

# Structure and Dynamics of the Antibiotic Peptide PGLa in Membranes by Solution and Solid-State Nuclear Magnetic Resonance Spectroscopy

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**ABSTRACT** PGLa, a 21-residue member of the magainin family of antibiotic peptides, is shown to be helical between residues 6 and 21 when associated with detergent micelles by multidimensional solution nuclear magnetic resonance (NMR) spectroscopy. Solid-state NMR experiments on specifically  $^{15}\text{N}$ -labeled peptides in oriented phospholipid bilayer samples show that the helix axis is parallel to the plane of the bilayers.  $^{15}\text{N}$  solid-state NMR powder pattern line shapes obtained on unoriented samples demonstrate that the amino-terminal residues are highly mobile and that the fluctuations of backbone sites decrease from Ala6 toward the carboxy terminus. The powder pattern observed for  $^{15}\text{N}$ -labeled Ala20 is essentially that expected for a rigid site. These findings are similar to those for the 23-residue magainin2 peptide in membrane environments.

## INTRODUCTION

PGLa (peptide starting with a glycine and ending with a leucine amide) is a member of the magainin family of antibiotic peptides found in frog skin and its secretions (Hoffman et al., 1983; Gibson et al., 1986; Zasloff, 1987). It is a 21-residue peptide with an amidated carboxy terminus (GMASKAGAIAGKIAKVALKAK). Like many other polypeptides associated with frog skin, the magainin family of peptides have close relationships with mammalian hormones and neurotransmitters (Bevins and Zasloff, 1990). Because of the interest in the antibiotic activities of these peptides, a major focus of research has been to provide a chemical and structural basis for the selective ability of these peptides to lyse a wide variety of bacteria, fungi, viruses, and tumor cells (Zasloff et al., 1988; Aboudy et al., 1994; Ohsaki et al., 1992; Baker et al., 1993). In contrast to other amphiphilic peptides, such as the bee venom melittin, magainins do not exhibit hemolytic activity (Bevins and Zasloff, 1990). Magainins have been shown to interact with acidic phospholipid membranes and to decouple transmembrane electrochemical gradients; when added to phospholipid model membranes they induce ion flux with some of the features associated with anionic-specific channel formation (Cruciani et al., 1992; Duclouhier, 1994). Antibiotic activity is correlated with their ability to decouple the respiratory chain of sensitive organisms as well as of liposomal systems reconstituted in the presence of cytochrome oxidase (Juretic et al., 1994; Westerhoff et al., 1989a,b).

Peptides in solution or associated with detergent micelles in solution may reorient rapidly enough in solution so that they can be investigated by multidimensional solution NMR spectroscopy. Magainin2 has been shown to form an  $\alpha$ -helix with this approach (Marion et al., 1988; Gesell et al., 1997). In contrast, peptides bound to phospholipid bilayers typically undergo slow anisotropic reorientation and are not suitable for solution nuclear magnetic resonance (NMR) spectroscopy. Therefore, solid-state NMR spectroscopy is the method of choice for these samples. In the absence of rapid molecular reorientation the anisotropy of the nuclear spin interaction tensor affects many spectral features. In solid-state (NMR) experiments on unoriented samples it provides valuable information about global and local dynamics. When polypeptides are incorporated into oriented membrane bilayer samples, the angular dependence of the NMR interactions can be used to determine the alignment of peptide planes, side chains, or secondary structure elements with respect to the direction of the bilayer normal (Bechinger et al., 1991, 1996).

## MATERIALS AND METHODS

PGLa peptide samples were synthesized by solid-phase peptide synthesis utilizing t-Boc chemistry.  $^{15}\text{N}$ - or  $^2\text{H}$ -labeled amino acids (Cambridge Isotope Laboratories, Andover, MA) were incorporated into specific positions in individual syntheses. Solution NMR spectroscopy was performed on a sample of 3.6 mM PGLa, 375 mM perdeuterated dodecylphosphocholine (Cambridge Isotope Laboratories) in 550  $\mu\text{l}$  of 5 mM citrate- $\text{d}_4$ , pH 3.2, in water ( $\text{H}_2\text{O}:\text{D}_2\text{O}$ , 90%:10%). An additional set of spectra was obtained using perdeuterated sodium dodecyl sulfate as the detergent (Cambridge Isotope Laboratories). Two-dimensional double quantum filtered correlation spectroscopy (DQF-COSY) (Aue et al., 1976) as well as MLEV-TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985) and nuclear Overhauser effect spectroscopy (NOESY) spectra in ( $\text{H}_2\text{O}:\text{D}_2\text{O}$ , 90%:10%) (Macura and Ernst, 1980) were acquired on Bruker AMX600 and AMX500 NMR spectrometers. Typical experimental parameters were as follows: 64 scans averaged for each  $t_1$  value following 2 dummy scans, spectral width of 10 kHz, 4000 data points, 512  $t_1$  values, selective water presaturation during the 1.5-s recycle delay, and mix times

Received for publication 8 August 1997 and in final form 5 November 1997.

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0006-3495/98/02/981/07 \$2.00

of 150 ms for the NOESY and 100 ms for the MLEV experiments. The sample temperature was maintained at 323 K.

For solid-state NMR experiments, 10–45 mg of labeled peptide were dissolved in water and the pH adjusted to 7 in Tris buffer. Phospholipids approximating the composition of bacterial membranes 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPE/POPG; 3:1; Avanti Polar Lipids) and peptide were co-dissolved in trifluoroethanol/dichloromethane/water. This solution was slowly applied to thin glass plates (11 mm × 22 mm). After removing residual solvent under high vacuum overnight, the samples were equilibrated in a closed chamber at 93% relative humidity. The glass plates were then stacked. The orientation of the resulting bilayer samples was monitored with  $^{31}\text{P}$  NMR spectroscopy. Solid-state  $^{15}\text{N}$  NMR spectra were obtained on spectrometers with  $^1\text{H}$  resonance frequencies of 360 MHz and 400 MHz. A conventional solenoidal coil probe was used for unoriented samples. A flat coil double-resonance probe was used for the oriented samples. Single contact cross-polarization with MOIST phase alternation (Levitt et al., 1986; Pines et al., 1973) was used to generate the  $^{15}\text{N}$  signals. Typical experimental parameters were as follows: 1.6-ms cross-polarization mix time, 6.4-ms acquisition time for 256 data points, >1.1 mT  $^1\text{H}$  decoupling field during acquisition, 3-s recycle delay, and 20,000 transients were acquired for signal averaging. For those samples with mobile  $^{15}\text{N}$  sites, direct pulsed spin-echo experiments were performed with an echo delay of 40  $\mu\text{s}$  and a recycle delay of 5 s (Rance and Bryd, 1983). The samples were maintained at room temperature with a stream of humidified air during data acquisition. The NMR data were processed using the program FELIX (Biosym, San Diego, CA) on Silicon Graphics workstation computers.

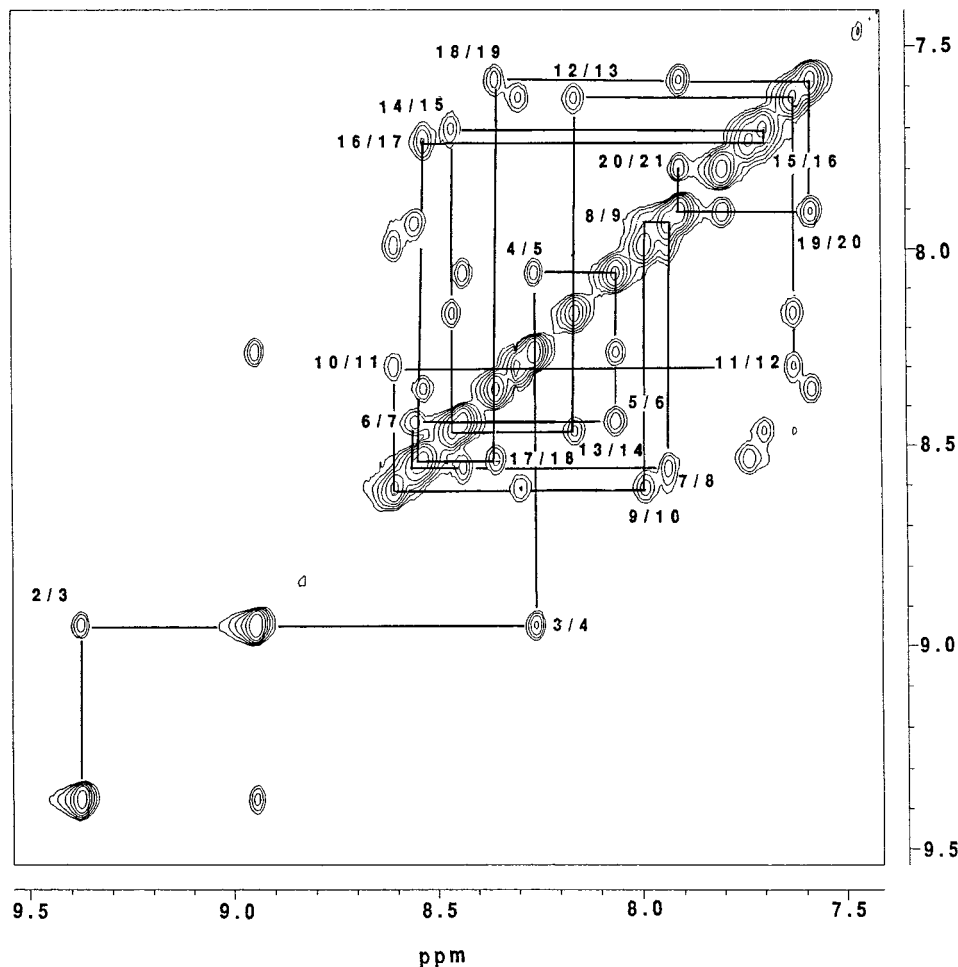
## RESULTS

The results of several multidimensional solution NMR experiments on micelle samples were used to describe the secondary structure of PGLa. Figs. 1 and 2 show the NH-NH and NH- $\text{C}_\alpha\text{H}$  regions, respectively, of a two-dimensional homonuclear NOE spectrum of PGLa in dodecylphosphocholine (DPC) micelles in  $\text{H}_2\text{O}$  solution. The  $^1\text{H}$  amide resonances in Fig. 1 have line widths of 15–20 Hz, typical of a large protein or a protein complex rather than a peptide in aqueous solution, and this indicates that PGLa is strongly associated with the micelles.

Individual spin systems were identified in two-dimensional TOCSY and DQF-COSY spectra in solution (Wuthrich, 1986). Starting with Met2, the continuous network of amide NH-NH NOE cross-peaks enables the direct sequential assignment of the amide N-H resonance for all residues. Spectra of  $^{15}\text{N}$ -Ala10 and [ $^{15}\text{N}$ -Ala20,  $\text{CD}_3$ -Ala6]-PGLa confirmed the assignments of this 21-residue peptide, which was reassuring because the amino acid composition (7 Ala, 4 Lys, and 3 Gly residues) presents some difficulties due to the high redundancy of spin systems.

Fig. 3 summarizes the short-range constraints obtained from the NMR data involving the amide hydrogens. The

FIGURE 1 The amide region of the two-dimensional homonuclear  $^1\text{H}$  NOE spectrum of PGLa in DPC micelles. The lines map the sequential assignments of the amide N-H resonances starting at Met2 (9.38ppm). The assignments of the  $\text{NH}_i\text{-NH}_{i+1}$  cross-peaks are labeled.



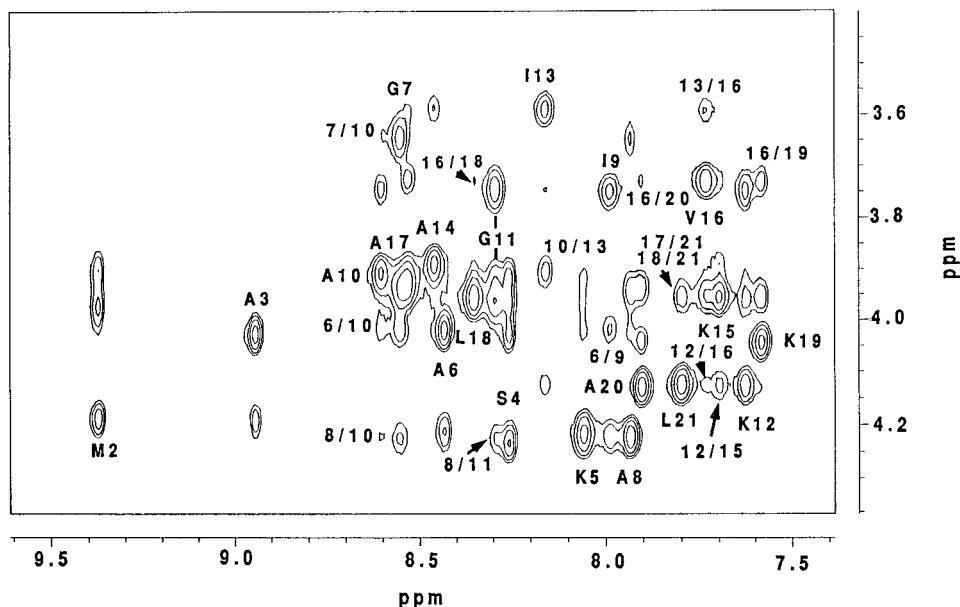


FIGURE 2 The fingerprint region of the two-dimensional homonuclear <sup>1</sup>H NOESY spectrum of PGLa in DPC micelles. The assignments of the intraresidue C<sub>α</sub>H-NH interresidue cross-peaks indicative of α-helical secondary structure are labeled.

observation of numerous NOEs between NH and C<sub>α</sub>H resonances from sites three (*i* + 3) and four (*i* + 4) residues apart are highly characteristic of an α-helical conformation. This pattern extends from Ala6 to the carboxamide terminus. The chemical shifts of C<sub>α</sub> hydrogens, with the exception of Gly11, have values consistent with helical but not extended conformations (Wishart et al., 1992) and are shown in Fig. 3 by their digital values. Spectral overlap in the methyl and methylene regions of the NOESY spectra, as well as interference from signals from residual hydrogens in the nominally perdeuterated detergent, precludes the assignment of all side chain hydrogen resonances. Nonetheless, a large number of cross-peaks between NH resonances of residue *i* and side chain hydrogens of residues *i* + 3 or *i* + 4, respectively, have been identified among residues 10–17 and confirm that they have an α-helical conformation.

Structured residues of peptides strongly associated with lipid bilayers are immobile on even the slowest relevant NMR time scales (10<sup>-4</sup> s) determined by the frequency breadth of the <sup>15</sup>N amide chemical shift anisotropy powder pattern. All orientations are present in unoriented samples, and the powder pattern line shape is characterized by a span of approximately 170 ppm (Fig. 4 D). The magnitude and orientation of the principal elements of the amide <sup>15</sup>N chemical shift tensor have been characterized in model peptides (Wu et al., 1995). The principal tensor elements have been shown to be largely independent of the chemical nature of the side chains; therefore the <sup>15</sup>N chemical shift interaction is well suited for analysis of the global and local dynamics as well as the structure of membrane-associated polypeptides.

The breadth of a powder pattern decreases when the labeled site undergoes large-amplitude motions fast enough

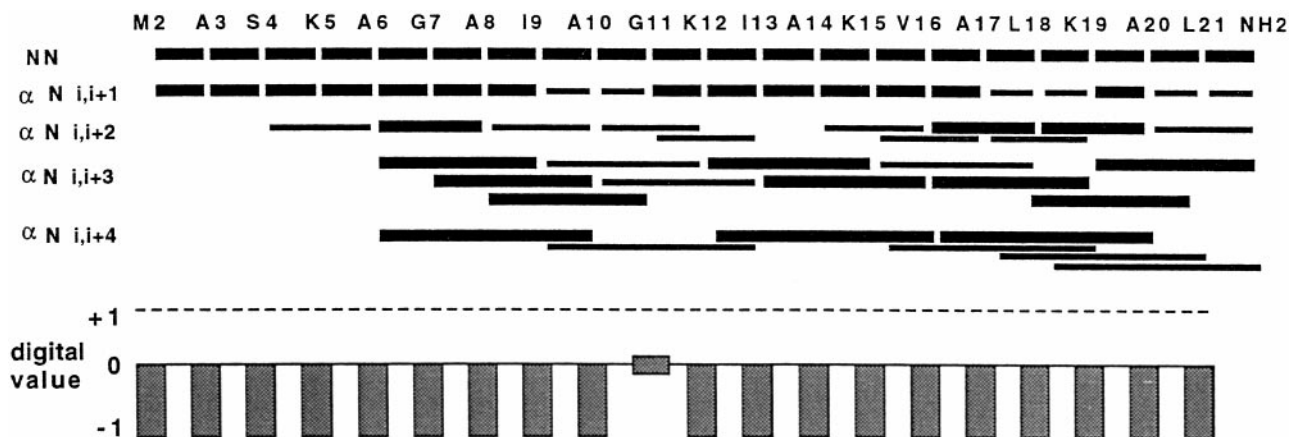


FIGURE 3 Summary of the short-range NMR parameters for PGLa in DPC micelles (cf. Figs. 1 and 2). Unambiguously assigned cross-peaks are shown as bold lines; spectral overlap in some regions meant that the most probable connectivities are indicated (thin lines). The high density of negative digital values in the chemical shift index in the middle portion of the figure has been correlated with α-helical conformations of proteins in aqueous solution (Wishart et al., 1992).

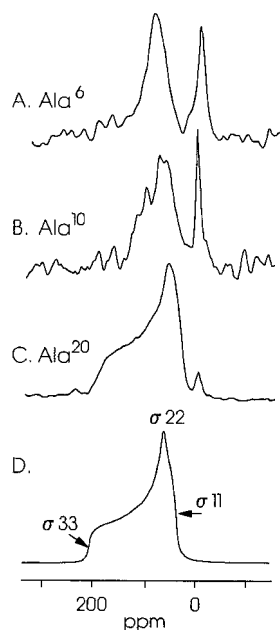


FIGURE 4 Solid-state NMR spectra of unoriented samples of  $^{15}\text{N}$ -labeled PGLa in lipid bilayers. The peptides are labeled at the indicated sites. (A) Ala6; (B) Ala10; (C) Ala20; (D) Simulated powder pattern line shape of a static  $^{15}\text{N}$  amide bond. The three discontinuities of the powder pattern corresponding to the principal chemical shift tensor elements are indicated.

to average the chemical shift interaction. Motional averaging affects dipole-dipole as well as chemical shift interactions; thus  $^{15}\text{N}$  amide sites undergoing extensive motional averaging are characterized by low cross-polarization efficiency as well as narrow resonance intensity at the isotropic frequency. An unoriented sample of PGLa with a  $^{15}\text{N}$  amide label at residue 20 incorporated into POPE/POPG (3:1) phospholipid bilayers and hydrated at 93% relative humidity yields a powder pattern line shape with a breadth of 152 ppm (Fig. 4 C), only slightly reduced from that of a static powder pattern observed for polycrystalline peptides and simulated in Fig. 4 D. In contrast, relatively narrow isotropic resonance intensity with approximately 40 ppm line width is observed for PGLa labeled at residues 3 (data not shown) or 6 (Fig. 4 A) under the same experimental conditions. The  $^{15}\text{N}$  solid-state NMR spectra of peptides labeled at residues in the central portion exhibit line shapes with intermediate breadths, increasing from the amino toward the carboxy terminus (Figs. 4 B and 5). The isotropic lines near 0 ppm arise from natural abundance  $^{15}\text{N}$  in the amino groups of phosphatidylethanolamine and lysine side chains. The extensive motional averaging observed for residues near the amino terminus of the PGLa lipid bilayers is consistent with the absence of interresidue NOE cross-peaks at the same positions in the micelle sample.

Representative solid-state NMR spectra of oriented bilayers containing specifically  $^{15}\text{N}$ -labeled PGLa peptides are shown in Fig. 6. The sample labeled at Ala3 exhibits a chemical shift value of approximately 100 ppm, the isotropic value of the  $^{15}\text{N}$  amide chemical shift tensor, in agree-

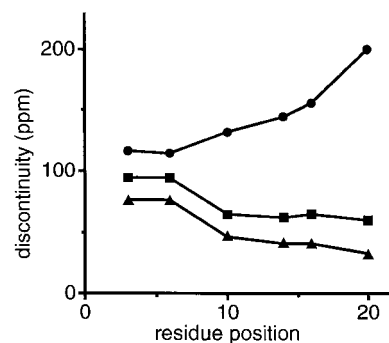


FIGURE 5 The observed values for the three discontinuities of the  $^{15}\text{N}$  chemical shift powder patterns, from PGLa in lipid bilayers.  $\sigma_{11}$  ( $\blacktriangle$ ),  $\sigma_{22}$  ( $\blacksquare$ ), and  $\sigma_{33}$  ( $\bullet$ ) are depicted as a function of residue position (see Fig. 4 for details). The magnitude of the difference between  $\sigma_{11}$  and  $\sigma_{33}$  corresponds to the breadth of the observed anisotropy powder pattern.

ment with the findings on unoriented samples (Figs. 4 A and 5). The high mobility of the amino-terminal amino acids is also reflected in the difficulty in obtaining signals through cross-polarization. The  $^{15}\text{N}$  NMR spectrum shown in Fig. 6 B was obtained using direct pulsed excitation followed by a spin-echo to reduce spectral distortion. The peptides labeled at the Ala10, Ala14, Val16, or Ala20 positions, respectively, exhibit  $^{15}\text{N}$  solid-state NMR spectra characteristic of structured peptides; the chemical shift frequencies occur near the high-field end of the  $^{15}\text{N}$  chemical shift anisotropy

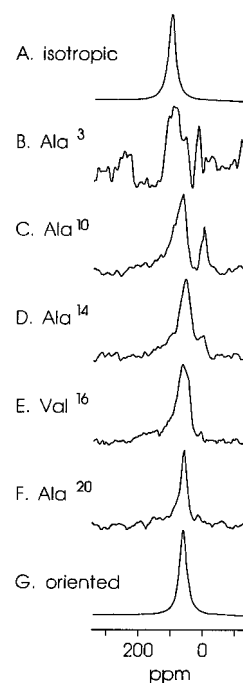


FIGURE 6 Proton-decoupled  $^{15}\text{N}$  solid-state NMR spectra of PGLa incorporated into oriented POPE/POPG 3/1 phospholipid bilayers. The PGLa concentrations in spectra B–E are 2.3 mol % and in spectrum F, 1.5 mol %. Also shown are simulated  $^{15}\text{N}$  solid-state NMR spectra in the presence of fast motional averaging (A) and of an amide site that is part of an  $\alpha$ -helix oriented parallel to the bilayer surface (E).

powder pattern and are indicative of an orientation of the individual N-H bonds approximately perpendicular to the magnetic field direction. As the solution NMR data show PGLa to be helical, these solid-state NMR data from oriented bilayer samples indicate that the helix axis of PGLa is parallel to the plane of the bilayers, which is arranged to be perpendicular to the direction of the applied magnetic field.

## DISCUSSION

The results from the multidimensional solution NMR experiments indicate that PGLa is highly helical in micelles, with the exception of a few residues near the amino terminus. This is in agreement with results obtained by circular dichroism and Raman and infrared spectroscopies (Duclohier et al., 1989; Williams et al., 1990; Jackson et al., 1992). The helical secondary structure results in a distinctly amphipathic molecule, with polar and hydrophobic residues on opposite sides. Like other magainin peptides, PGLa exhibits good solubility in aqueous buffers where it appears to be unstructured (Duclohier et al., 1989; Williams et al., 1990; Jackson et al., 1992). The strong interactions of the peptide with membranes are likely a consequence of its adopting an amphipathic helical structure in the presence of lipids. When arranged in an in-plane orientation, such a conformation reflects the distribution of hydrophobic and charged functional groups along the membrane interface in a manner that is fully consistent with the arrangement of polar and hydrophobic groups in the lipid bilayers (Hoffman et al., 1983).

The analysis of NOE cross-peaks as well as  $^{15}\text{N}$  solid-state NMR spectra of both oriented and nonoriented samples show that the amino terminus of PGLa is unstructured and mobile. In contrast, all of these same parameters are consistent in indicating that the  $\alpha$ -helix extends from Ala6 through to the carboxy terminus. The observed  $^{15}\text{N}$  powder pattern line widths depict a continuous decrease in mobility from Ala6 to Ile20. The large breadths observed for the carboxyl-terminal residues, therefore, show that this part of the polypeptide is fully immobilized by its interactions with the membrane. On the other hand, the partial averaging of the anisotropy in the central residues and the nearly complete flexibility of the amino-terminal residues suggest that more global motions, such as a wobbling or bending of the peptide, are present, in addition to local motions associated with the residues not in the stable helical portion of the molecule.

Previous analysis of the conformation and orientation of charged amphiphilic  $\alpha$ -helical polypeptides, such as magainin2 (Bechinger et al., 1993), melittin (Stanislawski and Ruterjans, 1987; Frey and Tamm, 1991), and designed amphipathic polypeptides (Bechinger et al., 1998), indicate that many of them orient parallel to the membrane surface. This is in contrast to the more hydrophobic sequences of the peptide corresponding to channel-lining fragment M2 $\delta$  of the acetylcholine receptor (Bechinger et al., 1991; Opella et

al., 1997), designed hydrophobic helices (Bechinger et al., 1996), the transmembrane helices of bacteriorhodopsin (Henderson et al., 1990), or the hydrophobic helices of the photoreaction center (Deisenhofer et al., 1985). When the physiological and biochemical properties of the magainins are compared with hydrophobic peptide systems, striking differences become apparent. These include the solubility in water and the channel-forming properties that are characterized by comparatively large conductance levels with pronounced variability even within an individual experiment (Duclohier, 1994; Cruciani et al., 1991).

It is unlikely that PGLa lyses bacterial cells through interactions with a protein receptor. A wide variety of lysine-rich amphipathic  $\alpha$ -helical peptides consisting of 10–25 residues either of L- or, notably, D-amino acids (Wade et al., 1990) show bactericidal activity at millimolar concentrations (Chen et al., 1988; Cuervo et al., 1990; Cornut et al., 1994). No primary sequence homology can be detected between the magainin family and many of its active derivatives (Bevins and Zasloff, 1990). And magainins and derivatives are active against a wide variety of organisms, including bacteria, fungi, and eukaryotic tumor cells (Zasloff et al., 1988; Baker et al., 1993; Cruciani et al., 1991; Ohsaki et al., 1992).

The possibility remains that magainin peptides have an orientation in-plane with the membrane surface under the experimental conditions used in the NMR experiments but that transmembrane electric fields or the lipid compositions of sensitive cells results in these peptides adopting transmembrane orientations concomitant with channel formation (Matsuzaki et al., 1995, 1996). Models of this kind have been suggested for other membrane-active polypeptides, such as alamethicin or hemolysin (Fox and Richards, 1982; Raghunathan et al., 1990). The structure and orientation of magainins have been examined as a function of the lipid to peptide ratio, temperature, and degree of hydration. None of the solid-state NMR spectra show significant signal intensities near the low-field frequencies of the chemical shift anisotropy powder pattern, which would indicate the presence of transmembrane orientations of helical peptides.

All of the NMR data are consistent in showing that the peptides are helical in membrane environments. The  $\alpha$ -helix is much too short to span the hydrophobic part of the lipid bilayer. Moreover, designed derivatives of the magainin family with as few as 12 residues have been shown to exhibit antimicrobial activity. Solid-state NMR spectroscopy indicates that these helices are also parallel to the membrane surface (Bechinger et al., 1998). Recent models of the magainin pore therefore suggest that these peptides assume in-plane orientations along the bilayer surface (Vaz Gomez et al., 1993; Matsuzaki et al., 1995; Ludtke et al., 1996) in agreement with the NMR results on all of these systems. In these models the transient reorientation of magainin peptides results in the formation of transmembrane pores sufficiently large for fluorescence marker dyes or ions to pass.

We thank Howard Eck for help with peptide synthesis and Jennifer Gesell for advice.

The research of S. J. Opella was supported by grants RO1GM29754 and R37GM24266 from the National Institutes of Health. The research of M. Zasloff was supported by grants from the G. Harold and Leila Y. Mathers Foundation. B. Bechinger was supported by an EMBO long-term fellowship (ALTF 454-1989). This research utilized the Resource for Solid-State NMR of Proteins at the University of Pennsylvania: An NIH-supported research center, supported by Grant P41 RR097393.

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