been observed. Instead, conductance has been observed that is similar to that of gA (Fonseca et al., 1992; Heitz et al., 1982; Koeppe and Andersen, 1996), although the channel lifetime is somewhat shorter and the conductance is less.

The lifetime of the conducting state has been argued to be the lifetime of the dimer, and because of the increased hydrogen bonding between the monomers, the double-stranded structures have a longer lifetime than the single-stranded structures. Koeppe, Andersen, and co-workers have demonstrated what appears to be double-stranded conductance by using a hybrid structure of des-Val_1-gC and gM^- (Durkin et al., 1992). The lifetimes are several orders of magnitude longer than in gA, and conductance can be observed by adding the peptides to one side of the membrane. gA flip-flops across the membrane at a very slow rate (Arumugam et al., 1996; O'Connell et al., 1990), and when it is added to one side of the membrane it requires a long time before conductance is observed. Durkin et al. (1992) argued that conducting heterodimers form at the membrane interface, and only then penetrate the membrane to form the membrane-spanning double-stranded channel. The observed conductance from gM is assumed to arise from single-stranded conformers. It is worth noting that in measuring the conductance of other hydrophobic gramicidin analogs, Fonseca et al. (1992) needed to increase the gramicidin concentration by 100-fold to observe conducting states. Therefore, as observed here, the dominant conformation of gM in the lipid environment is the nonconducting species 3 conformation, and the rare single-stranded conformer provides the conducting states.

Tryptophan has many roles in gramicidin, one of which is to provide the driving force for the formation of the single-stranded channel structure. It is well known that indoles prefer the bilayer interface (Wimley and White, 1993). In fact, based on the gA (Ketchem et al., 1993) and Photosystem Reaction Center (Michel and Deisenhofer, 1990) structures, they appear to prefer having the indole N-H oriented toward the bilayer surface and the six-membered ring oriented toward the bilayer center (Schiffer et al., 1992). Without the indoles being present, the double-helical structure of gramicidin does not unscrew to form the single-stranded helix. The indoles are distributed fairly evenly along the length of the antiparallel double-helical structure, whereas in the single-stranded helix the indoles are clustered at the bilayer interface. Burying the indoles in the hydrophobic domain of the lipid bilayer is very costly energetically, and therefore the helical monomers unscrew from each other, allowing the indoles to migrate toward the bilayer surface (Arumugam et al., 1996; Urry et al., 1975; Zhang et al., 1992). Without the indoles (i.e., gM), the double helix is the minimum energy conformational state in the bilayer.

In the hybrid gA/gM structures that also adopt a left-handed double-helical structure, there are at least two buried indole groups. This result suggests that a full contingent or nearly a full contingent of indoles is necessary to induce the structural interconversion to the single-stranded state. Moreover, it also suggests that the unfavorable interactions of the indole N-H with the bilayer interstices is somehow

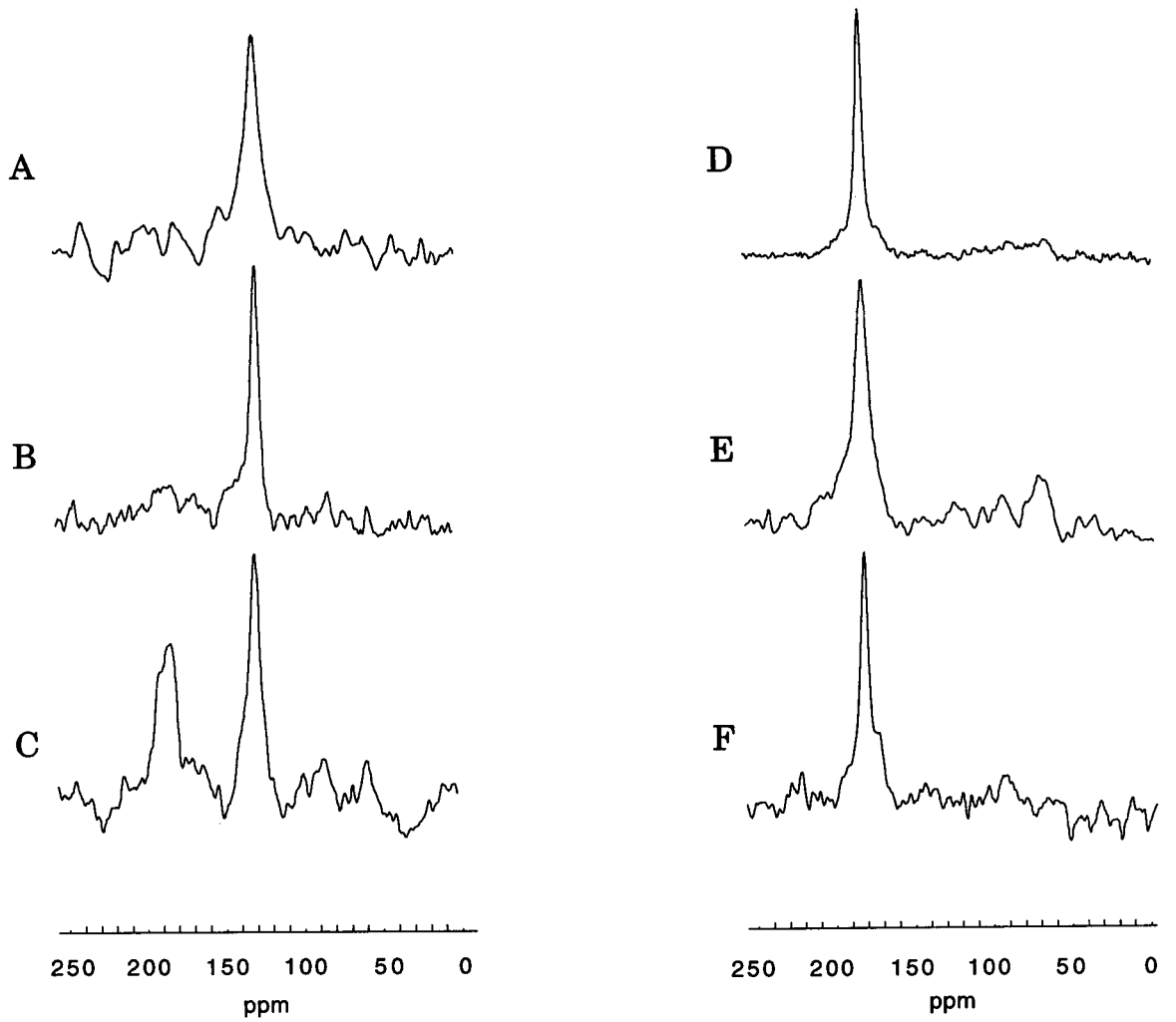

FIGURE 6 ^{15}N spectra single-site labeled gA and gM in uniformly aligned lipid bilayer preparations obtained at 40.6 MHz. (*A*) ^{15}N-Leu_{12}-labeled gA. (*B*) ^{15}N-Leu_{12}-labeled gA with unlabeled gM (1:1 molar ratio) after incubation at 48°C. (*C*) as in *B* after incubation at 68°C. (*D*) ^{15}N-Leu_4-labeled gM. (*E*) ^{15}N-Leu_4-labeled gM with unlabeled gA after incubation at 48°C. (*F*) as in *E* after incubation at 68°C.

compensated for in these hybrid interactions. Recently we showed that ethanol provides such compensation in the mixed benzene/ethanol (95:5) environment (Xu et al., 1996). Gramicidin A is not soluble in pure benzene, but when a few percent ethanol is added, it was observed that ethanol molecules bind to each of the indoles and to the exposed amide backbone sites. Therefore we speculate that a few water molecules may be sequestered on the outside of the gA/gM hybrid dimer to help solvate the indoles into the hydrophobic environment. Moreover, water molecules may be needed to catalyze the conformational interconversion. For instance, it is shown here that the minimum energy conformational distribution in the lipid environment when gA and gM are both added is achieved only after incubation of the sample at 68°C for 2 days. This kinetic trapping of conformational states in lipid bilayers has previously been demonstrated (Arumugam et al., 1996). Structural rearrangement in a nonpolar environment could be facilitated by the addition of a protic solvent that could destabilize intra- and interpeptide hydrogen bonds and stabilize broken hydrogen bonds. Although these roles for water in a membrane environment are speculation, conformational interconversion in a dry low dielectric environment has been shown to be exceptionally slow (Zhang et al., 1992); Xu and Cross, unpublished results). Furthermore, as stated above, it is known that a protic solvent can help solvate the peptide into a low dielectric environment. The peptide, gramicidin, is a feature of these experiments in organic solvents and lipid bilayers. But tryptophan and the role that this amphipathic amino acid plays may be important for many, if not most, membrane proteins. Tryptophan may facilitate the penetration of protic solvents into the lipid environment.

As membrane proteins and peptides are studied, it is important to recognize that the conformation of these molecules may be dependent on their environment. For gramicidin it was well accepted years ago that its structure was solvent history dependent (Bañó et al., 1988, 1989; Cox et al., 1992; Killian et al., 1988; LoGrasso et al., 1988), meaning that the structure in the lipid bilayer was dependent on the organic solvent history of the polypeptide before the

lipid environment was hydrated. This history dependence primarily reflected the ability of the membrane environment to stabilize nonminimum energy conformations in metastable states. Here it is shown that gM has the same minimum energy conformation in the organic solvent environment and in the bilayer environment, but gA has very different conformations in these two environments. So although it is generally recognized that the amino acid sequence dictates protein and polypeptide structures, it should be recognized that changes in the molecular environment may influence the peptide or protein structure. This suggests that mechanisms for membrane protein insertion must consider the influence of a changing environment on protein structure and that once in the bilayer environment, change in structure will be difficult to achieve without access to protic solvents.

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