Experimental Observation of Orientational Dispersion in the Peptide Backbone of the Gramicidin Cation Channel

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An approach for studying the structure and dynamics of proteins and other biological macromolecules in which uniaxial orientation can be achieved is presented here. Solid-state ^{15}N NMR has been used in this study to investigate specific sites in the peptide backbone of the gramicidin A transmembrane channel incorporated into uniformly oriented dimyristoylphosphatidylcholine (DMPC) bilayers. The temperature dependence of spectra obtained with ^{15}N-Gly_2-gramicidin and ^{15}N-Val_7-gramicidin shows an orientational dispersion in the polypeptide backbone for the two sites. These results represent the first experimental support for a motional model of peptide linkage fluctuations that is thought to be important for the cation transport activity of the gramicidin channel.

Gramicidin A, a highly hydrophobic polypeptide consisting of 15 alternating L and D amino acids (see Figure 1), forms, as a dimer, a helical cation selective channel when incorporated into lipid bilayers. The generally accepted structural model of the channel[1] is stabilized by β-type hydrogen bonding between the polar peptide linkages that form the channel walls. In this model, the peptide linkages containing the Val_7 and Gly_2 amide groups are each stabilized by two hydrogen bonds. Val_7 is located near the center of each monomer, while the Gly_2 site is located at the monomer-monomer junction. Despite decades of research, an atomic resolution determination of the channel structure and dynamics in a lipid environment has not been achieved and consequently a detailed mechanism of ion transport has not been elucidated. For a given peptide linkage a range of carbonyl orientations has been theoretically predicted[2-4] that would allow the carbonyl groups to swing into the channel lumen to solvate the largely dehydrated cation during ion transport.

A wide variety of solid-state NMR techniques have been used to obtain structural and dynamic information from proteins,[5-8] and specifically from gramicidin.[9-12] The solid-state ^{15}N NMR experiments reported here take advantage of the orientational dependence of the ^{15}N chemical shift interaction with respect to the magnetic field. This interaction is represented by a 2nd rank tensor that is fixed in the molecular frame. For samples in which molecules are uniaxially aligned, the frequency of the observed narrow resonance reflects the relative orientation of the molecule with respect to the field. Both global and internal motions of the molecule result in averaging the chemical shift tensor and can greatly affect the observed resonance. It is well established that the gramicidin channel undergoes global rotation about an axis parallel to the bilayer normal.[9,13,14] The frequency of this rotational diffusion is fast on the NMR time scale at temperatures above the gel-to-liquid crystalline phase transition of the sample (centered at 28 °C) and slow when the temperature is below the onset of the phase transition (13 °C).[9] For uniformly aligned samples positioned in the magnet such that the axis of global rotation is parallel to the field, cessation of channel rotation by lowering the sample temperature should not change the observed spectrum. Although rotational averaging no longer occurs, the component of the chemical shift tensor along the field should remain the same, and neither the frequency nor the line width of the observed resonance should be affected. Consequently, a situation can be created in which the averaging effects by internal motions on the chemical shift spectrum can be assessed directly.

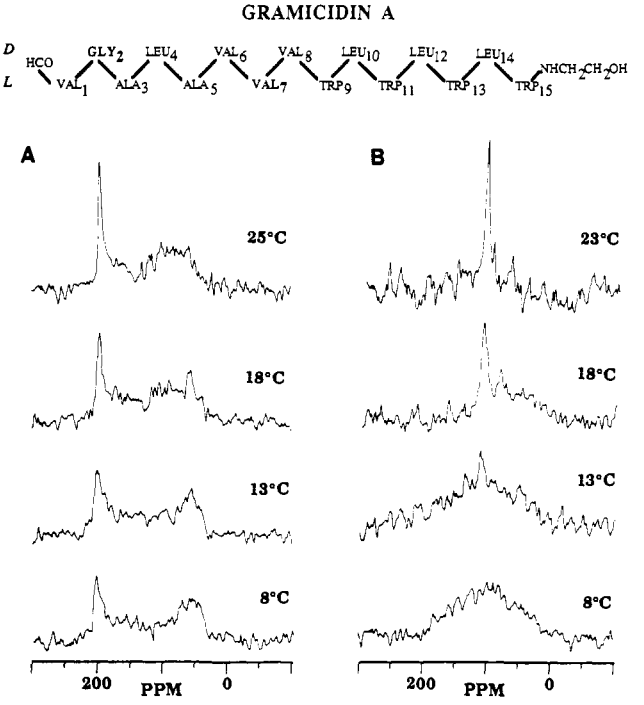

Figure 1. The temperature dependence of ^{15}N NMR spectra of oriented samples prepared as previously described[9,17] which contain gramicidin and DMPC in a 1:8 molar ratio with 60% hydration are shown. Samples were aligned with the bilayer normal parallel to the magnetic field. Experimental conditions were as previously reported using cross polarization and proton decoupling in a 4.7 T field.[9] The amino acid sequence of gramicidin A is also shown emphasizing the location of Val_7 and Gly_2. (A) ^{15}N-Val_7-gramicidin. This sample contains an underlying powder pattern that arises from defect structures and from sample that has seeped from between the glass plates. (B) ^{15}N-Gly_2-gramicidin.

Shown in Figure 1 is the temperature dependence of the ^{15}N resonances from samples of ^{15}N-Gly_2-gramicidin and ^{15}N-Val_7-gramicidin in oriented lipid bilayers. The ^{15}N chemical shift spectra of these gramicidins in oriented samples recorded at 23 °C show single sharp resonances for each labeled site. The spectrum of the Gly_2 site recorded at 8 °C shows a large dispersion of chemical shift frequencies. Since the gaussian broadening of ^{15}N-Gly_2-gramicidin powder pattern discontinuities is temperature insensitive, T_2 is not responsible for the broad resonance at low temperature presented here. Consequently, the large dispersion of frequencies observed in the inhomogeneously broadened resonance band at low temperature represents a dispersion of orientations for the Gly_2 site. A less dramatic broadening is observed at the Val_7 site when the temperature is lowered through the phase transition. Because chemical shift frequency and orientation with respect to the field are not linearly related, the very different frequency dispersions for the two sites do not necessarily imply very different amounts of orientational dispersion. A determination of the extent of orientational dispersion for a given site is dependent

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upon the orientation of the chemical shift tensor with respect to the molecular frame, and on the mean orientation with respect to the field of both the peptide plane containing the site and the axis about which the dispersion occurs. The data in Figure 1 support a qualitative interpretation for an orientational distribution about the C_α-C_α axis of ±10 to 20° for both of the isotopically labeled peptide linkages.

Several possible explanations for these observed results can be considered. Substantial tilting of the channel axis away from the magnetic field caused by distortions of the bilayer surface upon cooling through the phase transition is unlikely. Such distortions are anticipated to be small and consequently could not account for the broad dispersion of chemical shift frequencies observed at the Gly_2 site at low temperatures. Another possible explanation is that significant structural changes of the polypeptide backbone have occurred upon lowering the temperature. This would be expected to have a profound effect on channel conductance, but the gramicidin dimer is an effective ion conducting channel below the phase transition temperature.[15] The most probable explanation for the observation of the orientational dispersion at low temperature is that the peptide linkages are trapped in local potential energy minima. These minima represent conformational substates that have the same overall structure of the gramicidin channel but differ only in subtle structural details including the orientation of the isotopically labeled site with respect to the channel axis. This interpretation requires that the transition rate from one conformational substate to another is much faster than the NMR time scale (3.5 kHz) when the sample is at 23 °C, resulting in a narrow motionally averaged chemical shift resonance. At 8 °C, the transition rate is slower than the NMR time scale, allowing the chemical shift spectrum to reflect the orientation of each conformational substate. Such a change in transition rate could be based on differences in the environment for the amino acid side chains as the phase of the sample is changed.[16] Finally, it should be noted that a continuous motion of a peptide plane within a smooth and shallow potential energy well is unlikely, since, for such a model, a decrease in frequency requires a dramatic increase in amplitude, which is unreasonable considering the dynamic behavior of the lipid at low temperature.

A large number of experiments and computational studies have implicated the existence of conformational substates in protein systems.[18,19] For the gramicidin channel, conductance studies have detected functional substates,[16,20] suggesting that a variety of similar channel conformations may exist. Despite maximal hydrogen bonding in the model structure of the peptide backbone of gramicidin, computations have indicated that the backbone is very flexible[2-4] and the existence of helically librated states has been suggested.[2] From the experimental results presented here, the computationally predicted flexibility is due to transitions over a wide range of conformational substates. For the first time in the study of gramicidin, direct experimental evidence for these substates has been achieved. Moreover, solid-state NMR has for the first time been used to provide evidence for conformational substates. These ^{15}N chemical shift spectra of uniformly aligned samples at low temperature have provided a window through which to view the orientational dispersion of conformational substates, opening an exciting avenue for obtaining a detailed spatial description of the internal motions not only of the gramicidin channel but also of other bilayer bound systems.

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Synthesis of a Bacteriophage DNA Containing a Site-Specific Cis-Syn Thymine Dimer

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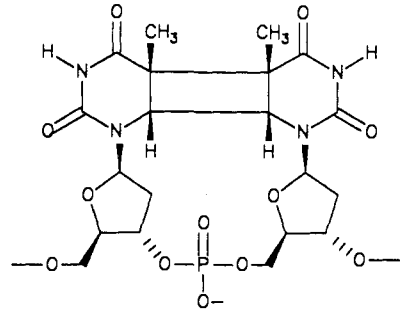
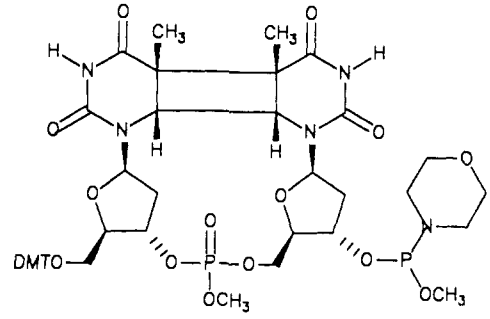
The cis-syn thymine dimer is the major sunlight-induced photoproduct of DNA[2] whose production has been linked with mutations and skin cancer.[3] The lethality, mutagenicity, and mutation spectrum of the cis-syn thymine dimer as a function of its location in a genome is unknown.[4] Attempts to derive such structure-activity relationships have been hampered by the lack of general methods for the preparation of DNA containing site-specific cis-syn thymine dimers for study. Recently, we reported the synthesis of the building block **1** for the site-specific incorporation of cis-syn thymine dimers into oligonucleotides by solid phase DNA synthesis technology.[5] Herein we report the synthesis and characterization of a bacteriophage DNA containing a site-specific cis-syn thymine dimer suitable for in vivo repair, replication, and mutagenesis studies.



cis-syn



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