Structure and Dynamics of a Membrane Bound Polypeptide

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INTRODUCTION:

Orientational constraints can be used to build-up three dimensional structures of biological macromolecules in much the same way that distance constraints are used today. In an anisotropic environment where molecular motions do not average NMR signals to their isotropic average the resonant frequencies become orientation dependent. Consequently, chemical shift frequencies, dipolar couplings, and quadrupolar interactions are all dependent on the specific orientation of the interaction tensor with respect to the magnetic field. In unoriented samples this results in broad spectral lineshapes, which are useful in their own right, but if the samples are aligned so that all molecules have the same orientation with respect to the magnetic field, then sharp line spectra can be obtained that reflect the orientation dependence of the spin interactions.

To interpret these frequencies for orientational constraints two pieces of information are critical. The first is a refined knowledge of the static tensor element magnitudes and orientation with respect to the molecular frame. The tensor element magnitudes represent the unique frequencies of the orthogonal axes of the interaction ellipsoid. In other words, the magnitudes represent the resonant frequency when the individual axes are aligned with the magnetic field. These values can, in many cases, be readily obtained from spectra of unoriented samples. The second critical characterization is a knowledge of the molecular motions that result in averaging both the tensor element magnitudes and orientation. Consequently, a detailed picture of dynamics is needed to characterize the axis about which motions are occurring, the amplitude of the motions and whether the motions are continuous or discontinuous, such as a flip of an aromatic ring. In fact, it is this separation of dynamics and structure that represents the most difficult challenge for the biological solid state NMR spectroscopist. By using low temperature (120 K) experiments, torsional motions are essentially eliminated except in specific cases such as methyl group three site jumps. Once both the static tensors and motional averaging of the tensors are characterized it is possible to achieve very high resolution quantitative constraints. Here we present both an overview of the structural constraints and the dynamic characterizations achieved for the channel forming polypeptide, gramicidin A in a fully hydrated lipid bilayer.

Gramicidin A is a hydrophobic polypeptide of 15 amino acid residues with both end groups blocked so that there are no formal charges. As a dimer it forms a helical channel with a 4Å pore that accommodates a single file of water molecules and cations that are stripped of all but two waters in the primary hydration sphere. The peptide linkages that line the channel are thought to help solvate the cations during transport by rotating the carbonyl oxygens toward the channel axis. While crystal structures of gramicidin A have been achieved in organic solvents (Langs, 1988; Wallace & Ravikumar, 1988; Langs et al., 1991) no crystal structure of the channel conformation has been achieved. However, much is known about the channel conformation. The backbone folding motif is a β-sheet type of structure that has been wound into a helix (Urry, 1971). This is possible because the amino acids of gramicidin alternate in stereochemistry between D and L configurations. The helix sense has been determined from the orientational constraints of the ^{15}N amide sites (Nicholson & Cross, 1989). Recently, the first backbone torsion angles for the channel conformation have been determined from orientational constraints (Teng et al., 1991). Complementing this work

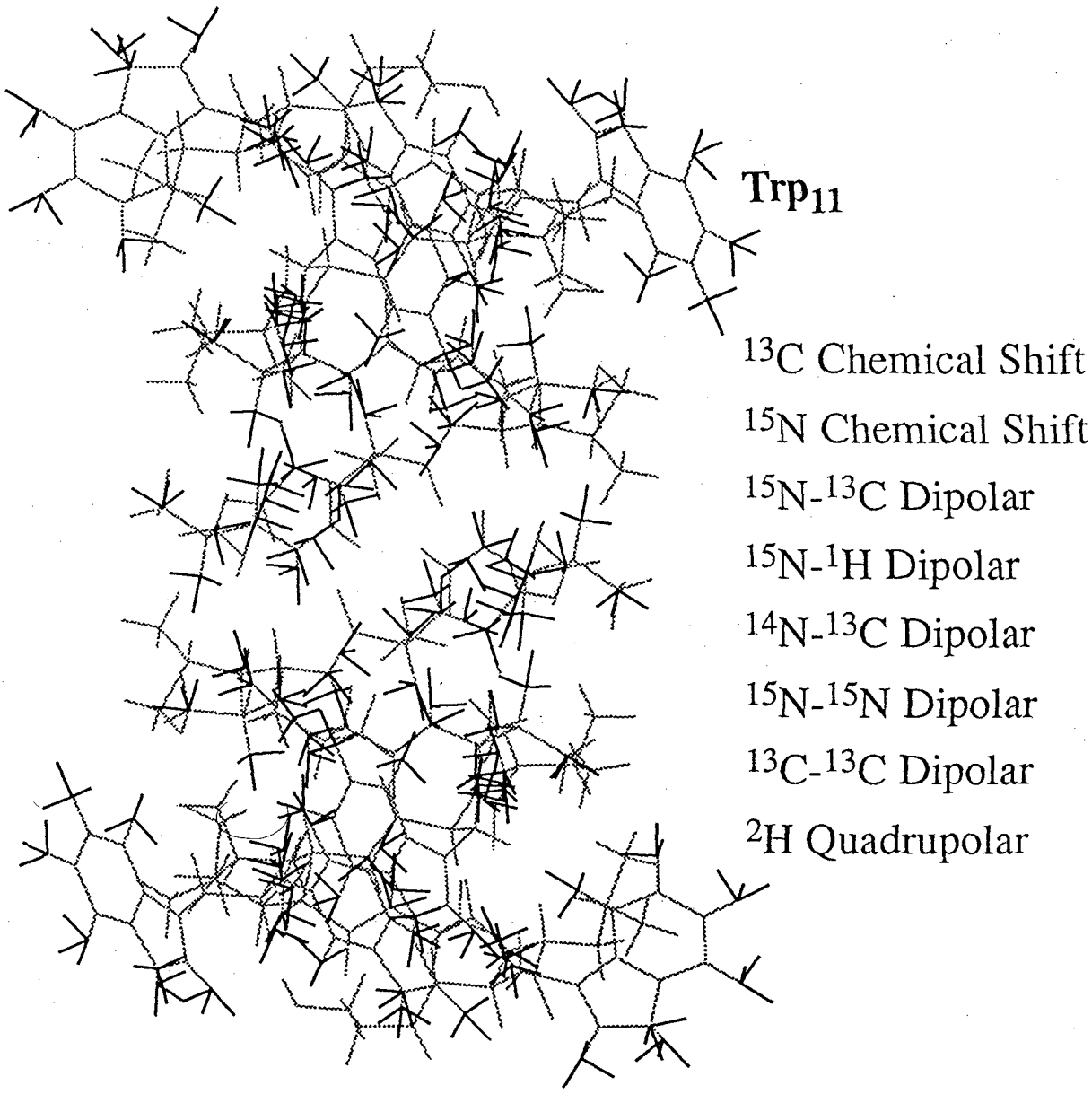

Figure 1: A model of the gramicidin channel dimer showing the interaction tensors that have been studied. The model structure is that of Lomize et al., 1992. Each tensor is represented by a orthogonal set of three unit vectors that have been oriented correctly for the specific site of interest. All backbone and tryptophan indole ^{15}N chemical shift and ^{15}N-^{1}H dipolar tensors have been studied with the exception of the ethanolamine blocking group. About half of the ^{15}N-$^{13}C_{\alpha}$ dipolar interactions in the backbone have been studied, four of the backbone carbonyl ^{13}C chemical shift tensors and in so doing the ^{14}N electric field gradient tensor has been characterized. Four sidechains have been deuterated and the quadrupole splittings obtained. ^{15}N spin diffusion has been observed between selectively labeled sites. To achieve this data approximately 50 separate syntheses of gramicidin A have been performed (Fields et al., 1989).

are solution NMR studies of gramicidin in SDS micelles that have also shown the same backbone folding motif (Arseniev & Barsukov, 1986; Lomize et al., 1992).

RESULTS & DISCUSSION:

Fig. 1 illustrates the large number of nuclear spin interactions that have been studied in gramicidin. For each interaction the tensor represented by an orthogonal set of unit vectors is placed on each atomic site that has been studied. More than 40 different isotopically labeled (Fields et al., 1989) gramicidins have been synthesized and more than 100 different structural constraints have been developed. The structural task is one of determining the torsion angles. If it is assumed that the peptide linkages are planar then there are two backbone torsion angles for each amino acid residue, one for valine sidechains and two each for leucine and tryptophan sidechains. There are two additional torsion angles in the ethanolamine blocking

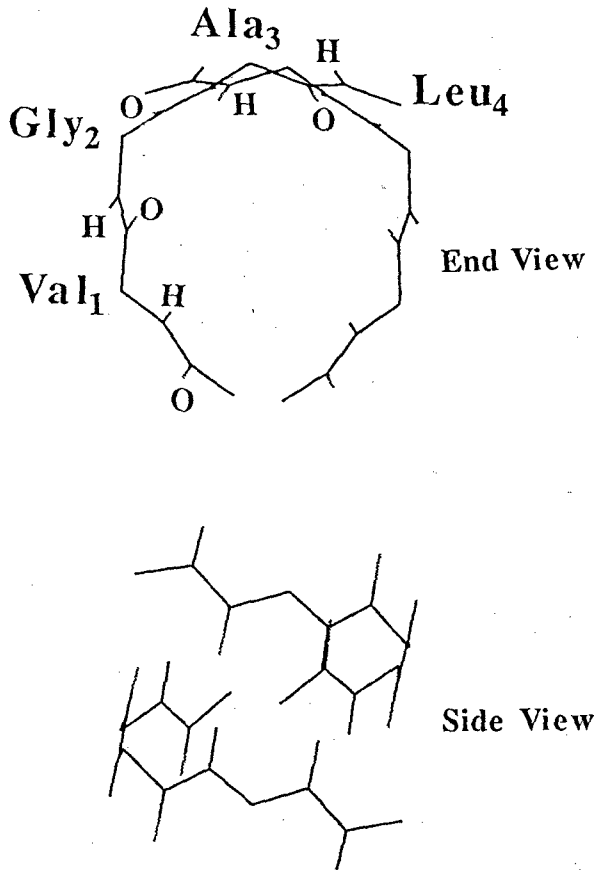

Figure 2: A partial structure for the gramicidin channel dimer, experimentally derived. The N-terminal four peptide planes have been oriented with respect to the bilayer normal through a combination of ^{15}N-^{1}H, ^{15}N-$^{13}C_1$ dipolar and ^{15}N chemical shift interactions in uniformly aligned bilayer samples. The experimentally verified symmetry of the two monomers has permitted the docking of these two partial structures to form a turn of the helix that supports previous models with 6.3 residues per turn of the channel helix. The amino acid residues have been identified for one of the monomers and the amide protons and oxygens labeled.

group of the carboxy terminus. Therefore, the structural problem comes down to finding the solution for 52 torsion angles.

Fig. 2 shows the N-terminal / N-terminal backbone junction for the channel as determined by orientational constraints. For each peptide linkage plane the ^{15}N - ^{1}H and ^{15}N - $^{13}C_1$ dipolar interactions, which have their unique static tensor elements directed along the internuclear vectors, were obtained. These two vectors define the orientation of the plane with some ambiguity that is minimized by an interpretation of the ^{15}N chemical shift for each plane. The orientation of the chemical shift tensor elements with respect to the molecular frame has been determined for each of these ^{15}N sites (Teng & Cross, 1989; W. Mai, W. Hu, C. Wang and T.A. Cross- unpublished results).

This structure clearly shows the right-handed helical sense and the β-helical class of torsion angles, in that the orientation of adjacent peptide planes alternates between parallel and antiparallel with respect to the channel axis. Furthermore, like many of the computationally refined structures (e.g. Roux and Karplus, 1988; Chiu et al., 1991) and unlike the original structural model (Urry, 1971) many of the carbonyl oxygens are rotated in toward the channel axis. The prime exception to this observation is the formyl oxygen of the amino terminal blocking group. Here we suspect that this is an artifact of the assumption that this peptide linkage is planar. Preliminary evidence suggests that the ω torsion angle for this plane is far from 180°, the value for normal trans-planar linkages.

Fig. 3 shows that we have also studied the sidechains of gramicidin A. In (A) the chemical shift powder pattern of a dry powder sample of ^{15}N indole labeled tryptophan is shown. The chemical shift tensor elements agree fairly well with those from the dry powder sample of gramicidin shown in (B). However the tensor elements for the fast frozen hydrated lipid bilayer preparation of gramicidin is very different. Both σ_{22} and σ_{33} tensor elements differ by 10 ppm from the dry samples. This sample was frozen in liquid propane to avoid distortions in the lipid bilayer which occur when a bilayer preparation is slowly cooled through its phase transition temperature. The sample was then transferred to liquid nitrogen and from there into our low temperature NMR probe. It is unlikely that the former two samples have the nitrogen bound hydrogen involved in a hydrogen bond. However, there is some electrophysiological evidence that these hydrogens for each of the tryptophan rings in the gramicidin channel state are hydrogen bonded to the carbonyl oxygens of the ester linked lipids. Consequently, it may be that the substantial changes seen in the chemical shift tensor element magnitudes reflect hydrogen bonding of the indole group. Further indirect evidence, given below, for hydrogen bonding comes from the orientation of the indole N-H with

respect to the bilayer surface.

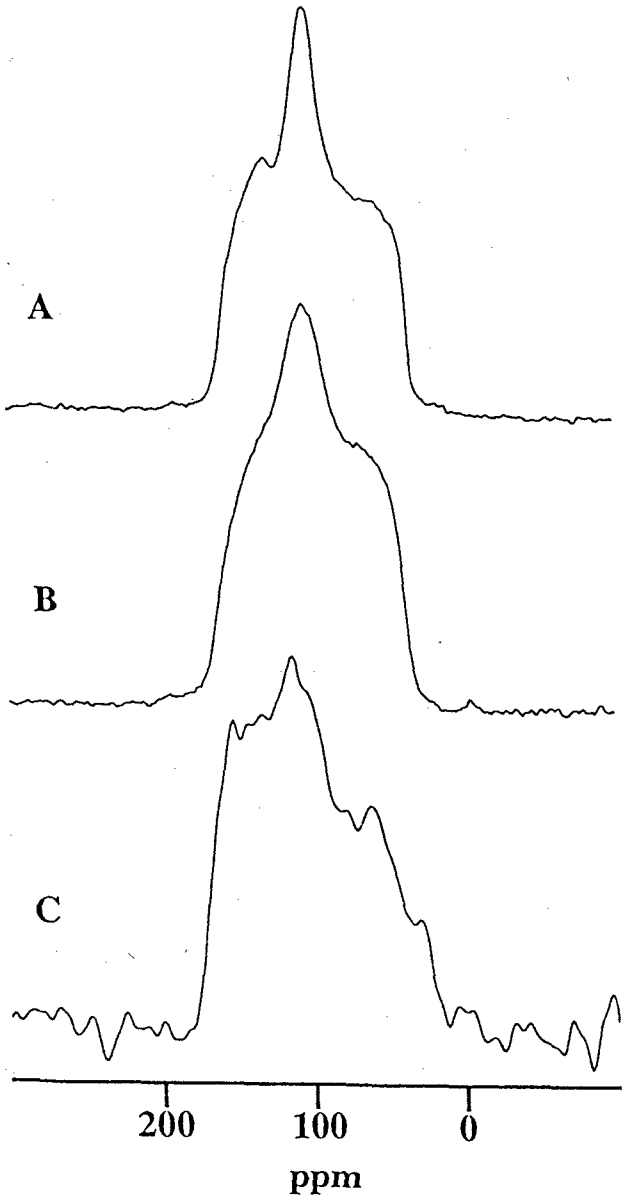

Figure 3: Powder pattern spectra of ^{15}N labeled indole. The spectra were obtained at 40 MHz for ^{15}N on a spectrometer that has been home built around a Chemagnetics data acquisition system and an Oxford 400/89 superconducting magnet. A] Dry powder sample of the amino acid, tryptophan obtained at room temperature. Spectral simulation yields tensor elements: $\sigma_{11} = 35$, $\sigma_{22} = 104$, and $\sigma_{33} = 158$ ppm. B] Dry powder of ^{15}N-Trp_9 gramicidin A: $\sigma_{11} = 36$, $\sigma_{22} = 106$, and $\sigma_{33} = 161$ ppm. C] Fast frozen sample of ^{15}N-Trp_9 gramicidin A in fully hydrated lipid bilayers: $\sigma_{11} = 36$, $\sigma_{22} = 116$, and $\sigma_{33} = 171$ ppm.

The ^2H quadrupolar spectrum (Fig. 4) of d_5-Trp_{11}-gA provides an example of the spectroscopic data that has been used for structural constraints. The arrows point to a linewidth of 1.4 kHz that represents an uncertainty for the orientation of ±0.2°. Not only does this spectrum describe a single conformation for this tryptophan ring, but it also dictates that the orientation for all of the gramicidin molecules in the sample is remarkably uniform (Moll & Cross, 1990). For this sample there are five deuterons on the tryptophan ring and five quadrupole splittings are observed. The analysis of this data combined with the ^{15}N chemical shift and ^{15}N-^1H dipolar interaction for the indole nitrogen (W. Hu, K.-C. Lee and T.A. Cross - unpublished results) yields two possible orientations for this ring. Each of these structures has the same orientation with respect to the channel axis and the orientation is similar to that shown in Fig. 1 for Trp_{11}, where the N-H points towards the bilayer surface. In recent computational (Meulendijks et al., 1989) and electrophysiological (O'Connell et al., 1990) studies it has been suggested that the indoles of gramicidin A are hydrogen bonded to the carbonyl oxygens of the ester-linked lipids. These results indicate that such hydrogen bonding may be present and this may be one of the prime reasons why this conformation is present in lipid bilayers rather than the double-helical structures that dominate organic solutions (Veatch and Blout, 1974; Zhang et al., 1992). Furthermore, the tryptophan dipole moment is oriented primarily along the channel axis, rather than radial to this axis. This has significant implications for cation transport by gramicidin A.

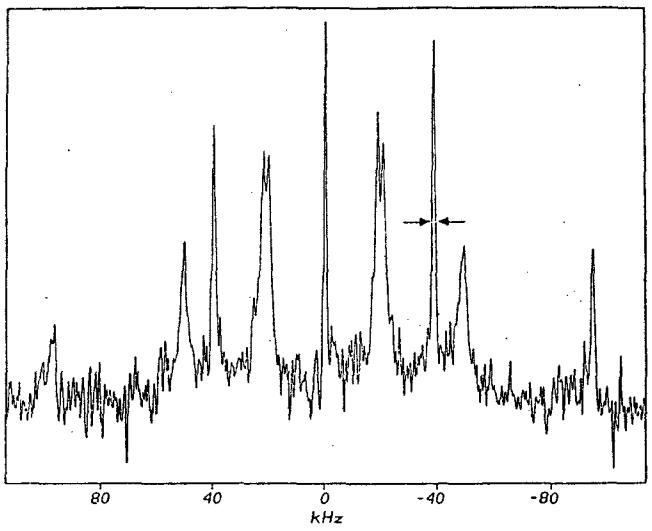

Figure 4: ^2H NMR spectrum of d_5-Trp_{11} gramicidin A in an oriented lipid bilayer preparation. The arrows indicate a resonance linewidth of 1.4 kHz. The assignment of two of these quadrupole splittings is clear from a knowledge of other spin interactions in this ring system: $\zeta_3 = 192$; and $\eta_2 = 99$ kHz.

Detailed dynamics have come from a combination of lineshape simulation and relaxation studies. Below the gel to liquid crystalline phase transition temperature of the bilayers all large amplitude motions cease. Both the global motion about the bilayer normal and the local backbone

motions become slower than the chemical shift frequency scale (Nicholson et al., 1989; 1991). This is because the backbone motions are dependent upon motion of the C_α-C_β axis and hence, if the sidechain conformations are frozen in the gel phase the backbone motions will be greatly impeded. When oriented samples are lowered through the phase transition temperature the broadened resonance represents a considerable orientational dispersion. Instead of the backbone having a single conformation in this static environment a range of conformational substates has been trapped (Frauenfelder et al., 1988; Nicholson et al., 1989). This set of substates represents the range of orientations over which local motions occur above the phase transition temperature. From the observed lineshape it has been possible to determine the orientation of the axis about which the local backbone motions occur with respect to the magnetic field (Nicholson et al., 1991). This axis has been shown to be coincident with the C_α-C_α axis for each peptide linkage. Since the number of conformational substates is greater than three, the local motion can be modeled as a diffusion within a gaussian well. Furthermore, the amplitude of the motion could be estimated.

Once such a motional model has been experimentally developed for a specific backbone site in the gramicidin channel conformation it is possible to interpret relaxation data for these same ^{15}N sites. T_1 relaxation times were obtained from oriented samples of fully hydrated lipid bilayers. To fix the frequency of the local motions it has been necessary to measure the relaxation times at two different field strengths. In Fig. 5 an analysis of such data is shown. The global correlation time (τ_p) and the local motion correlation time (τ_i) are variables as well as the amplitude of the local motion. However, we have a previous estimate of the motional amplitude (±15°, Nicholson et al., 1991) as well as the global correlation time (200 ns, Seelig and Macdonald, 1987). Consequently, it is possible to achieve a unique solution for the local motion correlation time (10 ns). A similar correlation time has been reported for an even numbered site (Leu_4) in the gramicidin channel (North and Cross, 1992)

This is a remarkably slow correlation time for a motion of a molecular group with nominally such a small molecular weight. Roux and Karplus (1988) have analyzed the normal modes in the gramicidin channel and concluded that fluctuations occur with frequencies of 4.6 to 20 cm^{-1} corresponding to harmonic oscillator periods of 2 to 8 ps, approximately, a factor of 10^3-10^4 slower than the determination reported here. Similarly, estimates from molecular dynamics indicate frequencies for these local motions that are in a similar range to those of the normal mode analysis.

The experimental evidence presented here is not unique for slow motions in polypeptide backbones, in fact most of the experimental evidence suggests much slower motions than the computational techniques. Analysis of T_1 and NOEs from the backbone of the filamentous virus fd suggested correlation times of 1 ns (Cross and Opella, 1982). A similar timescale has been reported for collagen

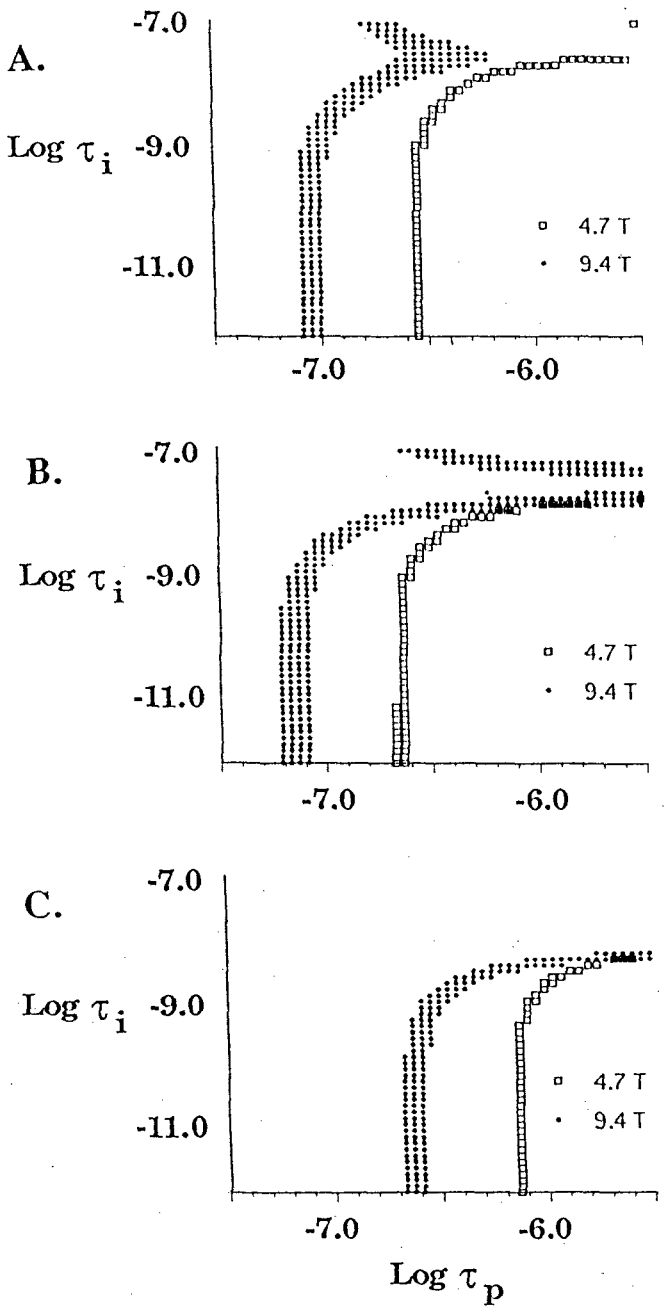

Figure 5: T_1 relaxation times that were obtained from ^{15}N-Gly_2 gramicidin A in oriented lipid bilayers at 20 and 40 MHz have been analyzed to achieve a solution for local and global correlation times as well as the local motional amplitude. A] calculated for 12° rms deviation; B] 15°; and C] 18°. Because of previous estimates for the amplitude and global correlation time, it is possible to determine the local correlation time as 10 ns with an rms amplitude of 15°.

(Sarkar et al., 1985). More recently, Cole and Torchia (1991) have reported local motional frequencies in the backbone of crystalline staphyloccal nuclease in the ns or near-ns timescale.

For the gramicidin channel there appear to be two primary reasons for the discrepancy between the computational and experimental frequencies. First, it maybe as suggested by Venkatachalam and Urry (1984) that the local motions are correlated along the polypeptide backbone. Computational studies have argued that the extent of such correlations are limited to nearest neighbors. The second reason is that the lipid environment may damp the backbone motions severely. Evidence has already been presented that the lipid environment can reduce the motional frequencies below the kHz range when the lipids are in the gel phase. Above the phase transition temperature it is well known that the lipid environment damps global motional frequencies. For instance, the 200 ns global correlation time for gramicidin (1880 daltons) would be consistent with a protein of 50,000 daltons or greater in aqueous solution. Furthermore, evidence of specific gramicidin - lipid interactions described here suggests an additional mechanism for damping by the lipid environment.

The transit time for cations to move between dipeptide carbonyl sites in the polypeptide backbone can be estimated from kinetic measurements (Anderson, 1983) and analyses of the energetics (Roux and Karplus, 1991) to be in the range of 10 ns. Consequently, it is now likely that there is a correlation between kinetics and the local dynamics of the polypeptide backbone. This unique correlation between structure, dynamics and function emphasizes the importance of pursuing such detailed studies of polypeptides and proteins in functional native-like environments.

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