Sodium Ion Binding in the Gramicidin A Channel. Solid-State NMR Studies of the Tryptophan Residues

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ABSTRACT Gramicidin A analogs, labeled with ^{13}C in the backbone carbonyl groups and the C-2 indole carbons of the tryptophan-11 and tryptophan-13 residues, were synthesized using t-Boc-protected amino acids. The purified analogs were incorporated into phosphatidylcholine bilayers at a 1:15 molar ratio and macroscopically aligned between glass coverslips. The orientations of the labeled groups within the channel were investigated using solid-state NMR and the effect of a monovalent ion (Na^+) on the orientation of these groups determined. The presence of sodium ions did not perturb the ^{13}C spectra of the tryptophan carbonyl groups. These results contrast with earlier results in which the Leu-10, Leu-12, and Leu-14 carbonyl groups were found to be significantly affected by the presence of sodium ions and imply that the tryptophan carbonyl groups are not directly involved in ion binding. The channel form of gramicidin A has been demonstrated to be the right-handed form of the $\beta^{6.3}$ helix: consequently, the tryptophan carbonyls would be directed away from the entrance to the channel and take part in internal hydrogen bonding, so that the presence of cations in the channel would have less effect than on the outer leucine residues. Sodium ions also had no effect on the C-2 indole resonance of the tryptophan side chains. However, a small change was observed in Trp-11 when the ether lipid, ditetradecylphosphatidylcholine, was substituted for the ester lipid, dimyristoylphosphatidylcholine, indicating some sensitivity of the gramicidin side chains to the surrounding lipid.

INTRODUCTION

Gramicidin A (GA) is a hydrophobic, 15-residue peptide that forms membrane-spanning, head-to-head dimers of right-handed, $\beta^{6.3}$ helices upon incorporation into lipid bilayers (Urry et al., 1983a; Arseniev et al., 1985; Cornell et al., 1988; Prosser et al., 1991; Mai et al., 1993; Hing and Schaefer, 1993). It selectively transports partly hydrated univalent cations across lipid membranes and is blocked by anions or multivalent cations.

The four tryptophan residues of GA play a central role in its conductivity. The indole nitrogens of the side chains are thought to lower the potential energy barrier of the approaching cation, and even the conservative replacement of tryptophan with phenylalanine causes a marked drop in the single-channel conductivity (Mazet et al., 1984; Sawyer et al., 1990; Becker et al., 1991). In earlier experiments directed at locating the ion-binding sites within the channel, Urry and colleagues (Urry et al., 1982a, b, 1983a, b) examined the changes in chemical shifts of the peptide carbonyl groups upon addition of Tl^+, K^+, and Na^+ to GA incorporated into lysophosphatidylcholine sheets at high temperatures. They concluded that these ions bound to two sites close to the mouth of the channel, centered on Trp-11 residues at each end of the channel and about 20 Å apart (Urry et al., 1980, 1982c, 1984, 1985). In later solid-state NMR experiments on GA incorporated into planar lipid bilayers, the carbonyl groups of D-Leu-10, D-Leu-12, and D-Leu-14, but not D-Leu-4 were shown to be affected by the addition of sodium ions, implicating them in ion binding in this system (Smith et al., 1990). Given the differences in the lipid systems into which the peptide was incorporated in the two studies, it was not clear whether there were different locations for the ion binding sites in the two systems, or whether the site involved both Trp and D-Leu residues. However, Urry and co-workers concluded that the gramicidin was a left-handed helix, whereas the results of Arseniev et al. (1985) for sodium dodecyl sulphate micelles and Nicholson et al. (1987) for dimyristoylphosphatidylcholine multilayers show that the gramicidin is a right-handed helix. For a right-handed helix, the outer leucine carbonyls would be available for ion binding, and the tryptophan carbonyls would hydrogen bond internally. We have now addressed this point directly by synthesizing two gramicidin analogs in which the carbonyl groups of Trp-11 (GA11) and Trp-13 (GA13) have been labeled with ^{13}C. The response of these labeled groups to the addition of sodium ions has been examined using solid-state NMR spectroscopy of the peptides incorporated into multilayers of phosphatidylcholine.

The effect of sodium ions on the tryptophan side chains, ^{13}C-labeled at the C-2 indole carbon (Separovic et al., 1991), was also investigated. In addition, the effect of changing from an ester to ether linkage at the lipid hydrocarbon-water interface, on the tryptophan side chains was examined. It has been proposed (O'Connell et al., 1990; Becker et al., 1991; Hu et al., 1993) that the tryptophan side chains anchor the gramicidin A at the membrane surface, and perhaps hydrogen bond to the lipid carbonyls.

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MATERIALS AND METHODS

L-[1-^{13}C]-Trp was isolated from a racemic mixture (Icon, Inc., Summit, NJ) by N-acetylating the amino acid with 0.6 equivalents of acetic anhydride in 0.5 N NaOH, followed by enzymatic, stereospecific de-acetylation of the L-isomer using mold acylase from *Aspergillus niger* (Sigma Chemical Co., St Louis, MO). The L-Trp was purified on a 20 μm Aquapore C18 250 × 10 mm Brownlee column (Applied Biosystems, Foster City, CA), using a gradient of acetonitrile in 0.1% TFA. The yields of L-Trp were increased by racemizing the *N*-acetyl-D-Trp in 2 N NaOH with addition of a further equivalent of acetic anhydride at 40°C for 2 h, and re-isolating the L-Trp as described. The L-Trp was reacted with ditertiarybutyl dicarbonate to protect the amino group. L-tryptophan [indole-2-^{13}C] was obtained from Cambridge Isotopes Laboratories (Woburn, MA) and similarly modified.

Manual peptide synthesis was carried out following the procedure of Cornell et al. (1988) with several modifications. Synthesis was initiated on t-Boc-Trp(CHO) Pam-resin (Applied Biosystems) with 0.1 mmol of the starting amino acid. t-Boc-protected D-Leu and L-Trp(CHO) were from Auspep (Melbourne, Australia), and L-Val, D-Val, Gly, and L-Ala were from the Peptide Institute (Osaka, Japan). The optical purity of all amino acids was verified using a Perkin-Elmer 141 polarimeter.

O-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexaflurophosphate (HBTU) in dimethyl formamide (DMF) was used for amino acid activation for the first coupling (Dourtoglou and Gross, 1984), whereas the re-coupling step used dicyclohexylcarbodiimide (DCC) in dichloromethane (DCM), to optimize coupling yields. Labeled L-[1-^{13}C]-Trp was added in a 1.1-fold molar excess; all other amino acids were added at 2.5-fold excess. The couplings were monitored using a quantitative ninhydrin assay (Sarin et al., 1981).

Amino-terminal formylation was carried out on the side chain protected, resin-bound peptide. A 2.5-fold excess of formic acid was activated with DCC in DCM for 15 min and coupled for 1 h. This procedure minimizes losses generally encountered during formylation after peptide cleavage. The peptide was cleaved from the resin using distilled ethanolamine as previously described (Cornell et al., 1988). Crude yields from the syntheses were 100 mg (77% of the theoretical yield) for GA11 and 120 mg (65%) for GA13.

The GA analogs were purified in the dimeric form at 12 mg/ml, using isocratic reverse-phase HPLC with 25% water in methanol, on a C18 column (8 mm × 10 cm). The purified analogs were then passed several times down a mixed-bed, AG 501-X8(D) ion exchange column (100–200 mesh, Bio-Rad, Richmond, CA) equilibrated in 90% methanol/H_2O to remove sodium and potassium ions, which were found to be present before this treatment. Peptide purity was assessed by analytical reverse-phase HPLC, electrospray mass spectrometry, and ^{13}C high-resolution NMR in d_4-methanol, at 50.323 MHz on a Bruker AC 200 NMR spectrometer.

The gramicidin A analogs were incorporated into phosphatidylcholine bilayers at 1:15 molar ratios, hydrated, and aligned on glass coverslips (Cornell et al., 1988). Ditetradecylphosphatidylcholine (DTPC) or dihexadecylphosphatidylcholine (DHPC), which contain ether-linked hydrocarbon chains, allow observation of the peptide carbonyl resonance without interference from the lipid carbonyl resonances of the usual ester-linked lipids. DTPC was synthesized as described previously (Ruocco et al., 1985), and DHPC was purchased from Sigma. The peptide/lipid multilayers were shown to be well aligned and hydrated using ^{1}H and ^{31}P NMR spectroscopy, as described previously (Cornell et al., 1988). In addition, the side chain-labeled gramicidin A was incorporated into ester-linked dimyristoylphosphatidylcholine (DMPC; Sigma).

^{13}C NMR were recorded on Bruker MSL300 and CXP300 spectrometers, operating at 75.46 MHz for ^{13}C, using probes modified to allow rotation of the sample about an axis perpendicular to the magnetic field without removal from the spectrometer. Cross-polarization from protons to the ^{13}C was used to enhance the intensity of the latter (Pines et al., 1973). Typical NMR parameters at 75 MHz were: 90° carbon pulse of 9 μs, 1 ms contact time, 2 s repetition delay, 62.5 kHz sweep width, and an 8.5 ms acquisition time. Spectra were also acquired on a Bruker MSL400 at 100 MHz for ^{13}C. Most spectra were obtained by Fourier transformation of the sum of 15,000–80,000 free induction decays, with 100 Hz line broadening. All chemical shifts were referenced to the resonance of adamantane.

Reduced chemical shift anisotropies were derived from the observed peak positions with the bilayers oriented parallel and perpendicular to the spectrometer magnetic field, orientations that were shown to yield the extreme downfield and upfield shifts for the aligned samples. The principal values of the rigid lattice chemical shift tensor were obtained from simulations, on a Sun SparcStation 2, of the spectra obtained from GA powder.

RESULTS AND DISCUSSION

The tryptophan [indole-2-^{13}C] powder patterns have been described previously (Separovic et al., 1991). Powder pattern spectra obtained with the solid, 1-^{13}C-labeled peptides gave principal values for the rigid lattice tensor close to the figures of −74 ± 3, −7 ± 3, and 82 ± 3 ppm obtained previously with other analogs (Cornell et al., 1988; Smith et al., 1989).

Both backbone-labeled peptides showed reduced chemical shift anisotropies (Table 1) above the gel-to-liquid crystalline transition temperature, T_c, that were similar to values recorded previously for other carbonyl-labeled analogs, again demonstrating that there is considerable motion of the peptide within the bilayers. As noted in earlier studies of other analogs, the chemical shift for samples aligned with the bilayer normal parallel to the spectrometer field did not change on passing through T_c, whereas with the bilayer normal at 90° to the field the sharp resonance above T_c was broadened to almost the full powder CSA of over 150 ppm (Fig. 1). These observations reinforce the conclusion (Cornell et al., 1988; Smith et al., 1989) that above T_c the peptide reorients rapidly (on the NMR timescale) around the bilayer normal, leading to averaging of the shielding about this axis. The reduced CSAs are consistent with the right-handed β-helical conformation that has been proposed for the channel form of the peptide, with angles between the carbonyl bonds and the molecular long axis of near zero for even residues and about 22° for odd residues. On the basis of ^{15}N NMR results, the amide NH bonds, to which the CO groups are of course hydrogen-bonded, have also been proposed to be about 17° off the bilayer normal (Nicholson et al., 1987; Fields et al., 1988).

The reduced chemical shift anisotropies for the side-chain C-2 resonances for Trp-9, Trp-11, Trp-13, and Trp-15 gramicidin A analogs in DMPC were +8, −3, −4, and −9 ppm,

TABLE 1 Peak positions and reduced chemical shift anisotropies for aligned multilayers of the GA11 and GA13 analogs of gramicidin A in DTPC in the presence and absence of 0.16 M NaCl

	Temperature			
	274K	308K		
Sample	0°	0°	90°	CSA
GA11	178	178	169	−9
GA11 + NaCl	177	178	168	−10
GA13	179	179	169	−10
GA13 + NaCl	180	178	169	−9

The data were recorded below (274 K) or above (308 K) the lipid phase transition temperature, and with the bilayer normal parallel (0°) or perpendicular (90°) to the applied magnetic field. All values are in ppm with an estimated uncertainty of ±2 ppm.

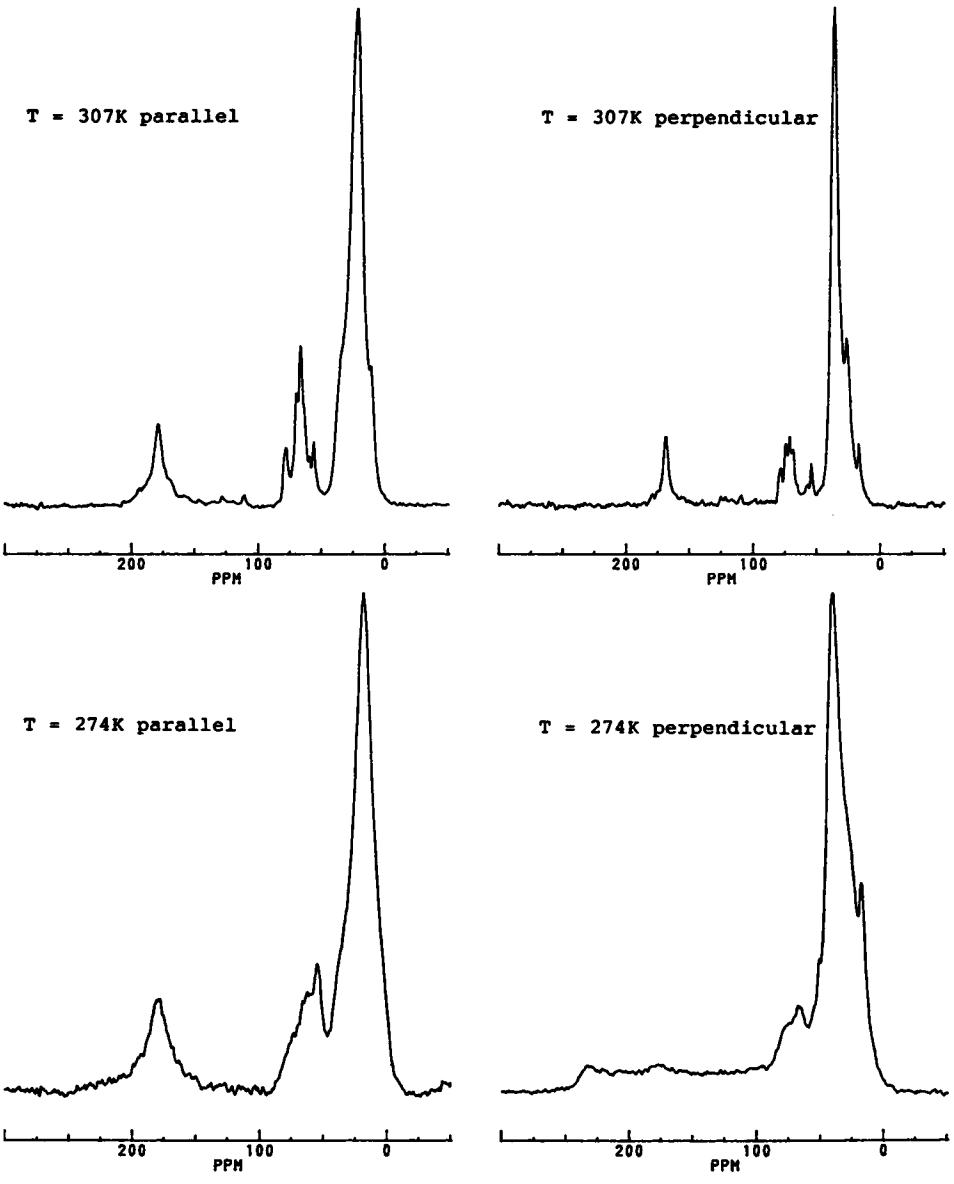

FIGURE 1 ^{13}C NMR spectra of GA13 at 75.48 MHz recorded above (*top*) and below (*bottom*) the lipid phase transition temperature for multilayers aligned with the bilayer normal parallel (0°, *left*) and perpendicular (90°, *right*) to the magnetic field direction. The spectra were processed with 100 Hz line-broadening. The peptide carbonyl resonances are near 175 ppm, and those further upfield are natural abundance signals arising from peptide and lipid.

respectively. When DHPC was the supporting lipid, no change was seen for Trp-9, Trp-13, and Trp-15. However, Trp-11 showed a small change in anisotropy, from −3 to 0 ppm, suggesting that the Trp-11 side chain is sensitive to a change in the lipid carbonyl (Fig. 2). A unique role for Trp-11 has also been proposed from conductance measurements in which substitution of this residue with Phe resulted in a marked increase in the single-channel lifetime (Becker et al., 1991). This change was attributed to direct interactions between the indole side chain of Trp and the adjacent lipid molecules (or water). In the current experiments, no changes were seen in the chemical shift anisotropy of the C-2 resonance when Na^+ was present (Fig. 3).

Earlier experiments with a series of gramicidin analogs, including those labeled in the peptide carbonyls of D-Leu-4, D-Leu-10, D-Leu-12, and D-Leu-14, had shown that all residues are not affected equally by ion binding (Smith et al., 1990). For the Leu residues near the channel entrance, but not D-leu-4 or several other carbonyl groups in the N-terminal half of the peptide, there was a reduction in the magnitude of the negative residual CSA of 5–9 ppm on the addition of 0.16 M NaCl, which was interpreted as resulting from a 10–15° movement of these carbonyls toward the center of the helix. It was suggested that the change in molecular structure resulted from binding of the cations to these carbonyl oxygen atoms within the lumen of the channel. By contrast, the addition of salt to samples containing the analogs labeled in the Trp-11 and Trp-13 caused no changes in the spectra at any angle (Fig. 4). Thus, it appears that the orientations of the Trp carbonyl groups are not altered in the presence of salt and that they do not actively participate in the ion binding. These observations can be rationalized in terms of the currently accepted structure of gramicidin A in lipid bilayers. In α-helices the peptide carbonyls are all directed toward the carboxy terminus, hydrogen bonding with NH protons that have the reverse orientation. However, in GA the alternation

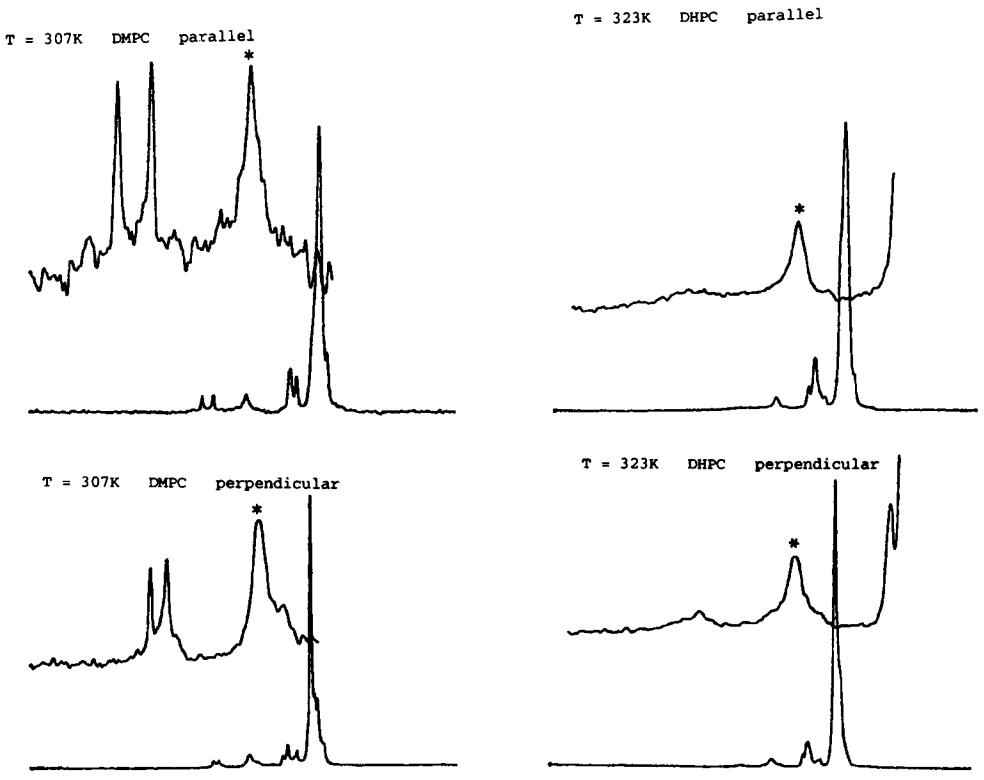

FIGURE 2 ^{13}C NMR spectra for Trp-11 [indole-2-^{13}C] gramicidin A at 0° (*top*) and 90° (*bottom*) to the magnetic field, in DMPC at 307 K (*left*; similar spectra were obtained at 323 K), and DHPC at 323 K (*right*). The plot width is 600 ppm, with the magnified region expanded by a factor of three. The indole resonance is marked with an asterisk in each spectrum: the other resonances are natural abundance signals arising from peptide and lipid.

of D- and L-amino acids results in the formation of a β-helix. In this structure, neighboring amino acids having their carbonyls in opposite directions, with the carbonyls from even residues pointing outwards and aligned along the helix axis, and those from odd residues pointing toward the center of the bilayer and tipped slightly toward the center of the lumen for a right-handed helix. In this conformation, the Leu carbonyl oxygen atoms are most likely to interact with ions in the channel, in accord with our observations. This conclusion is at variance with the earlier experiments of Urry et al. (1984), in which the Trp carbonyl carbon resonances were shifted in the presence of univalent cations. The differences may arise from the substantially different systems employed in the two studies, in our experiments, aligned lipid multilayers, and in

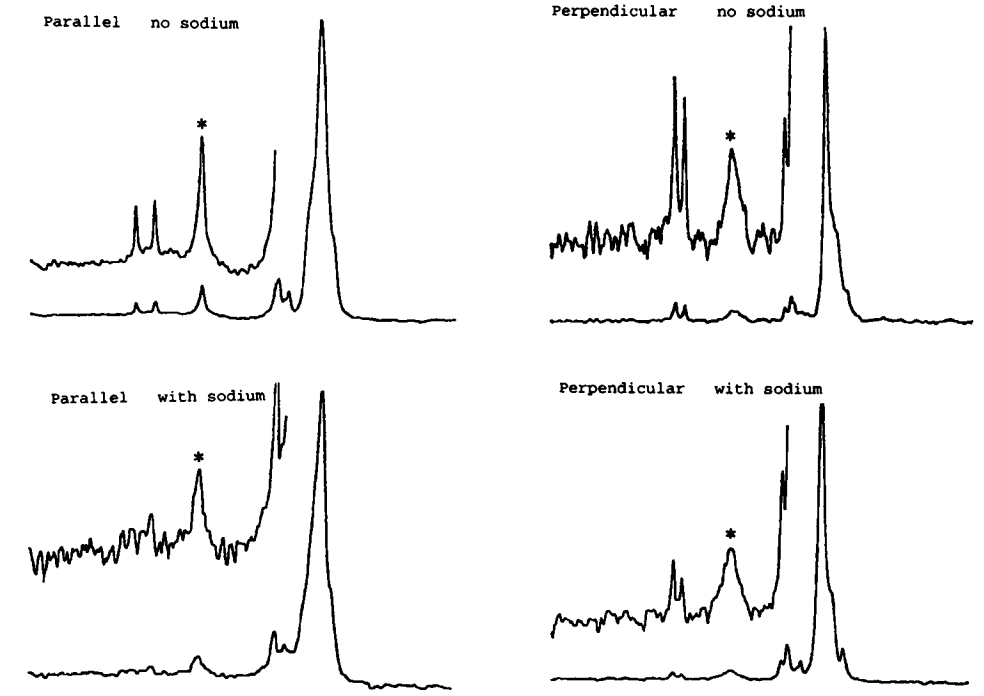

FIGURE 3 ^{13}C NMR spectra for Trp-15 [indole-2-^{13}C] gramicidin A at 0° (*left*) and 90° (*right*) to the magnetic field in DMPC at 307K with Na^+ (*bottom*) and without Na^+ (*top*) present. The plot width is 400 ppm. The indole resonance is marked with an asterisk in each spectrum: the other resonances are natural abundance signals arising from peptide and lipid.

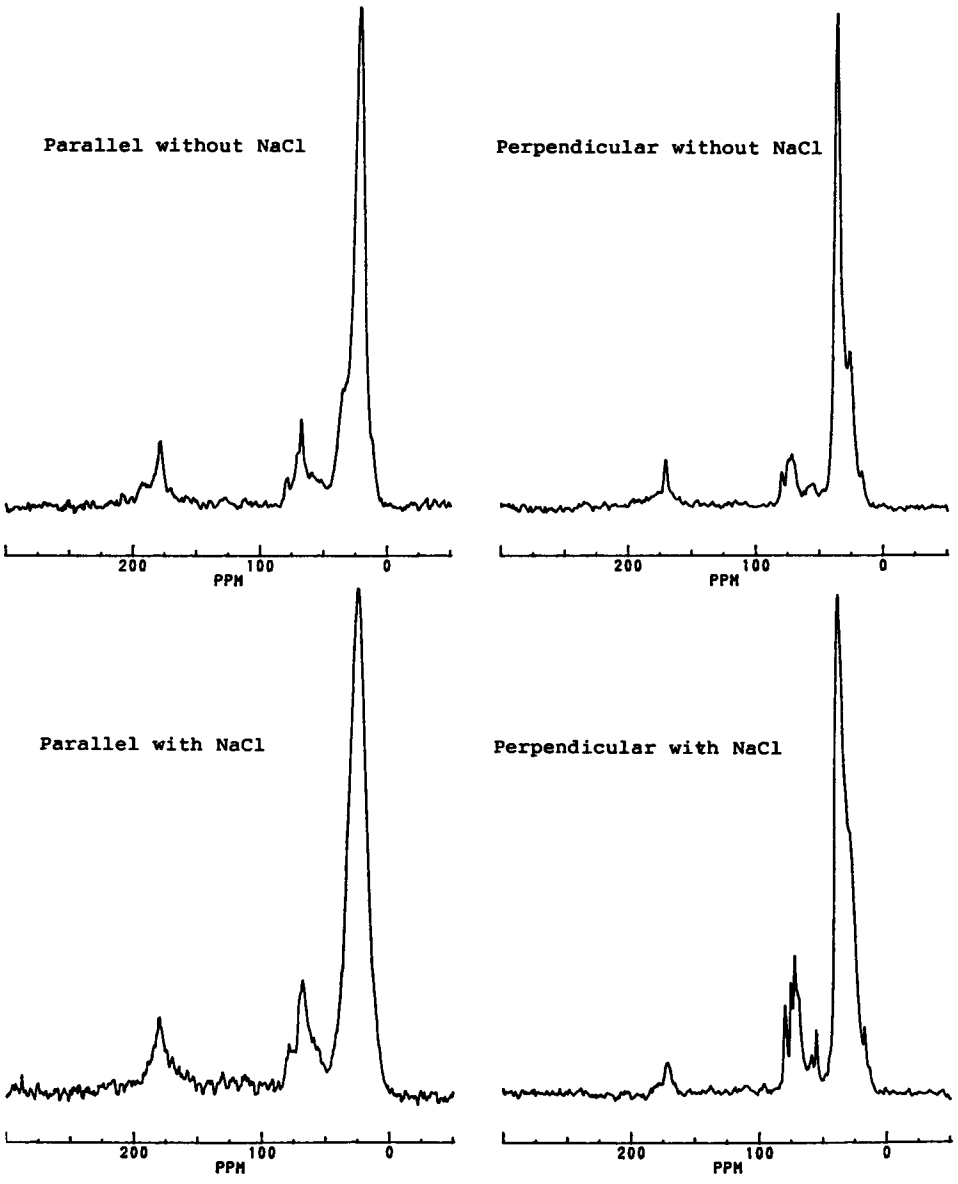

FIGURE 4 ^{13}C NMR spectra recorded for GA11 at 75.48 MHz and 308 K, with (*bottom*) and without (*top*) 0.16 M NaCl present. Spectra were recorded with the lipid multilayer normal aligned parallel (*left*) or perpendicular (*right*) to the spectrometer field. The spectra were processed with 100 Hz line-broadening. The peptide carbonyl resonances are near 175 ppm, and those further upfield are natural abundance signals arising from peptide and lipid.

the other, lysophosphatidylcholine sheets at high temperatures. Our results reaffirm the right-handed form of the $\beta^{6.3}$ helix in PC membranes and that the tryptophan side-chain N-H dipole alignments are not greatly affected by the presence of the cation.

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