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Solid-state ^{13}C-NMR studies of the effects of sodium ions on the gramicidin A ion channel

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End-to-end helical dimers of gramicidin A form transmembrane pores in lipid bilayers, through which monovalent ions may pass. The groups within the peptide that interact with these ions have been studied by application of solid-state spectroscopic methods to a series of gramicidin A analogues synthesized with ^{13}C in selected peptide carbonyl groups. The resonances of D-Leu^{10}, D-Leu^{12} and D-Leu^{14} analogues were perturbed in the presence of 0.16 M sodium ions, whereas the resonances of the carbonyls of Gly^{2}, Ala^{3}, D-Leu^{4} and Val^{7}, which are closer to the formylated N-terminal end of the peptide, were unaffected. The observed changes in chemical shift anisotropy are indicative of a change in orientation of the abovementioned leucine carbonyls.

Introduction

Gramicidin A, a pentadecapeptide, forms pores in lipid bilayers. Monovalent ions pass through the pore at rates which are inversely related to their diameter, whereas divalent ions bind to the peptide, blocking the channel [1–3]. Numerous studies have led to the conclusion that the channels are formed by end-to-end association of two $\beta^{6.3}$ single helices, which possess a 4 Å diameter solvent-filled lumen (see for example Refs. 4–6). Some controversy remains however over the handedness of the helices; Urry and his colleagues have presented evidence for formation of left-handed helices, whereas Arseniev et al. [7] using 2D NMR spectroscopy on gramicidin A packaged in SDS micelles, have concluded that the peptide is right-handed. This conclusion is supported by recent solid-state NMR studies [8,9].

Experimental and theoretical studies of the mechanism of ion transport through the peptide channel have led to the conclusion that the head-to-head dimer possesses two ion binding sites located close to the channel mouths and separated from each other by about 20 Å [4–6]. These locations have been defined by shifts in the carbonyl ^{13}C resonances of Trp^{9}, Trp^{11}, Trp^{13}, Trp^{15} and D-Leu^{14} residues in the presence of Tl^+ and Na^+ ions [10–14]: by contrast, there were no ion-induced shifts in the formyl, Val^{1} and Val^{8} carbonyls. These NMR experiments suggested that the binding site for these ions is centred on Trp^{11} [14,15] at each end of the dimeric channel. A variety of evidence (reviewed in Ref. 1) supports the view that the positions of the binding sites should be independent of the ion bound, though there is experimental evidence that the calcium ion binding site is about 1.5 Å closer to the channel mouth than the sodium binding site [6].

In earlier experiments [8,16] we used solid-state NMR spectroscopy of aligned lipid bilayers containing ^{13}C-labelled gramicidin analogues to examine the channel. The orientation and magnitude of the chemical shielding tensor for the carbonyl group in the peptide bond has previously been measured for glycylglycine [17] and other di- and tripeptides (Separovic, F., Cornell, B.A. and Smith, R., unpublished data). Given this information, measurement of reduced chemical shift anisotropies in oriented gramicidin samples may be used to deduce the orientation and dynamics of the labelled carbonyl groups. Therefore, using a series of analogues bearing single labels in positions along the backbone, a comparison can be made of the structural and dynamic properties of different segments of the molecule, and

Abbreviations: CSA, chemical shift anisotropy; DMPC, dimyristoylphosphatidylcholine; DHPC, dihexadecylphosphatidylcholine; T_c, gel to liquid-crystalline phase transition temperature.

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their responses to changes in the molecular environment studied.

Using this approach, additional evidence has been gained for adoption of a $\beta^{6,3}$ helix conformation by the peptide in forming the channel [8,16]. The lack of effect of variation of the lipid composition of the multilayers on the molecular structure and orientation has also been demonstrated [18]. We report here the effects consequent upon ion occupation of the channel.

Materials and Methods

Gramicidin A analogues, enriched with ^{13}C in selected peptide carbonyl groups, were synthesized and purified as previously described [16]. Each peptide gave a single dominant peak accounting for over 95% of the integrated intensity on reverse-phase high-performance chromatography on a C-18 column (Bondapak Radialpak 0.46 cm × 10 cm, Waters Associates, Waltham, MA, U.S.A.) in methanol/water (83:17, by vol.). High-resolution ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra confirmed the purity of the peptides.

For solid-state NMR spectroscopy aligned multilayers of dimyristoylphosphatidylcholine (DMPC) or dihexadecylphosphatidylcholine (DHPC) (Sigma Chemical Co., St. Louis, MO, U.S.A.) containing a 1:15 peptide/lipid molar ratio were prepared on glass slides [16]. Proton-enhanced ^{13}C spectra were recorded at 75.46 MHz on a Bruker CXP-300 spectrometer. Typical operating conditions were: 90° pulse, 9–9.5 μs; contact time, 2 ms; acquisition time, 8.5 ms; repetition time, 2 s; sweep width, 62.5 kHz. The sample tube was mounted in a probe which allowed measured rotation of the sample about an axis perpendicular to the magnetic field without removal of the probe from the spectrometer. Spectra were recorded in the absence of sodium chloride and in samples containing 0.16 M salt: 50–60 μl of saline were added to an equal weight of solid sample, resulting in a ratio of 2 Na^+ per gramicidin molecule. Chemical shifts are expressed relative to TMS.

Results and Discussion

Spectra for the Val^7 analogue in the presence and absence of 0.16 M sodium chloride are shown in Fig. 1 for samples aligned with the bilayer normal parallel to the spectrometer magnetic field. Salt induced no change in these 0° spectra, nor in those obtained at other angles: the minor differences in the spectra in Fig. 1 arise from variations in the Hartmann-Hahn conditions used for spectral acquisition. Comparable results were also obtained with gramicidin analogues labelled in the carbonyl groups of Gly^2, Ala^3 and D-Leu^4: the orientation dependencies and hence the CSA values were unaltered in the presence of sodium chloride. It may therefore be concluded that ion occupation of the channel has no observable influence on the section of each monomer from Val^7 to the formylated amino-terminal; this segment remains in a $\beta^{6,3}$ helical conformation and continues to rotate rapidly around the bilayer normal. Consequently, there is no evidence from these experi-

TABLE I

Comparison of the reduced chemical shift anisotropies from the labelled carbonyl groups in gramicidin A analogues in the presence and absence of 0.16 M sodium chloride

The gramicidin was present at a 15:1 lipid/peptide molar ratio in dimyristoylphosphatidylcholine multilayers. The CSA values are assigned negative values using the convention that the 0° orientation resonance is shifted further from the TMS standard than is the 90° peak.

Gramicidin analogue	CSA values	
	without NaCl	with NaCl
Gly^2	−11±2	−11±2
Ala^3	−14±1	−14±1
D-Leu^4	−12±2	−12±2
Val^7	−16±1	−16±1
D-Leu^{10}	−9±1	0±1
D-Leu^{12}	−12±1	−7±1
D-Leu^{14}	−13±1	−7±1

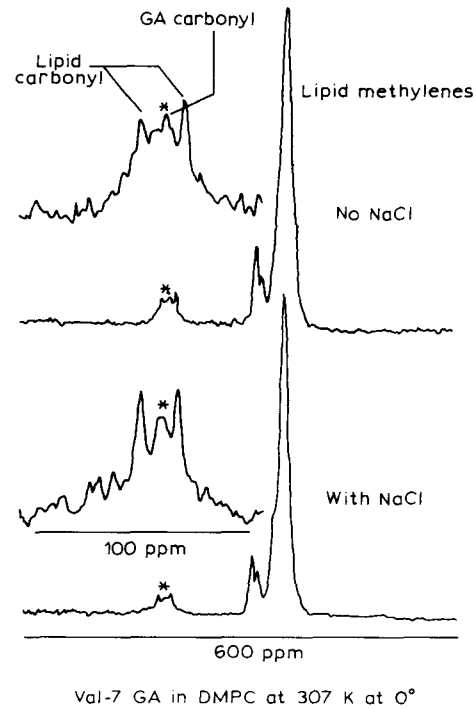

Fig. 1. Proton-enhanced spectra of gramicidin A ^{13}C-enriched in the carbonyl group of the Val^7 residue. The peptide was present at a 1:15 (peptide/lipid) mole ratio in dimyristoylphosphatidylcholine (DMPC). The spectra were recorded at 307 K with the multilayers aligned normal to the direction of the spectrometer magnetic field (i.e., at 0° angle). The top and bottom spectra required acquisition of 7200 and 28400 free induction decays, respectively. The spectra were plotted using 100 Hz linebroadening.

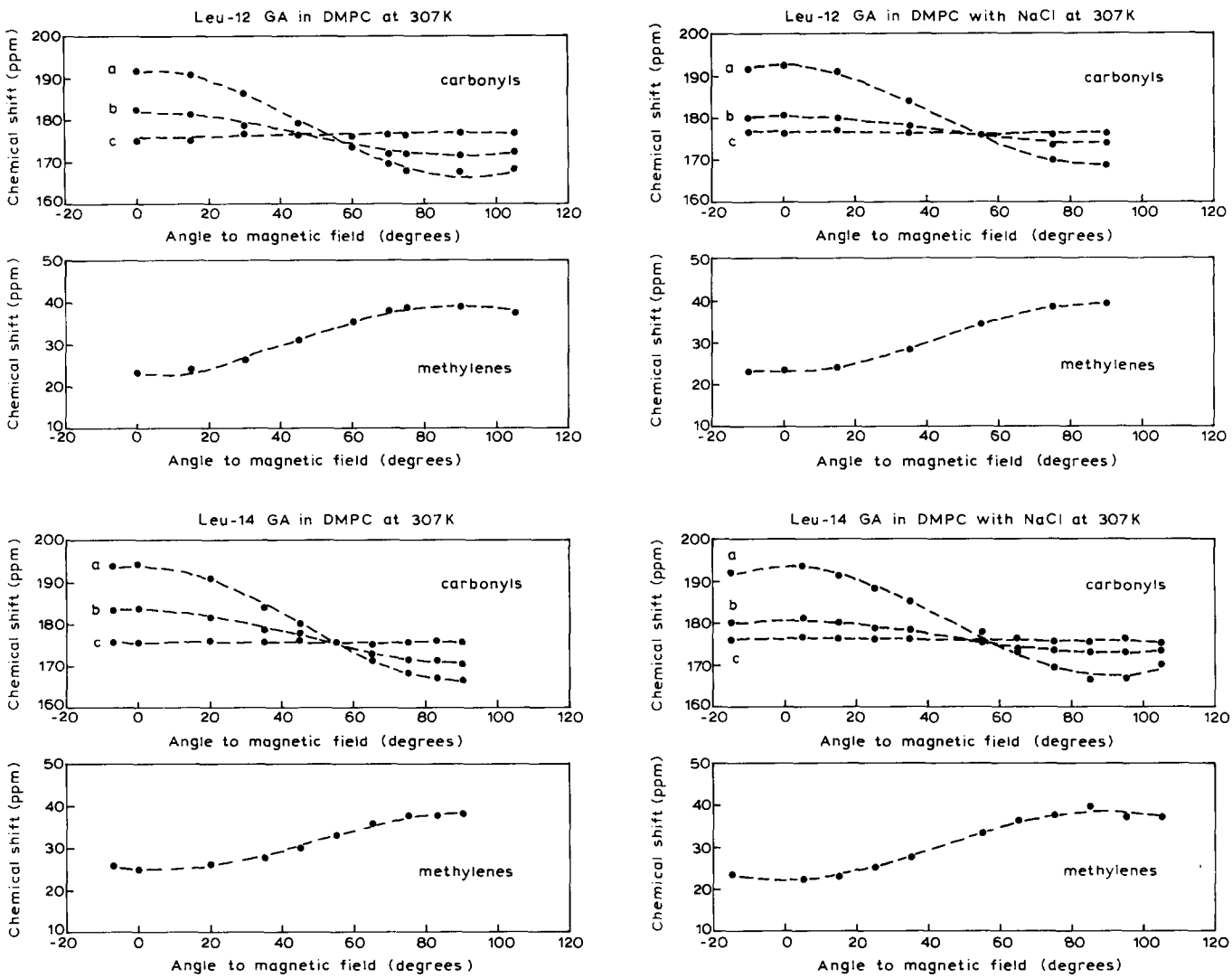

Fig. 2. Orientation dependence of the chemical shift for the peptide (b) and lipid (a, c) carbonyl resonances and the lipid methylene peak of aligned multilayers of the ester-linked lipid, DMPC, containing gramicidin enriched with ^{13}C in the carbonyls of D-Leu^{12} and D-Leu^{14}. All spectra were recorded at 307 K using the parameters specified in the legend to Fig. 1. The estimated errors in the chemical shift measurements are comparable to the symbol size. The dashed lines represent computer fits to the equation ($A + B \cos 2\theta + C \sin 2\theta$), where θ is the angle of the bilayer normal to the magnetic field.

ments for participation of the carbonyls of the Val^7 D-Leu^4, Ala^3 or Gly^2 residues in ion-binding or movement of ions through the channel.

On the other hand, there is clear evidence for perturbation of D-leucine residues closer to the membrane surface. Spectra for D-Leu^{12} and D-Leu^{14}-labelled gramicidins have also been recorded in aligned multilayers of DMPC in the presence and absence of 0.16 M sodium chloride (Fig. 2). The CSA values of both analogues of approx. −12 ppm in the absence of salt are reduced to −7 ppm by the presence of ions in the channel. Two mechanisms may lead to such a reduction in the CSA: either the chemical shift tensor is rotated on the carbon nucleus, or the peptide carbonyl orientation is modified in the presence of ions. The former explanation is unlikely to be correct as the magnitude of the rigid lattice tensor, seen with D-Leu^{14} (and D-Leu^{10}) gramicidin immobilized in gel-phase lipid, is not changed by the addition of salt. In the absence of changes in the chemical shift tensor, the magnitude of the change in the CSA is consistent with a 10–15° rotation of the carbonyl groups of the D-Leu^{12} and D-Leu^{14} residues. The direction of this CSA change, towards positive values, implies movement of σ_{33} towards the centre of the helix in a plane perpendicular to the membrane surface (Fig. 3), a reorientation which molecular modelling shows to result from movement of the D-Leu carbonyl oxygens towards the centre of the channel

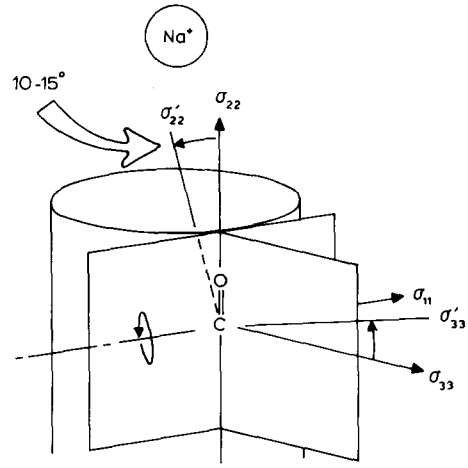

Fig. 3. Orientation of the carbonyl chemical shift tensors of the carbonyls of the D-Leu[10], D-Leu[12] and D-Leu[14] residues in the $\beta^{6.3}$ helix of gramicidin A. Addition of 0.16 M sodium chloride results in a reorientation in the direction indicated by the arrows. The initial and final orientations of D-Leu[10] differ slightly from those of the other two D-Leu residues. The gramicidin molecule is depicted as a cylinder with the carbonyl bond almost parallel to the helix axis. The position of the chemical shift tensor after addition of 0.16 M NaCl is indicated by the principal components σ'_{22} and σ'_{33}. The orientation of σ_{11}, tangential to the cylinder, is unchanged.

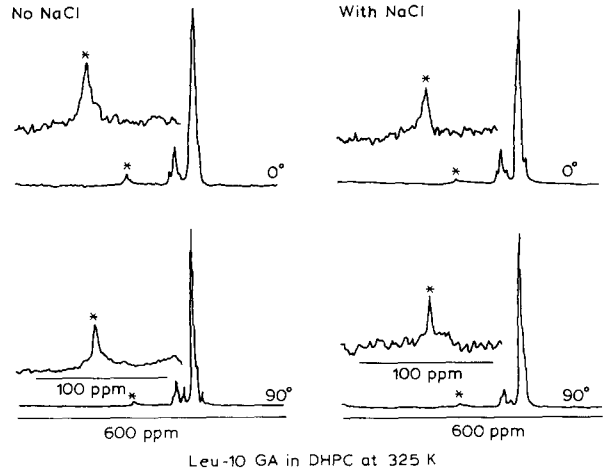

Fig. 4. ^{13}C proton-enhanced spectra of gramicidin A labelled in the carbonyl group of D-Leu[10], in dihexadecylphosphatidylcholine (DHPC) multilayers aligned with the bilayer normal parallel (top) and perpendicular (bottom) to the spectrometer magnetic field. The spectra were plotted using 100 Hz linebroadening. 23000–46000 transients were accumulated at 325 K for each spectrum. The 90° pulse was 9.5 μs for the spectrum obtained for salt-free sample and 11 μs for that recorded in the presence of sodium chloride. The corresponding mixing times were 2 ms and 0.5 ms.

lumen. Salt induced no changes in the carbonyl or methylene resonances of the lipid molecules (Fig. 2), hence it does not perturb the bilayer organization.

As noted earlier [8,18], the D-Leu[10] analogue has a lower CSA than the carbonyls of D-Leu[12] and D-Leu[14], possibly as a consequence of its binding to the ethanolamide hydroxyl group [19]: it also responds differently to the addition of sodium ions. In Fig. 4 representative spectra for the D-Leu[10] analogue with and without sodium chloride in multilayers of the ether-linked lipid, DHPC, are shown. The smaller CSA of this analogue caused overlap of the peptide and lipid carbonyl resonances with DMPC. These spectra again reveal changes in the chemical shift anisotropy on the addition of sodium chloride. These changes are demonstrated more clearly in Fig. 5, in which the chemical shift is plotted as a function of the angle between the bilayer normal and the direction of the spectrometer magnetic field. For this analogue the CSA drops from −9 ppm to ≈ 0 ppm. This loss of CSA is not caused by

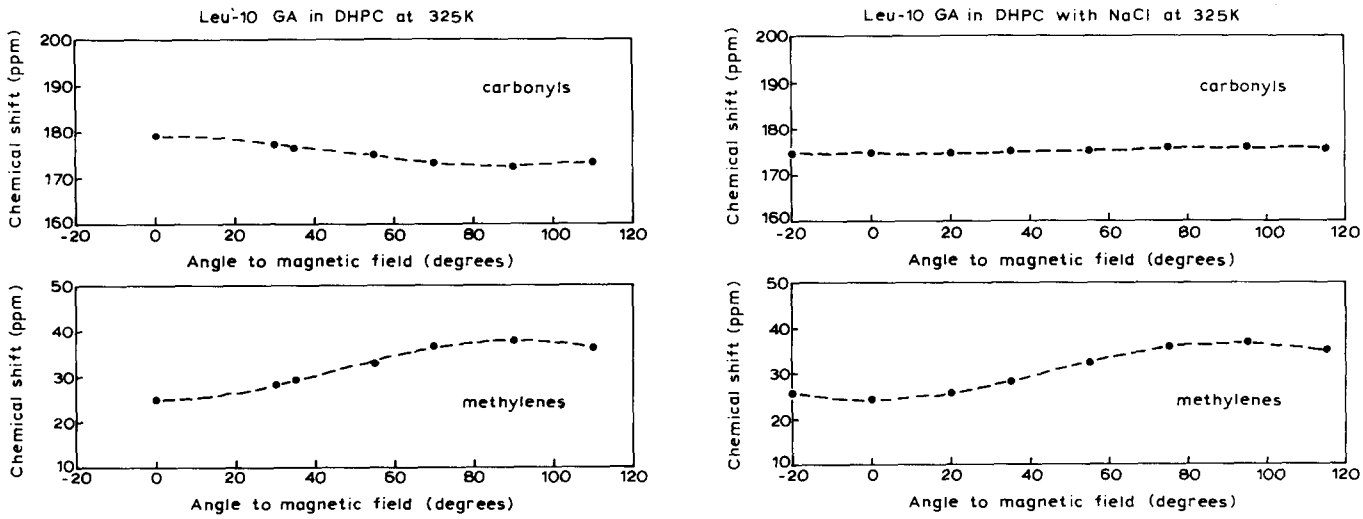

Fig. 5. Orientation dependence of the chemical shift for the peptide carbonyl and lipid methylene resonances of a sample containing D-Leu[10]-labelled gramicidin in aligned multilayers of the ether-linked lipid. The spectra were recorded in the absence (left) and presence (right) of 0.16 M sodium chloride. The parameters used for data collection and processing are given in the legend to Fig. 4. The estimated errors in the chemical shift measurements are comparable to the symbol size.

generation of an isotropic lipid-peptide phase as the usual bilayer CSA of the methylene groups in the lipid acyl chains has been retained (Figs. 2 and 5): formation of an isotropic phase would have eliminated the CSA of these groups. This conclusion is supported by the observation that the 0° angle resonance of the D-Leu^{10}-labelled peptide is not shifted on passage of the lipid from the liquid-crystalline to the gel phase. Had the low CSA been attributable to isotropic motion of the D-Leu^{10} the full rigid-lattice chemical shift anisotropy of 156 ppm should have been manifested upon elimination of gramicidin motion in the gel phase. Finally, the linewidths at 0° and 90° orientations would have been identical, rather than displaying the 2 : 1 ratio evident in Fig. 4. Nor can the low CSA be attributed to molecular motion about the magic angle, as this mechanism would also result in spectral changes as a result of loss of rotational averaging of the chemical shift tensor components on elimination of the free rotation of the gramicidin molecules below T_c.

The observed results may, however, arise if the σ_{22} principal component of the ^{13}C chemical shift tensor is oriented along the molecular long axis and thus perpendicular to the bilayer surface and parallel to the gramicidin rotation axis, as discussed more fully in Refs. 8 and 16. As σ_{22} is oriented at approximately 13° to the carbonyl bond direction, towards the alpha carbon atom, this orientation in the presence of sodium ions would represent a minor perturbation of the structure which exists in the empty channel [8].

As previously observed [8,18], the outer D-Leu residues have resonances which are half the width of those arising from other carbonyls, indicating greater motional freedom for the former groups. Below T_c the outer D-Leu resonances increase approximately 4-fold in width and become equal to those of the other residues. Sodium ion occupation of the channel did not influence the D-Leu linewidths in either the gel or liquid-crystalline states, thus although the D-Leu carbonyls change their average orientation in 0.16 M NaCl they appear to do so without reduction in their motional freedom.

Earlier NMR relaxation and chemical shift studies are consistent with the view that the gramicidin A channel may bind two ions simultaneously, the first with a greater dissociation constant than the second. For $^{23}Na^+$ these constants are 10–15 mM and 300–1000 mM [4,20]: comparable values have also been obtained for $^{87}Rb^+$, $^{39}K^+$, $^{7}Li^+$, $^{133}Cs^+$ and Tl^+, [13,21–24]. Similarly, two sites for Ca^{2+} ions have been postulated on the basis of data from circular dichroism and ^{13}C NMR spectroscopic measurements [25].

Previous crystallographic experiments have defined the location of the sites in the antiparallel, double helical form of the peptide obtained by crystallization from organic solvents. This crystalline form differs from the $\beta^{6.3}$ single-helix form of channel in that it undergoes a large conformational change on addition of Cs^+ [26–28]. However, it also has the peptide carbonyls parallel to the helix axis in the absence of ions, with several of the carbonyls tilting by up to 40° towards the centre of the pore in the presence of Cs^+ [27].

In lipid membranes the predominant form of the channel is considered to be the dimer of $\beta^{6.3}$ single helices and thus the mode of ion transport of this form is arguably of greater interest. The CSA changes observed in the current work are consistent with the inward movement of the carbonyl oxygens of D-Leu^{10}, D-Leu^{12} and D-Leu^{14} in response to ion occupation of the channel. This conclusion compares with that of Urry et al. [5] who proposed that the carbonyls of the Trp residues moved towards the cation in the channel, with a consequent movement of the D-Leu carbonyls away from the lumen. The strongest interaction in the molecule, which was considered to be a left-handed helix, was with Trp^{11} [5,29]. Comparison of these results is however complicated by the differences in the environment of the gramicidin in the two studies: Urry et al. [6,10,11] obtained their results with the peptide packaged in lysophosphatidylcholine sheets at 70°C, whereas in the current study the gramicidin was inserted in lipid bilayers at maximum temperatures which just exceeded the gel-to-liquid crystalline phase transition temperature.

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