Determination of the structure of a membrane-incorporated ion channel

Solid-state nuclear magnetic resonance studies of gramicidin A

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ABSTRACT Solid-state nuclear magnetic resonance (NMR) measurements on ^{13}C-labeled analogues of the ion channel-forming peptide, gramicidin A, have been used to directly determine the structure of this peptide in lipid membranes. Seven gramicidin analogues, each labeled in a single carbonyl group of gly^2, *L*-ala^3, *D*-leu^4, *L*-val^7, *D*-leu^{10}, *D*-leu^{12}, or *D*-leu^{14} were synthesized by the solid-phase method. These gramicidin analogues were incorporated into aligned multilayers of dimyristoylphosphatidylcholine, or diether lipid bearing 14- or 16-carbon chains, at a 1:15 peptide:lipid mole ratio. Proton-enhanced, ^{13}C, solid-state spectra were obtained at several temperatures and over a range of sample orientations with respect to the spectrometer magnetic field to permit accurate measurement of the chemical shift anisotropies. The observed anisotropies indicate that all of the labeled carbonyl bonds are oriented almost parallel to the molecular long axis and perpendicular to the lipid bilayer plane. These orientations are consistent with gramicidin forming a $\beta^{6.3}$ single-strand helix that is oriented parallel to the methylene chains of the lipid molecules. Comparison of the linewidths from labeled residues that are in the innermost turn of the helix (gly^2, ala^3, and *D*-leu^4), in the center of the molecule (val^7), and in the turn nearest the lipid bilayer surface (*D*-leu^{10}, *D*-leu^{12}, and *D*-leu^{14}) suggests that although the peptide behaves largely as a rigid barrel, segments of the peptide close to the membrane surface possess greater motional freedom. At temperatures above the gel-to-liquid crystalline transition temperature (T_c) the gramicidin molecules rotate, with a less than millisecond correlation time, about the bilayer normal: several degrees below T_c they become immobile on the NMR timescale, without change in the channel conformation. In the L_β' phase the linewidths of the *D*-leu^{10}, *D*-leu^{12}, and *D*-leu^{14} resonances become equal to those of the other labeled sites, indicating reduced but equivalent motion for all of the peptide carbonyl groups.

INTRODUCTION

Much has been learned about the functions of water-soluble, globular proteins from consideration of their structures, as revealed by x-ray crystallography. In attempts to understand the functioning of membrane proteins, methods have been sought for elucidation of the detailed three-dimensional structures of this class of proteins. Initial successes with crystallization of membrane proteins from solutions containing detergents such as octyl glucoside have not, however, led to development of general methods for obtaining crystals suitable for diffraction analysis. Thus, methods such as nuclear magnetic resonance spectroscopy (NMR), which yield less complete structural information are still valuable in advancing our knowledge of the structure-function relationships of intrinsic membrane proteins. For membrane proteins, spectroscopic methods have added advantages: they can provide information on proteins in lipid bilayers, an environment which mimics the membrane, they yield information on the consequences of modifying this environment, and they yield not only information on structure but also on the modes and rates of molecular motion.

High-resolution NMR has been widely applied in structural studies of small molecules in solution and has recently been used for *de novo* determinations of the structures of small water-soluble proteins. A few membrane proteins have been studied by this method in the presence of detergents or in organic solvents (e.g., Brown et al., 1982; Lee et al., 1987; Bazzo, et al., 1988): although the proteins alone have low aqueous solubility, the protein-detergent complexes are sufficiently soluble to yield high-resolution spectra, but frequently under conditions (high temperature, often with methanol present) far removed from the native environment.

By contrast, proteins incorporated into lipid bilayers, which more closely mimic the *in vivo* environment, yield broad spectra that are unsuitable for the analysis applied to high-resolution spectra but contain, nevertheless, information on molecular structure, orientation and dynamics. Because of the large line widths and the chemical shift anisotropy (CSA), resonances from different groups overlap considerably and useful information is generally only

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gained by selective labeling, which enhances the signal from the labeled group relative to the background, natural-abundance signal. Using this approach Lewis et al. (1985) have, for example, studied the structure of bacteriorhodopsin biosynthetically labeled in all leucine residues. Other groups have taken a similar approach in studying the structure and dynamics of both the backbone and the side-chains of intrinsic membrane proteins (e.g., Kinsey et al., 1981; Keniry et al., 1984; Schiksnis et al., 1987), and Pauls et al. (1985), Datema et al. (1986) and Davis (1988) have studied gramicidin, labeled on peptide amide groups by chemical exchange in ^2H_2O, in an oriented lyotropic nematic phase and in multilayers. The structural information gained in such experiments is limited by the inability to assign resonances to unique sites within the protein: a more complete analysis would require labeling of the protein at single, well-defined sites, a requirement which currently cannot be met by biosynthetic incorporation of label, but is feasible in chemically-synthesized peptides.

Here, seven analogues of the intrinsic membrane peptide, gramicidin A, have been synthesized with ^{13}C substituted in single peptide bonds. Solid-state NMR spectra have been recorded for these analogues after incorporation into macroscopically-aligned multilayers of the lipid, dimyristoylphosphatidylcholine (DMPC), or lipid containing *C*-14 or *C*-16 ether-linked chains. Analysis of these spectra allowed determination of the orientation of each labeled carbonyl group, and the nature of their motion. From these data the structure of the peptide in the lipid membrane, the extent of intramolecular motions, and the nature of the whole-molecule motion has been deduced in both liquid-crystalline and gel-phase lipid. Preliminary accounts of this work, including data from three of the analogues, labeled in the *N*-terminal half of the molecule have been discussed in earlier publications (Smith and Cornell, 1986; Cornell et al., 1988).

MATERIALS AND METHODS

Unlabeled *t*-BOC amino acids, including *t*-BOC-*L*-(CHO)-trp, were purchased from the Protein and Peptide Research Foundation (Osaka, Japan), Pam-(CHO)trp resin from Applied Biosystems (Foster City, CA), 1-^{13}C-DL-leu from Cambridge Isotope Laboratories (Woburn, MA), 1-^{13}C-*L*-val and 1-^{13}C-*L*-ala from CEA (Saclay, France), and 1-^{13}C-gly from KOR Isotopes (Cambridge, MA). The amino acids were reacted with di-*t*-butyl-pyrocarbonate (Sigma Chemical Co., St. Louis, MO) to convert them to the *t*-BOC derivatives (Prasad et al., 1982). 1-^{13}C-D-leu was prepared from the racemic mixture after the method of Prasad et al. (1982).

Gramicidin synthesis

Labeled gramicidin analogues were prepared by the solid-phase method either manually (Cornell et al., 1988), or on an Applied Biosystems 430A peptide synthesizer using standard *t*-BOC protection for the amino groups and formyl protection on the imidazole side chain of tryptophan. Amino acid coupling was monitored using a quantitative ninhydrin method (Sarin et al., 1981): coupling yields were generally above 99%. In the automated syntheses double coupling was routinely used for all residues beyond val[8]. For all syntheses the peptide was cleaved from the resin by reaction with redistilled ethanolamine (Prasad et al., 1982), which simultaneously introduces the COOH-terminal ethanolamide group of gramicidin and removes the formyl protecting groups from the four tryptophan residues. The liberated peptide was isolated by ion-exchange chromatography on Dowex AG-50W X-2 (Dow Chemical Company, Midland, MI), formylated, and rechromatographed on the Dowex ion-exchange resin. The crude gramicidin was purified by chromatography on a column of Sephadex LH-20 (Pharmacia LKB Biotechnology, Uppsala, Sweden) in methanol, and isocratic reverse-phase HPLC on a C_{18} Radialpak column (Waters Associates, Waltham, MA) in 17% (vol/vol) water in methanol. The purity of the peptides was established by ^{1}H- and ^{13}C-high resolution NMR spectroscopy, HPLC and, for several of the peptides, by circular dichroism spectroscopy.

Sample preparation

Aligned lipid multilayers containing gramicidin at a peptide/lipid mole ratio of 1:15 were prepared as previously described (Cornell et al., 1988).

NMR spectroscopy

Spectra were acquired using a Bruker CXP-300 spectrometer (Bruker Instruments, Inc., Billerica, MA) operating at 75.46 MHz for ^{13}C and 300.066 MHz for ^{1}H. Proton-enhanced ^{13}C, cross-polarization spectra were obtained using the pulse sequence of Pines et al. (1973). Typical operating conditions were: Hartmann-Hahn 90° pulse, 7 μs; repetition delay, 2 s for ^{13}C and 10s for ^{1}H; contact time, 2 ms; acquisition time, 8.5 ms; sweep width, 60 kHz. The tube was mounted to permit rotation through an angular range of 180° about an axis perpendicular to the spectrometer magnetic field using a goniometer that allowed the angle to be adjusted with the probe and sample in the field. The angle, which was reproducible to ± 1°, was calibrated from the proton resonance "magic angle" effect. The water content of samples was checked before and after acquisition of ^{13}C spectra by integration of the ^{1}H spectrum measured at the magic angle (Cornell et al., 1988).

Spectral simulations were performed on an HP1000 computer using a time domain representation of the tensor distribution followed by Fourier transformation to obtain the frequency domain spectrum.

RESULTS

Interpretation of the NMR data from aligned samples requires knowledge of the principal values and orientation of the rigid-lattice chemical shielding tensor. The powder spectra for each of the dry, labeled peptides in the absence of lipid were superimposable on the spectrum for the *D*-leu[10] analogue, shown in Fig. 1: the principal values of the chemical shift tensor deduced from these spectra were $\sigma_{11} = -74 \pm 3$ ppm, $\sigma_{22} = -7 \pm 3$ ppm, and $\sigma_{33} = 82 \pm 3$ ppm relative to the isotropic position, values which are close to those derived for the peptide carbonyl in glycyl-

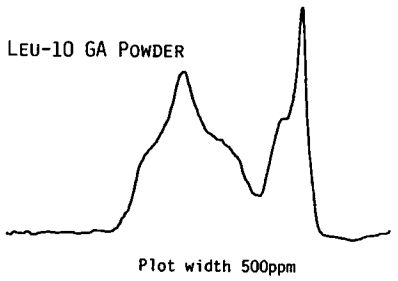

FIGURE 1 75 MHz, ^{13}C cross-polarization spectrum of a dry powder of the *D*-leu^{10}-labeled analogue of gramicidin A. The spectrum, which was obtained at 273 K, was plotted with a line broadening of 50 Hz. The carbonyl resonance (*left*) and the unresolved natural-abundance signal arising from aromatic and aliphatic carbons (*right*) appear in this rigid-lattice spectrum.

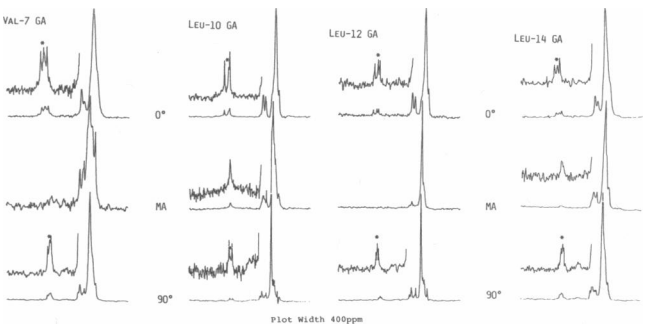

FIGURE 2 Cross-polarization spectra of gramicidin analogues in aligned dimyristoylphosphatidylcholine multilayers. Spectra were recorded with the bilayer normal aligned along (0°), at 54.73° (the magic angle, MA), or perpendicular to (90°) the direction of the spectrometer magnetic field. The asterisks mark the gramicidin carbonyl resonances. The number of aquisitions (in thousands) were val^7-GA, 10 (0°), 15 (MA) and 8 (90°); *D*-leu^{10}, 7, 35 and 6; *D*-leu^{12}, 52, 17 and 11; *D*-leu^{14}, 41, 48 and 24.

glycine (Stark et al., 1983). The orientation of the chemical shielding tensor has been assumed to be identical for each carbonyl in gramicidin and the same as in glycylglycine, with σ_{22} in the plane of the peptide bond making an angle of 13° from the carbonyl bond direction towards the alpha carbon, σ_{11} also in the peptide plane and at 77° to the carbonyl bond towards the peptide nitrogen. This assumption is supported by studies of other small peptides (D. E. Thomas, F. Separovic, R. Smith and B. A. Cornell, unpublished observations), including the sequences gly-ala and val-gly-ala, which are found in gramicidin: these studies indicate that the chemical shift tensor orientation for the carbonyl carbon in a peptide bond is relatively insensitive to the nature of the amino acid side chains. A similar conclusion was also reached by Oas et al. (1987) from studies of a series of small peptides, though they observed some varation in σ_{22} and in the tensor orientation which they attributed to lattice-dependent effects; such effects should be minor for gramicidin immersed in the hydrocarbon core of lipid bilayers.

Although the chemical shift anisotropy can in theory be deduced from the positions of the shoulders and the peak of a powder spectrum, in practice this is only possible at high signal-to-noise ratios, in the absence of overlapping resonances, for relatively immobile nuclei. The spectra of unaligned dispersions of gramicidin, especially in diester lipids, are such that one cannot directly derive accurate values of the CSAs: satisfactory values can only be obtained by following the chemical shift of the resonance as molecularly aligned samples are swept through all angles with respect to the external magnetic field. Oriented samples also yield additional information on the nature of the molecular motions that average the chemical shift tensor, including the macroscopic orientation of any dominant molecular rotation axis.

^{13}C NMR spectra were recorded as a function of the angle between the bilayer normal and the direction of the

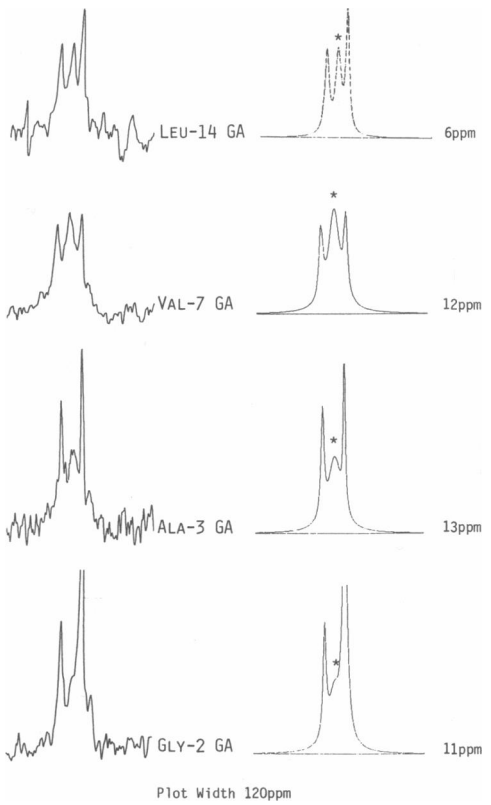

FIGURE 3 Comparison of experimental and simulated spectra for gramicidin analogues in aligned multilayers of DMPC. The experimental spectra were recorded at 307 K with the bilayer normal along the spectrometer magnetic field. The spectra were plotted using a line broadening of 100 Hz (except 50 Hz for ala^3) and were simulated using triple Lorentzians. The numbers of acquisitions were, from top to bottom, 41,000, 10,000, 23,000, and 71,000.

spectrometer magnetic field for oriented multilayers containing each of the gramicidin analogues. Representative spectra obtained with several of the analogues in multilayers formed from ester-linked lipids in the L_α phase are shown in Fig. 2. Each sample shows three peaks in the carbonyl region, two from the inequivalent lipid acyl chains and the third primarily from the label introduced into the peptide, with a small contribution from natural-abundance ^{13}C present in the unlabeled peptide carbonyls. These spectra were simulated with three Lorentzians as shown for several samples oriented at 0° (Fig. 3), and the resultant chemical shifts plotted as a function of the orientation angle to deduce the chemical shift anisotropies (Fig. 4). Also included in Fig. 4 are CSA plots for the lipid carbonyl and methylene resonances. The observed carbonyl CSAs are consistent with known rigid lattice chemical shift tensors and disposition of the phospholipid ester bonds; they demonstrate the small mosaic spread of the lipid molecules. The minor variations of these CSAs are possibly a reflection of the presence of small, but variable, amounts of nonaligned lipid that contribute an underlying powder pattern. The methylene CSAs afford a measure of the order of the lipid chains, which appear not to be affected significantly by the gramicidin.

Ether-linked lipids display very similar properties to the ester lipids, though with slightly higher gel-to-liquid crystalline transition temperatures: 300 K and 317 K for the ditetradecyl (DTPC) and dihexadecyl (DHPC) compounds, respectively, cf. 296 K and 315 K for the corresponding ester lipids. However, as the ether lipids do not possess carbonyl groups, the spectra of the peptide-containing multilayers, examples of which are presented

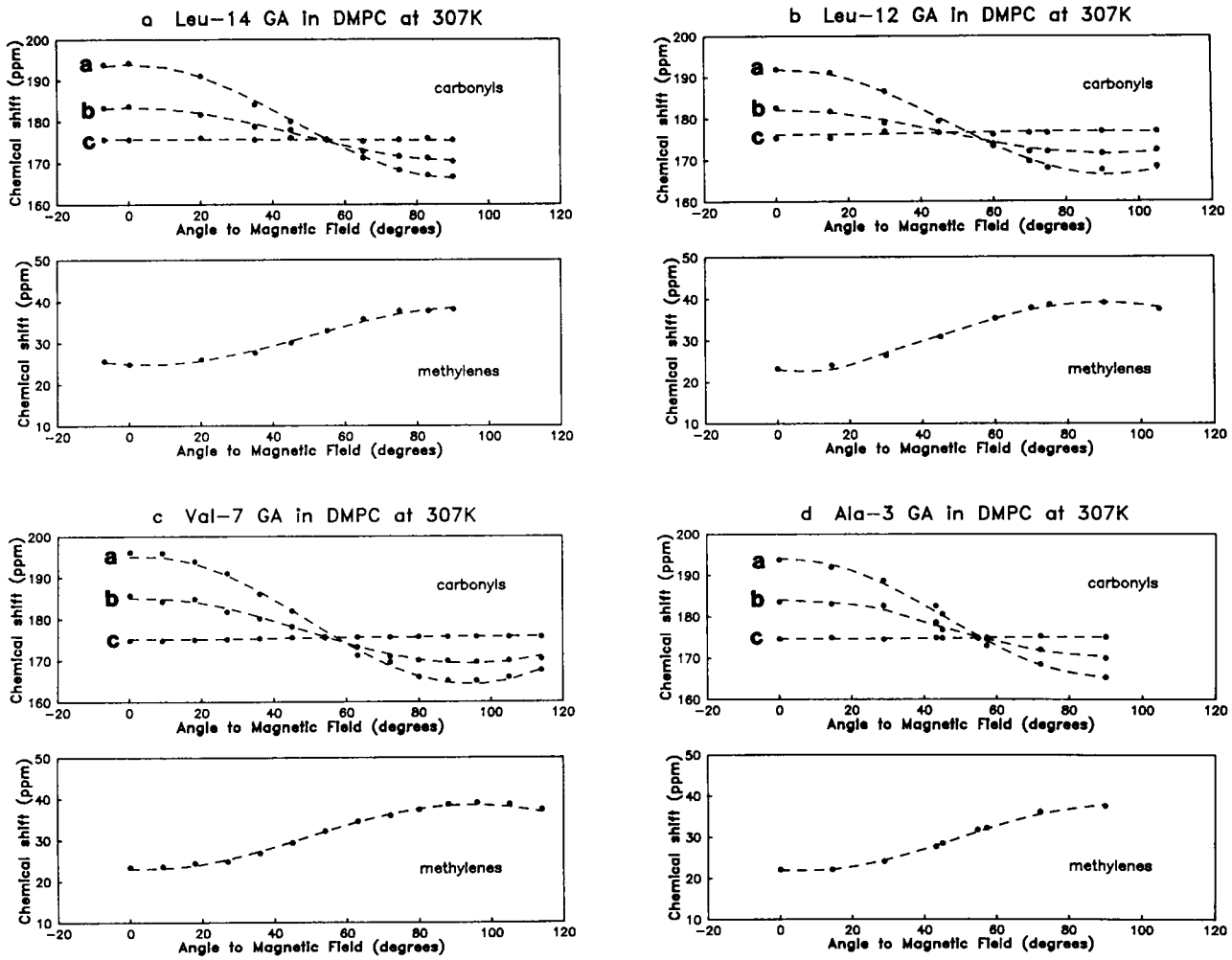

FIGURE 4 Representative plots of chemical shift anisotropies for gramicidin in aligned multilayers of DMPC, above the gel-to-liquid crystalline transition temperature. Data are presented for the resolved peaks assigned to the labeled gramicidin carbonyl carbon (*b*), the carbonyls of the two inequivalent sn-1 (*a*), and sn-2 (*c*) lipid acyl chains, and the bulk methylene resonance.

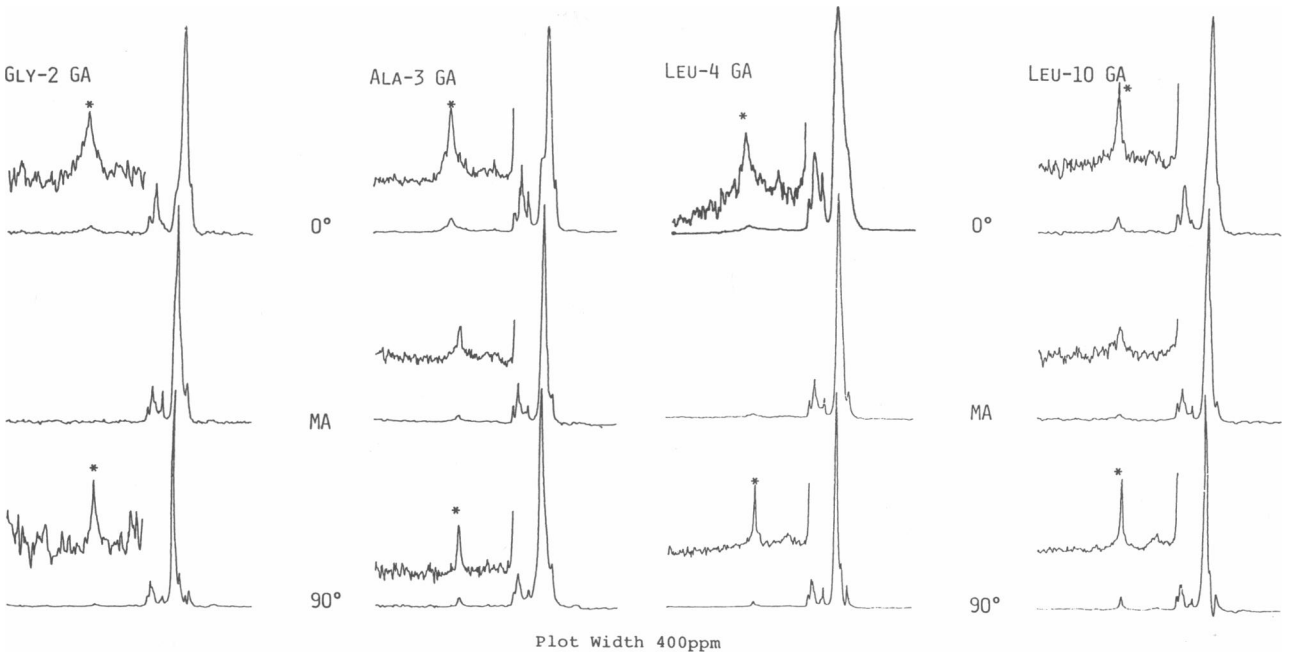

FIGURE 5 Cross-polarization spectra of gramicidin analogues in aligned multilayers of ether-linked lipids. The gly^2 analogue was studied in DTPC and the other gramicidins in DHPC. Spectra are presented for three orientations of each sample The numbers of acquisitions (in thousands) were: gly^2, 32 (0°), 48 (magic angle) and 39 (90°); ala^3, 35, 90 and 40; *D*-leu^4, 45, 15 and 26; *D*-leu^{10}, 14, 25 and 29.

in Fig. 5, manifest only the peptide resonance and thus do not require deconvolution to determine the chemical shift (Fig. 6).

The CSAs derived from plots such as those in Figs. 4 and 6 are given in Table I. Experimentally indistinguishable chemical shift anisotropies were derived from samples prepared in ether- and ester-linked lipids. All of the carbonyl CSAs in even amino acids, with the exception of *D*-leu^{10}, which has the lowest CSA, are similar and below the values for the amino acids in odd positions. The resonances of the *D*-leu^{10}, *D*-leu^{12}, and *D*-leu^{14} analogues have a width at half-height of 5–6 ppm, approximately half the width of the gly^2 and *D*-leu^4 resonances and those of the gramicidins with ^{13}C in the carbonyls of the odd amino acids, ala^3 and val^7.

Carbonyl spectra recorded at temperatures several degrees below the lipid gel-to-liquid crystalline phase transition, for bilayers oriented with the long axis of the lipid molecules parallel to the spectrometer field (Fig. 7), did not differ significantly from those shown in Figs. 2 and 5 for the gly^2, ala^3, *D*-leu^4, and val^7 analogues: there were no changes in the peak positions and little or no change in the peak widths. But the peaks of the other leucine analogues approximately doubled in width, to 18 ± 2 ppm, in gel-phase lipid (Fig. 7), suggesting that all labeled carbonyls possess similar motional freedom in lipid in the gel phase.

DISCUSSION

In a recent paper we had shown that gramicidin rapidly rotates about the lipid bilayer normal and that the orientation of the peptide carbonyl bonds is consistent with the $\beta^{6.3}$ helix model for gramicidin. These conclusions were based on spectra obtained with analogues labeled in gly^2, ala^3 and val^7, residues which are located towards the center of the lipid bilayer in the head-to-head dimer of single helices. These initial observations have been extended by synthesis of additional analogues labeled in each of the *D*-leu residues, three of which are located close to the bilayer surface. The data from these additional analogues allows a clearer distinction between the behavior of odd and even amino acids and shows a clear difference between the motion of residues close to the bilayer center and surface: the lower linewidths of the *D*-leu^{10}, *D*-leu^{12}, and *D*-leu^{14} resonances are not attributable to effects of the large aliphatic sidechain, but to greater motional freedom compared with *D*-leu^4.

The broad conclusions drawn in the earlier work (Cornell et al., 1988) are supported by the results presented here: thus, the CSAs from all labeled regions are consistent with the carbonyl bond orientation expected for a $\beta^{6.3}$ helix undergoing rapid rotation about the bilayer normal. From recent ^{15}N NMR studies Cross and his colleagues

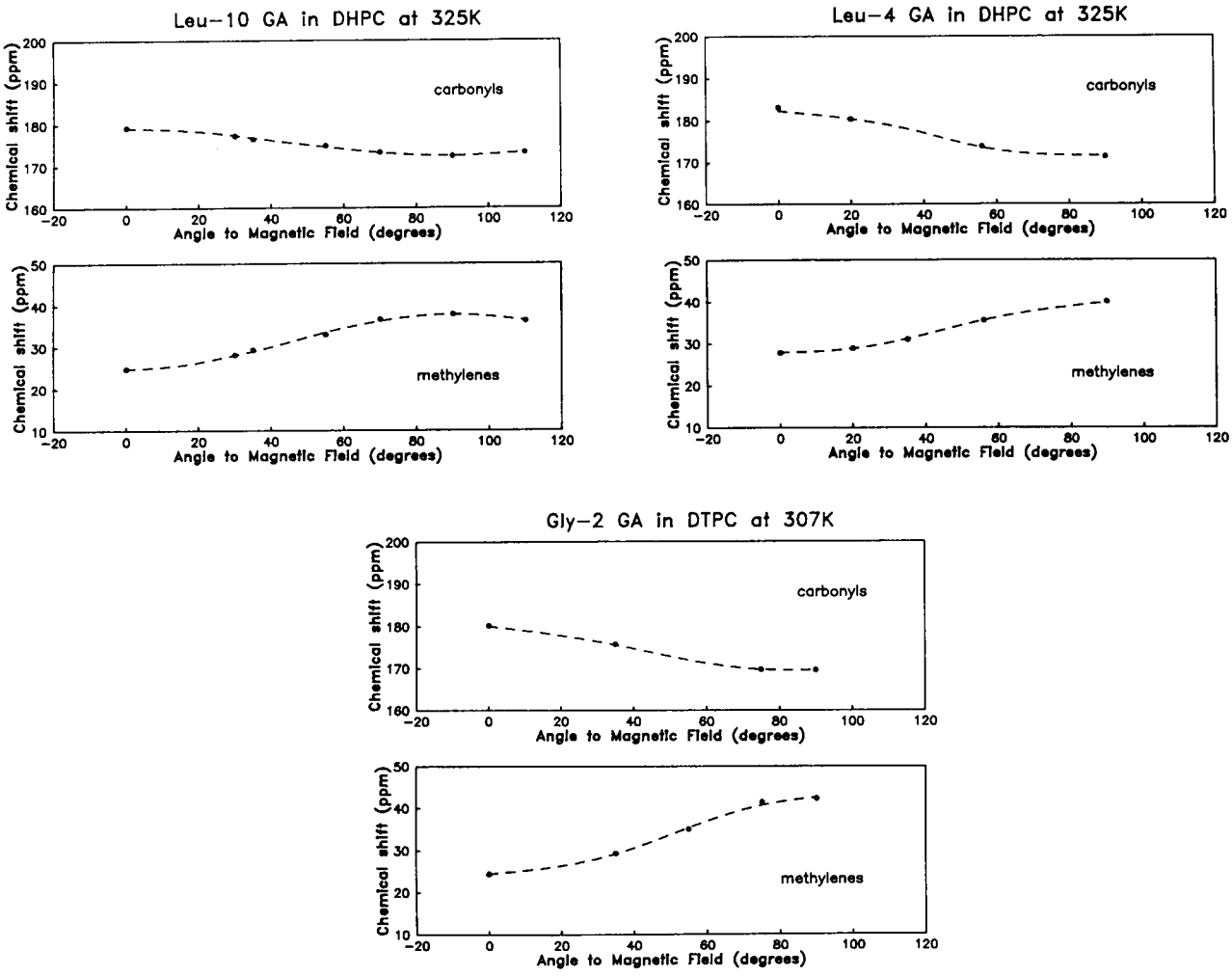

FIGURE 6 Representative plots of chemical shift anisotropies for gramicidin in aligned multilayers of the ether-linked lipids, DTPC and DHPC. Data are presented for the gramicidin carbonyl resonances and for the bulk methylene resonance which arises from nuclei present at natural abundance in both the peptide and the lipid.

(Nicholson et al., 1987; Fields et al., 1988) have also concluded that gramicidin rotates about an axis perpendicular to the bilayer plane, with the peptide bond *N-H* axis approximately parallel to the molecular rotation axis. The close correlation between the alignment and motion in different segments of the peptide is a reflection of the relative rigidity of the molecule that is maintained by extensive intramolecular hydrogen bonding along the helix axis. Davis (1988) reached the same conclusion from observations of the temperature independence of the quadrupole splittings of deuterium-exchanged gramicidin in a liquid crystalline phase formed by potassium laurate/decanol/KCl/water. However, the molecule is not without flexibility; the narrower linewidths of the *D*-leu[10], *D*-leu[12], and *D*-leu[14] resonances implies that either these carbonyls are more mobile or that this outer segment of the gramicidin molecule does not display the conformational heterogeneity postulated to explain the broad 0° angle peak observed for the gly[2], ala[3], and val[7] analogues (Cornell et al., 1988). As on transition of the lipid to the gel state the linewidth of these *D*-leucine analogues increased to the value observed for the other gramicidins, the latter explanation for the linewidth would require induction of configurational heterogeneity in the outer half of the molecule on passing from the liquid crystalline phase to the gel phase: thus, the former explanation appears more plausible. As the CSAs are similar for all of the leu-labeled analogues (except *D*-leu[10]) it is unlikely that the angles through which the carbonyls move differ substantially along the peptide chain, and the reduced linewidth is possibly attributable to differences in the rates of motion.

TABLE 1 Reduced chemical shift anisotropies (CSA) and linewidths from the labeled carbonyl sites in gramicidin A analogues, and CSAs for lipid carbonyl and methylene resonances

Label position	Lipid	Gramicidin CSA	Gramicidin linewidth	Lipid CSAs	
				Carbonyl	Methylene
		ppm	*Hz*		
gly[2]	DMPC, DTPC, DMPC	11 ± 2	800 ± 100	27 ± 1	17 ± 1
ala[3]	DTPC, DHPC	16 ± 1	800 ± 100	28 ± 1	15 ± 1
D-leu[4]	DMPC	12 ± 2	800 ± 100	25 ± 1	14 ± 1
val[7]	DMPC	16 ± 1	800 ± 100	30 ± 1	15 ± 1
D-leu[10]	DMPC, DHPC	9 ± 2	400 ± 70	25 ± 1	16 ± 1
D-leu[12]	DMPC	11 ± 1	400 ± 70	25 ± 1	16 ± 1
D-leu[14]	DMPC, DHPC	13 ± 1	400 ± 70	27 ± 1	14 ± 1

Measurements were performed on samples containing a 1:15 peptide/lipid molar ratio at 307 K in DMPC and DTPC, and at 325 K in DHPC.

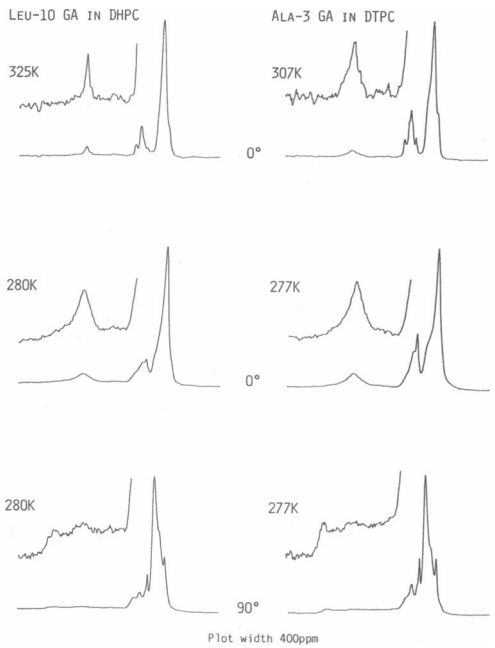

FIGURE 7 Comparison of representative spectra recorded at temperatures above and below the gel-to-liquid crystalline transition temperature. Spectra below the transition temperature are shown for samples aligned at 0° and at 90°.

D-leu[10] has a reproducibly lower CSA than the remaining *D*-leu residues. The reason for this lower CSA has not been established, but it may be significant that if the peptides adopt a right-handed helical geometry the leucine carbonyls would point outwards from the bilayer center with the possibility of *D*-leu[10] hydrogen bonding to the COOH-terminal ethanolamide group, which would place this residue in a unique environment.

The higher mobility of segments of the peptide closer to the bilayer surface contrasts with the order and rates of motion of the groups in the lipid acyl chains that are consistent with greater flexibility near the center of the bilayer. Vogel et al. (1988) have however similarly observed, using time-resolved fluorescence anisotropy measurements, that the ends of a helical peptide traversing a lipid bilayer are conformationally more flexible than the center of the helix. The carbonyl groups located close to the channel mouth are perturbed in the presence of univalent ions (Urry et al., 1983; Urry et al., 1984; R. Smith, D. E. Thomas, F. Separovic and B. A. Cornell, unpublished results) in a manner which suggests that they form the ligands binding cations in the channel. The greater motional flexibility of these carbonyls may contribute to their accommodation of a wide range of cations.

This work has been partly funded by a Australian Research Grants Scheme grant to R. Smith, and a Generic Industrial Research and Development Board Grant to B. A. Cornell.

Received for publication 29 December 1988 and in final form 10 April 1989.

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