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Solid-state ^{15}N-NMR evidence that gramicidin A can adopt two different backbone conformations in dimyristoylphosphatidylcholine model membrane preparations

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Using [^{15}N-Val$_7$]gramicidin A it is shown by solid state ^{15}N-NMR that in dimyristoylphosphatidylcholine model membrane preparations evidence is obtained for two different backbone conformations of gramicidin. One of these conformations is the familiar channel state while a second conformation possesses very different dynamic and structural characteristics. The relative amounts of the conformations depend upon the solvent used to initially codissolve peptide and lipid. Furthermore, by incubation of the samples at modestly elevated temperatures a conversion can be induced from the non-channel to the channel state in a lipid environment.

Protein–lipid interactions are generally assumed to play a decisive role in membrane structure and functioning [1,2]. In view of the complexity of biological membranes a fruitful approach to investigate such interactions and to obtain detailed structural information on both types of membrane constituents, is by the use of model membrane systems, in which a selected protein is incorporated. A polypeptide which has been widely used as a model for the hydrophobic part of intrinsic membrane proteins is the linear pentadecapeptide, gramicidin [3–5]. In model as well as biological membranes it forms cation selective transmembrane channels [6–8]. In its channel conformation gramicidin is generally considered to exist as an amino to amino terminal hydrogen bonded dimer in the single-stranded $\beta^{6.3}$ helical configuration [8–10]. Gramicidin also has an ability to modulate the phase behavior of membrane lipids. In bilayer forming lipids it induces hexagonal H_{II} phase formation when the lipid-acyl chain length exceeds 16 carbon atoms [5,11]; when mixed with lysophosphatidylcholines, which on their own prefer a micellar organization, bilayers are formed [12–14]. The conformation of the peptide is thought to play an essential role for gramicidin's ability to form transmembrane channels [15,16] and for its effect on lipid phase formation [17,18].

In organic solvents the conformation and aggregation state of gramicidin is highly dependent upon the specific solvent used, as shown by a variety of circular dichroism (CD) and solution

Abbreviations: CD, circular dichroism; CSA, chemical shift anisotropy; DMPC, dimyristoylphosphatidylcholine; DMSO, dimethylsulfoxide; NMR, nuclear magnetic resonance; PC, phosphatidylcholine.

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NMR studies. Even for a given solvent such as ethanol it has been demonstrated that four different, rapidly interconverting conformations of a gramicidin dimer are present, the dominant conformation being an antiparallel double helix [19,20]. In trifluoroethanol and dimethylsulfoxide (DMSO) gramicidin appears to be a structured monomer and the conformations in these two solvents may be similar [21,22]. A recent detailed ^{1}H-NMR study of gramicidin in DMSO has shown that in this solvent the predominant structure is that of the $\beta^{6.3}$ helix [23]. CD studies showed that when gramicidin was added to sonicated vesicles of phosphatidylcholine (PC) from an organic solution of either trifluoroethanol or DMSO, it directly incorporated in the channel state, but that a non-channel configuration was obtained when the polypeptide was added from an ethanolic solution [18,24]. Surprisingly, a similar solvent dependence of the conformational behavior of gramicidin was observed, when gramicidin/lipid dispersions were prepared by first cosolubilizing the polypeptide and lipid in an organic solvent, followed by removal of the solvent and hydration of the dry gramicidin/lipid film [25,26]. When trifluoroethanol was used, the channel configuration was obtained directly upon hydration, as determined by CD and ^{23}Na-NMR studies [25,26], but when ethanol was used to cosolubilize gramicidin and lipid the polypeptide initially was present in a different conformation, possibly an antiparallel double helix [25]. Upon heat incubation this latter conformation slowly converted to the channel configuration, which appeared to be the most thermodynamically stable conformation in a lipid environment. The rate of interconversion, but not the interconversion itself appeared to depend upon the temperature of incubation [25].

Although CD is a useful technique to monitor differences in conformation, unambiguous interpretation of CD data of gramicidin in terms of backbone structure is not yet possible, due to the major contribution of the tryptophan side chains to the CD spectrum [27]. Solid-state NMR is a technique well suited for the study of structural and dynamic properties of proteins and polypeptides in membranes [28–32]. The orientation-dependent nuclear spin interactions, such as the chemical shift interaction, provide detailed information for characterizing the structural and dynamical state or states of molecules which are not isotropically averaged. A solid state ^{15}N-NMR study is presented here on both randomly dispersed and oriented samples of site specific labeled gramicidin, incorporated into model membranes using trifluoroethanol and ethanol to codissolve the polypeptide and lipid. These solvent systems were chosen because their use resulted in the most distinctive conformational differences of the polypeptide in the membrane upon hydration as determined by CD [25].

[^{15}N-Val$_7$]Gramicidin A was prepared via solid phase synthesis as will be described elsewhere. A 1:8 molar ratio of polypeptide to lipid was used for all samples studied in this report. For the preparation of randomly dispersed samples approx. 75 mg of peptide was codissolved with 1,2-dimyristoyl-*sn*-3-glycerophosphocholine (DMPC from Avanti Biochemicals, Birmingham, AL), in either 1 ml of ethanol or 40 ml of trifluoroethanol. The different amounts of organic solvent were used to duplicate conditions in which extreme differences in CD spectra were observed [25]. After incubation for one hour at room temperature the solvent was removed on a rotary evaporator and the dry lipid/peptide film was hydrated with an amount of water equal to the sample weight. The hydrated sample was allowed to incubate for 1 h at room temperature. It was then centrifuged at $12000 \times g$ at 4°C for 15 min, after which the pellet was transferred to a small glass container ($6 \times 6 \times 12$ mm). ^{15}N-NMR measurements were carried out as described elsewhere [31]. Oriented samples were prepared using 10–15 mg of peptide and the appropriate amount of lipid. Samples were dissolved in 300 μl solvent and, after one hour incubation at 25°C, deposited in small quantities on glass microscope cover slips. The procedure was essentially as described previously [31], except that, in order to facilitate the hydration process, the lipid/peptide films were hydrated prior to stacking. On each glass plate approx. 5 μl water was deposited, followed by incubation for several hours at 25°C in an environment saturated with water. Next the samples were air dried for 10 min to remove excess water and then they were stacked. Finally the amount of hydration water was determined by weight and enough water was

added to equal the total sample dry weight. The alignment of the lipid bilayers was assayed with ^{31}P-NMR as described previously [31]. Methods of orientation which include long term incubation at elevated temperatures could not be used because of the concomittant conformational changes of gramicidin [25]. In all samples ^{31}P-NMR data were consistent with a pure bilayer organization of the lipids. No isotropic component was detected and a reduced chemical shift anisotropy was observed upon incorporation of the polypeptide, in agreement with earlier data [31].

The ^{15}N chemical shift powder pattern of [^{15}N-Val$_7$]gramicidin and DMPC using trifluoroethanol as the cosolubilizing agent is shown in Fig. 1A. This is the static spectrum of the Val$_7$ site obtained from a sample that has not been

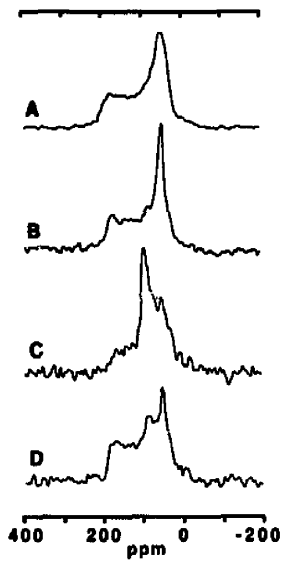

Fig. 1. ^{15}N-NMR powder patterns of [^{15}N-Val$_7$]gramicidin A/DMPC mixtures in a 1/8 molar ratio at 25°C. (A) Dry mixture, prepared using trifluoroethanol for cosolubilization, 16000 acquisitions. (B) 50% hydrated random dispersion, prepared using trifluoroethanol for cosolubilization, 22000 acquisitions. (C) 50% hydrated random dispersion, prepared using ethanol for cosolubilization, 18000 acquisitions. (D) Sample in (C) after incubation at 40°C for 3 days, 19000 acquisitions. Spectra were recorded on a modified IBM/Bruker WP200 SY spectrometer with a solids package, using cross polarization conditions: 5.0 μs 90° pulse, mixing time of 1 ms, and an increased ^{1}H decoupling field during acquisition of 2.0 mT. Other conditions include a sweep width of 62.5 kHz, a 16 ms preacquisition delay, and a 7 s recycle delay.

hydrated. Consequently, the principal elements of the chemical shift tensor can be determined from this spectrum as $\sigma_{11} = 34$, $\sigma_{22} = 55$ and $\sigma_{33} = 201$ ppm relative to external $^{15}NH_4NO_3$. Upon hydration the chemical shift tensor becomes motionally averaged resulting in an axially symmetric powder pattern with tensor elements of $\sigma_{\parallel} = 194$ and $\sigma_{\perp} = 50$ (Fig. 1B). These observations are consistent with previously published results from uniformly ^{15}N-labeled gramicidin [31]. When ethanol is used to codissolve the polypeptide and lipid a dramatically different powder pattern spectrum is obtained (Fig. 1C). The lineshape appears to consist of the superposition of two components, one axially symmetric component, similar to that observed in Fig. 1B, and one with a much reduced linewidth centered at 98 ppm, which is approximately the isotropic chemical shift. In principle, such a complex lineshape could be the result of motional averaging of the static chemical shift tensor if the motional frequency approximated the width of the interaction (3.4 kHz) [33]. That this is not the situation here is supported by the following observations. (1) Upon raising the temperature from 25°C to 40°C there is no significant change in the ^{15}N powder pattern spectrum. If the motional frequency was on the order of the interaction width then such a change in temperature should affect the motional frequency and lineshape significantly. (2) Following incubation at 40°C for 3 days the ^{15}N spectral lineshape obtained at 25°C was converted to a spectrum (Fig. 1D) similar to that shown in Fig. 1B. These observations suggest that the spectrum, shown in Fig. 1C, is composed of two separate components that reflect two different situations for the Val$_7$ site in gramicidin. One situation is the same (by ^{15}N-NMR) as that observed in the trifluoroethanol preparation which is considered to be the channel conformation.

For a single nitrogen site in molecules uniformly aligned with respect to the magnetic field the ^{15}N-NMR powder pattern spectrum is reduced to a single sharp resonance reflecting the orientation of the chemical shift tensor relative to the field. When trifluoroethanol is used as the cosolubilizing solvent such a sharp resonance is observed at 194 ppm (Fig. 2B). There is also a significant amount of powder pattern intensity in

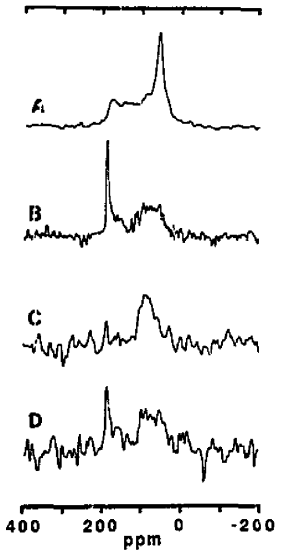

Fig. 2. ^{15}N-NMR spectra of 50% hydrated DMPC bilayers containing [^{15}N-Val$_7$]gramicidin A at 25°C. (A) Unoriented sample, as in Fig. 1B. (B) Oriented sample prepared using trifluoroethanol for cosolubilization, 24000 acquisitions. (C) Oriented sample prepared using ethanol for cosolubilization, 24000 acquisitions. (D) Sample used for (C) after incubation at 40°C for 7 days, 21000 acquisitions. Spectra were recorded as described in Fig. 1. Spectra of oriented samples were obtained with the bilayer normal aligned parallel to the static magnetic field.

this spectrum resulting from unoriented sample that has seeped from between the glass cover slips due to the high hydration level used. Since the frequency of the oriented resonance corresponds exactly with the value of $\sigma_{\parallel}$ in Fig. 2A the axis of rotation for gramicidin in the bilayer is parallel to the bilayer normal, consistent with an earlier determination [34]. Assuming collinearity of the chemical shift (CSA) tensor and the molecular symmetry axis frame, an angle of 11° between the ^{15}N-H bond and the bilayer normal is indicated by these results. When an oriented sample is prepared from ethanol, two spectral components are again observed in the ^{15}N-NMR spectrum (Fig. 2C). One is a low intensity narrow resonance at 194 ppm similar to that in Fig. 2B. Most of the intensity is centered in a much broader resonance at 98 ppm which has essentially the same width and center frequency as one of the components in the spectrum obtained from the randomly dispersed sample (Fig. 1C). A similar resonance at much lower intensity is observed in the oriented sample of the trifluoroethanol preparation (Fig. 2B). Both of the oriented samples showed the same high degree of orientation when characterized by ^{31}P-NMR. The relative amount of the two components in the oriented and the randomly dispersed samples prepared from ethanol is different. This is probably due to minor differences in sample preparation such as the rate of organic solvent removal [25]. The symmetric distribution of intensity of this second component about the isotropic frequency in the oriented preparation suggests that the Val$_7$ site has no preferred orientation with respect to the magnetic field and the lipid bilayer. Upon heating the oriented sample for 7 days at 40°C the intensity of the second component decreased and an increased intensity was observed for the peak at 194 ppm (Fig. 2D) similar to the behavior of the unoriented sample. Apparently, it requires very little thermal energy to convert this other conformation to one which is consistent with the channel state and while there is a change in the gramicidin conformation, no similar change occurs for the lipids upon incubation at 40°C. ^{31}P-NMR spectra recorded before and after the incubation at 40°C showed no change in the degree of alignment of the bilayers or in the averaging of the ^{31}P chemical shift tensor which is a very sensitive measure of the amount of gramicidin incorporated in the bilayer [35].

Upon preparation of samples with trifluoroethanol as the cosolubilizing agent gramicidin primarily adopts the channel conformation directly after removal of the solvent and hydration of the dry film. In this conformation gramicidin is oriented with the rotation axis parallel with the bilayer normal and the N-H bond of the Val$_7$ site has a well defined orientation with respect to the bilayer. When samples are prepared from ethanol two spectral components are observed prior to heating the sample for an extensive period of time. One spectral component represents the channel conformation and the other component has very different spectral features. The ^{15}N chemical shift anisotropy is much more extensively averaged as seen in the powder pattern spectra. There is no evidence that the Val$_7$ site has a preferred orientation with respect to the lipid bilayers. These features in conjunction with a knowledge that the

gramicidin in both preparations is incorporated into the lipid bilayers forces a conclusion that this second spectral component must arise from gramicidin that is structurally and dynamically different from the channel conformation. The structure and dynamic properties of this component are not completely resolvable with the data available. However, it is clear that neither a pure structural change (e.g. change of N-H orientation with respect to the bilayer) nor a purely dynamic change (e.g. increased local motion amplitude) will account for the spectral results. From a structural point of view there are multiple N-H orientations observed, but this does not fully explain the reduced powder pattern width. From a dynamics point of view this component clearly shows an increased averaging of the powder pattern, but this does not account for the broad linewidth in the oriented spectrum. Furthermore, it is not possible to account for the dynamic observations by simply considering global motions of the gramicidin molecule within the lipid bilayer. This would require nearly isotropic tumbling of the gramicidin molecule which is not substantiated by either the lipid dynamics or dynamic modeling. Therefore, these results suggest substantial differences in local dynamics, which are predicated on local conformational changes in the molecule, such as hydrogen bonding rearrangements. Consequently, it is reasonable to consider that the second gramicidin component has both a different gramicidin backbone structure and different dynamics in the vicinity of the Val_7 site.

The results presented here are consistent with the CD data of vesicularized systems [25] and with ^{15}N-NMR experiments utilizing uniformly ^{15}N-labeled gramicidin [26,31]. They clearly indicate two very different sets of characteristics for gramicidin in a phospholipid preparation. Furthermore, the non-channel conformation can be converted to the channel conformation upon modest heating. These unique observations illustrate the usefulness of solid state NMR for obtaining information on the structural and dynamic properties of polypeptides and proteins in membranes.

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