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Structure and orientation of the antibiotic peptide magainin in membranes by solid-state nuclear magnetic resonance spectroscopy

BURKHARD BECHINGER,[1] MICHAEL ZASLOFF,[2,3] AND STANLEY J. OPELLA[1]

[1] Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104
[2] Departments of Pediatrics and Genetics, Division of Human Genetics and Molecular Biology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

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Abstract

Magainin 2 is a 23-residue peptide that forms an amphipathic α-helix in membrane environments. It functions as an antibiotic and is known to disrupt the electrochemical gradients across the cell membranes of many bacteria, fungi, and some tumor cells, although it does not lyse red blood cells. One- and two-dimensional solid-state ^{15}N NMR spectra of specifically ^{15}N-labeled magainin 2 in oriented bilayer samples show that the secondary structure of essentially the entire peptide is α-helix, immobilized by its interactions with the phospholipids, and oriented parallel to the membrane surface.

Keywords: α-helix; antibiotic peptide; magainin; membrane protein; oriented bilayers; protein structure; solid-state NMR spectroscopy

Antibiotic peptides are abundant in many organisms, forming a defense system exclusive of, or complementary to, the immune system (Bevins & Zasloff, 1990; Boman, 1991). Magainins are a family of 21–26-residue peptides that protect frogs from infections, even after severe injury, with a broad spectrum of antibacterial, antifungal, and tumoricidal activities (Zasloff, 1987; Cruciani et al., 1991). Although these peptides are water soluble, they interact with bacterial (Zasloff, 1987; Zasloff et al., 1988) and acidic (Matsuzaki et al., 1991) model membranes, suggesting that they function at membranes. Even stronger evidence that these peptides exert their effects at membranes comes from the finding that synthetic all-D-magainin has the same activities as synthetic or natural all-L-magainin (Bessalle et al., 1990; Wade et al., 1990), as well as the fact that they disrupt the electrochemical ionic gradient across cell membranes (Duclohier et al., 1989; Westerhoff et al., 1989; Cruciani et al., 1991).

Structural studies of magainins and other peptides that interact with membranes rely primarily on NMR spectroscopy. Multidimensional solution NMR experiments show that magainin 2 is unstructured and highly flexible in aqueous solution and nearly completely α-helical in the model membrane environments of dodecylphosphocholine micelles, dodecylsulfate micelles, and trifluoroethanol/water solutions (Marion et al., 1988; J. Gesell, M. Zasloff, & S.J. Opella, unpubl.). These experimental NMR results differ considerably from those of CD, infrared, and Raman spectroscopies, which suggest the presence of considerably lower amounts (0–60%) of α-helix in these same systems (Chen et al., 1988; Wade et al., 1990; Williams et al., 1990; Jackson et al., 1992).

There are questions outstanding concerning the mechanism of action and selectivity of magainins that can only be answered through detailed studies of their structures and interactions with phospholipids in bilayer systems. Peptides associated strongly with lipid bilayers are not amenable to multidimensional-solution NMR experiments because of their slow overall reorientation rates. Therefore, solid-state NMR methods, which are are well suited for determining the structures and describing the dynamics of immobilized biopolymers, must be employed (Smith & Peersen, 1992; Opella, 1993). Solid-state NMR structural studies utilizing oriented samples have been applied successfully to a variety of helical proteins and peptides

Reprint requests to: Stanley J. Opella, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104.
[3] Present address: Magainin Pharmaceuticals Inc., 5110 Campus Drive, Plymouth Meeting, Pennsylvania 19462.

associated with lipid bilayers (Bechinger et al., 1991, 1992; Shon et al., 1991a,b; McDonnell et al., 1993; Opella et al., 1993). Solid-state NMR spectroscopy has also been used to measure distances for structural studies of unoriented samples (Smith & Griffin, 1988), including magainins (Blazyk et al., 1991). This paper substantially extends our previous NMR studies of magainin 2 (Bechinger et al., 1991, 1992; Shon et al., 1991b; Opella et al., 1993; Gesell et al., unpubl.), focusing on the determination of the secondary structure and orientation of the peptide in hydrated phospholipid bilayers.

Results

The anisotropies of chemical-shift and dipole–dipole interactions are averaged out by rapid molecular reorientation in solution. However, they are present in immobile samples, such as hydrated phospholipid bilayers, where they serve as valuable sources of both structural and dynamic information. In the one-dimensional ^{15}N solid-state NMR spectra presented in Figure 1, the heteronuclear $^{1}H/^{15}N$ dipole–dipole interactions are decoupled by high-power radiofrequency irradiation at the ^{1}H resonance frequency, therefore all of the observed spectral features arise from the ^{15}N chemical-shift interaction. The magnitudes and orientations in the molecular frame of the principal elements of the amide ^{15}N chemical-shift tensor have been characterized for several model peptides and proteins (Harbison et al., 1984; Hartzell et al., 1987; Oas et al., 1987; Shoji et al., 1989; Teng & Cross, 1989), providing a basis for the interpretation of the observed ^{15}N chemical-shift spectral parameters. In an oriented sample, the ^{15}N resonance frequency can have a value between ~30 and 200 ppm, depending on the orientation of the peptide group with respect to the direction of the applied magnetic field in an oriented sample. All of these frequencies are present in an unoriented sample, as long as the peptide group is immobile on the timescale of the chemical-shift interaction (approx. 10^4 Hz), with the intensities distributed statistically in a characteristic powder pattern lineshape. If the peptide undergoes large-amplitude motions more frequently than 10^4 Hz, then narrow resonance intensity at the isotropic frequency is observed in both oriented and unoriented samples.

The ^{15}N NMR spectrum of ^{15}N-Ala 9-labeled magainin 2 in unoriented lipid bilayers is shown in Figure 1J. It is a powder pattern whose breadth is reduced slightly from that for a typical amide site in a rigid polycrystalline peptide, simulated in Figure 1K. This comparison indicates that only very limited motional averaging occurs when magainin 2 interacts with phospholipids. In the absence of phospholipids, the peptide is highly flexible in aqueous solution, and the ^{15}N resonance is a narrow single line (Shon et al., 1991b).

Because the peptide is immobilized by its interactions with the lipid bilayers, the chemical-shift and heteronu-

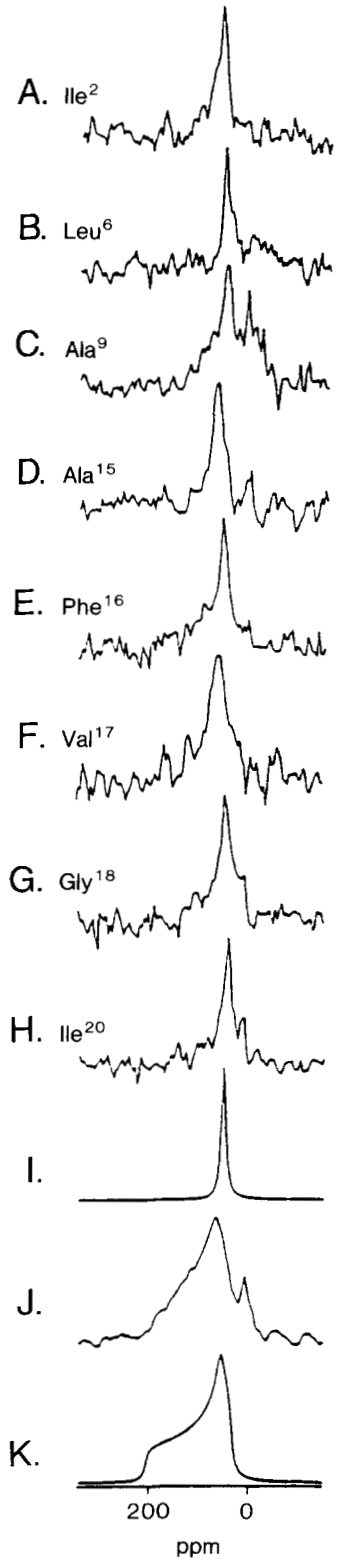

Fig. 1. The ^{15}N NMR spectra of magainin in phospholipid bilayers. **A–H:** Experimental spectra of magainin 2 peptides in oriented bilayers specifically ^{15}N-labeled at the designated residues. **I:** Simulated spectrum for a ^{15}N-labeled peptide oriented with its helical axis perpendicular to the direction of the applied magnetic field. **J:** Experimental spectrum of ^{15}N-Ala 9-labeled magainin 2 in unoriented bilayers. **K:** Simulated rigid lattice ^{15}N-amide powder pattern.

clear-dipolar interactions provide angular information, which can be interpreted in terms of structure, in oriented samples (Opella et al., 1987; Opella & Stewart, 1989). The ^{15}N NMR spectra obtained from samples of specifically ^{15}N-labeled magainin 2 peptides in lipid bilayers oriented between glass plates are presented in Figure 1A–H. Each spectrum has a single line from the one ^{15}N-labeled residue in the magainin 2 peptide whose resonance frequency is determined by the orientation of the labeled peptide group with respect to the applied magnetic field. The additional smaller upfield peak with a resonance frequency near 15 ppm, present in some of the spectra, arises from the natural abundance of ^{15}N in the amino groups of the phospholipid headgroups; the intensity of this peak is variable because of the sensitivity of the cross-polarization matching conditions to motional averaging at the amino sites, and it does not interfere with the analysis because it is recognized readily as separate from the amide resonance of interest.

All of the spectra in Figure 1 have ^{15}N resonance frequencies near the upfield principal element (σ_{33}) of the ^{15}N-amide chemical-shift tensor. Although a single spectral parameter, such as the ^{15}N chemical shift, is rarely sufficient to determine unambiguously the orientation of a peptide plane, only a limited set of orientations is consistent with a measured value (Opella et al., 1987; Opella & Stewart, 1989; Chirlian & Opella, 1990). Each experimental measurement restricts the possibilities for the orientations of a peptide plane, described by the two polar angles, α and β, and defined in Figure 2E. The dark areas in the restriction plots in Figure 2A–C indicate the α, β-angle pairs (corresponding to peptide-plane orientations) that are consistent with the ^{15}N resonance frequencies observed in the spectra (Fig. 1D–G) of the four different oriented magainin 2 samples. Although the precision and reproducibility of the experimental measurements are quite good (approx. 1 ppm), in order to ensure that no plausible orientations are missed in the initial steps in the analysis performed by the restriction plots, the calculations utilize very conservative estimates of experimental errors and uncertainties in the measurement of both the resonance frequencies in spectra from oriented samples and the magnitudes of the principal elements derived from spectra of unoriented samples. The combined restriction plot in Figure 2D shows the maximum angular dispersion consistent with data for any of the observed sites. Because the resonance frequencies for all of the sites are near σ_{33}, it is possible to qualitatively interpret these results, which indicate that the peptide planes have their N–H bonds approximately perpendicular to the direction of the applied magnetic field. Although magainin 2 has been shown to be an α-helix in model membrane environments by multidimensional solution NMR spectroscopy (Marion et al., 1988; Gesell et al., unpubl.), the data shown in Figure 1 and analyzed in Figure 2 enable the peptide to be modeled as an α-helix in the plane of the bilayer (Bechinger

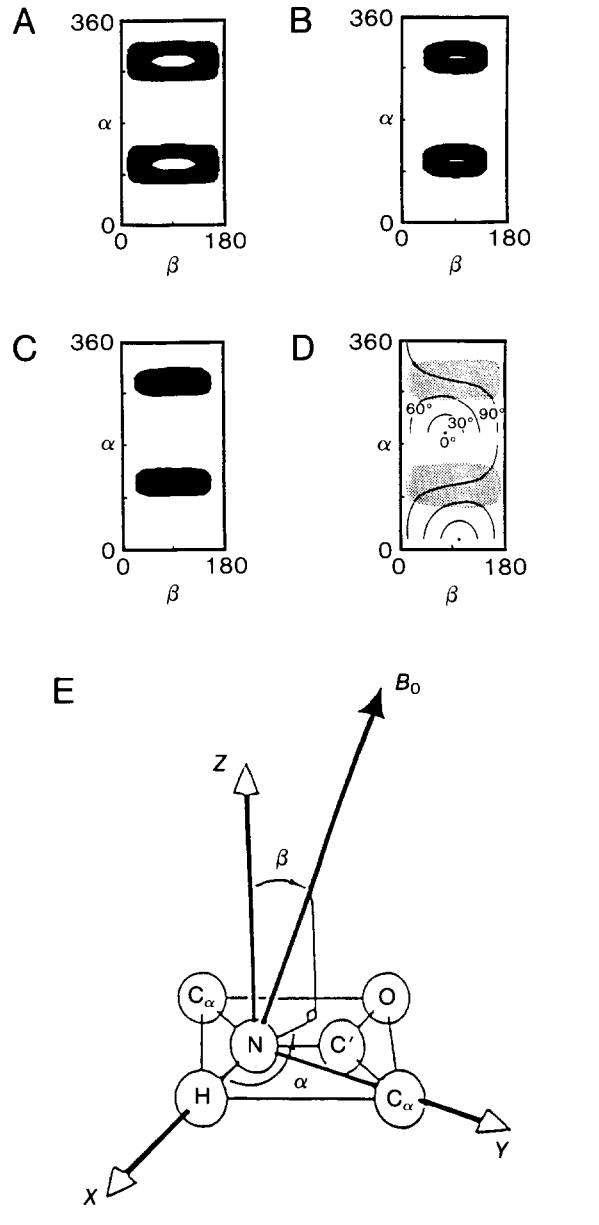

Fig. 2. Restriction plots for the ^{15}N chemical-shift measurements of **(A)** ^{15}N-Ala 15 and ^{15}N-Val 17, 57 ± 5 ppm; **(B)** ^{15}N-Phe 16, 47 ± 5 ppm; and **(C)** ^{15}N-Gly 18, 42 ± 5 ppm. The magnitudes of the principal values of the ^{15}N chemical-shift tensor used in these calculations were determined from four powder patterns of unoriented ^{15}N-magainin/lipid mixtures hydrated at 93% relative humidity. A set of line fits that resulted in noise after subtraction from the experimental spectrum was obtained for each powder pattern. The resulting values were: σ_{11} = 33.5 ± 5.5 ppm, σ_{22} = 68.5 ± 5.5 ppm, and σ_{33} = 188.5 ± 16.5 ppm. The angle between the σ_{11}-axis of the tensor and the N–H bond is 18.5 ± 6.5°. **D:** Graphical display of the pairs of α, β angles that describe α-helices oriented with their helix axis at 0°, 30°, 60°, and 90° with respect to the bilayer normal. The dark areas show all α, β-angles that are in agreement with chemical-shift values determined experimentally (42 ± 5 to 57 ± 5 ppm). **E:** Molecular axis system for the peptide plane. The X-axis is coincident with the N–H bond, the Z-axis is perpendicular to the plane, and the Y-axis is perpendicular to both X and Z. The orientation of the peptide plane with respect to the magnetic field, B_0, is given by the angles α and β as follows: β is the angle between B_0 and the Z-axis and, α is the angle between the X-axis and the projection of B_0 in the XY plane.

et al., 1991). Nonetheless, it is highly desirable to determine both the structure and the orientation of the peptide in the phospholipid environment itself.

In order to obtain the additional restrictions on the orientations of individual peptide planes necessary to determine independently the structure of the peptide in phospholipid bilayers by solid-state NMR spectroscopy, the magnitudes of the heteronuclear $^{15}N/^{1}H$ dipole–dipole couplings associated with each ^{15}N-labeled site were measured with two-dimensional separated local field experiments (Waugh, 1976). The results of these experiments for peptides specifically ^{15}N-labeled at Ala 15, Phe 16, Val 17, and Gly 18, four contiguous peptide linkages, are shown in Figure 3. The two-dimensional spectra are presented as contour plots, with the oriented ^{15}N chemical shift along one frequency axis and the oriented heteronuclear $^{15}N/^{1}H$ dipolar coupling along the other frequency axis. The one-dimensional chemical-shift spectra for these sites are aligned along the top of the two-dimensional spectra, and the projection of the doublet due to the heteronuclear dipolar coupling is aligned along the side for comparison. Two spectral parameters are available for each amide site from these experiments. For example, the data associated with Gly 18 indicate that its amide nitrogen has a heteronuclear $^{15}N/^{1}H$ dipole–dipole coupling of 7.5 kHz and a ^{15}N chemical shift of 42 ppm. The measured values of the heteronuclear $^{1}H/^{15}N$ dipolar couplings and the chemical shifts for all four sites are listed in Table 1.

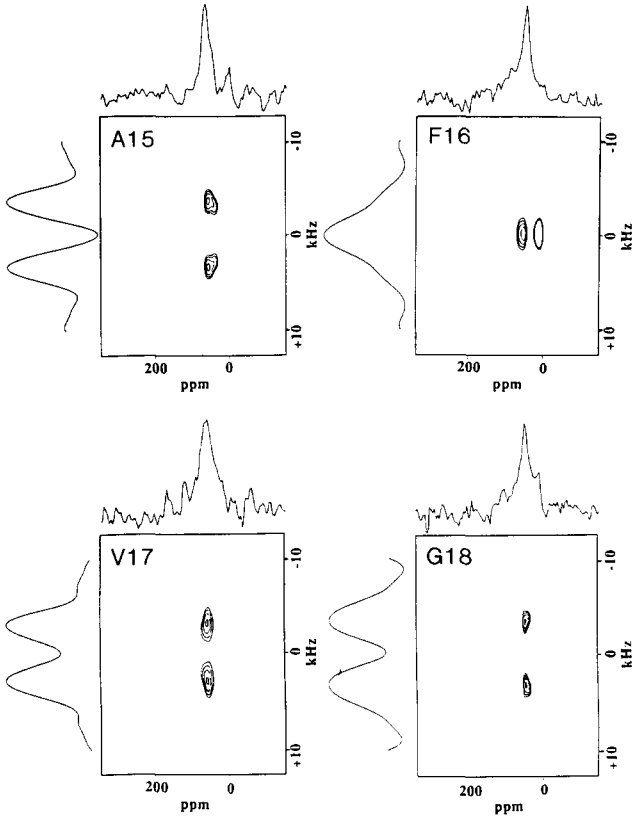

Fig. 3. Experimental results from two-dimensional separated local field experiments on four contiguous residues of magainin 2.

The dipolar and chemical-shift interactions are evaluated separately in terms of the restrictions they place on the orientations of the individual peptide planes. However, the orientation of each peptide plane has to be consistent with both experimental measurements; as a result, the intersection of the restriction plots has greatly reduced area. This is illustrated in Figure 4 for the experimental data shown in Figure 3, and listed in Table 1 for four contiguous residues in magainin 2.

The orientations of individual peptide planes with respect to the external axis system, where the direction of the magnetic field and the bilayer normal are arranged to be colinear by the sample geometry (Bechinger & Opella, 1991), provide the basic structural information for the computer program TOTLINK (Opella et al., 1987), which assembles the three-dimensional structures of polypeptides. In addition to the pairs of angles in the intersection of the individual restriction plots, this program takes into account the proper bond chemistry, spatial requirements of side-chain and backbone atoms, and the preferences of residues in proteins to adopt low-energy (secondary) structures in order to arrive at complete structures. The input to the program consisted of all the orientations for residues 15–18 consistent with the experimental data (Fig. 4). The program selected right-handed α-helix as the lowest energy secondary structure for these residues; significantly, no fit could be obtained for alternative left-handed α-helix or β-sheet conformations. This result, based solely on solid-state NMR experiments, is in agreement with two-dimensional solution NMR experiments for these same residues (Marion et al., 1988; Gesell et al., unpubl.) and provides strong evidence that magainin 2 has the same helical structure whether associated with micelles or bilayers. The TOTLINK evaluation of the solid-state NMR data not only finds the secondary structure of these four residues to be helical, but also that the orientation of the helix axis is perpendicular to the direction of the applied magnetic field, which is in agreement with our earlier results based on a qualitative interpretation

Table 1. *Experimental ^{15}N solid-state NMR interactions of magainin 2 oriented in phospholipid bilayers*

Residue	Chemical shift (ppm)	^{1}H–^{15}N dipolar splitting (kHz)
Ala 15	57 ± 5	7.9 ± 1.5
Phe 16	47 ± 5	0 ± 1.5
Val 17	57 ± 5	6.3 ± 1.5
Gly 18	42 ± 5	7.5 ± 1.5

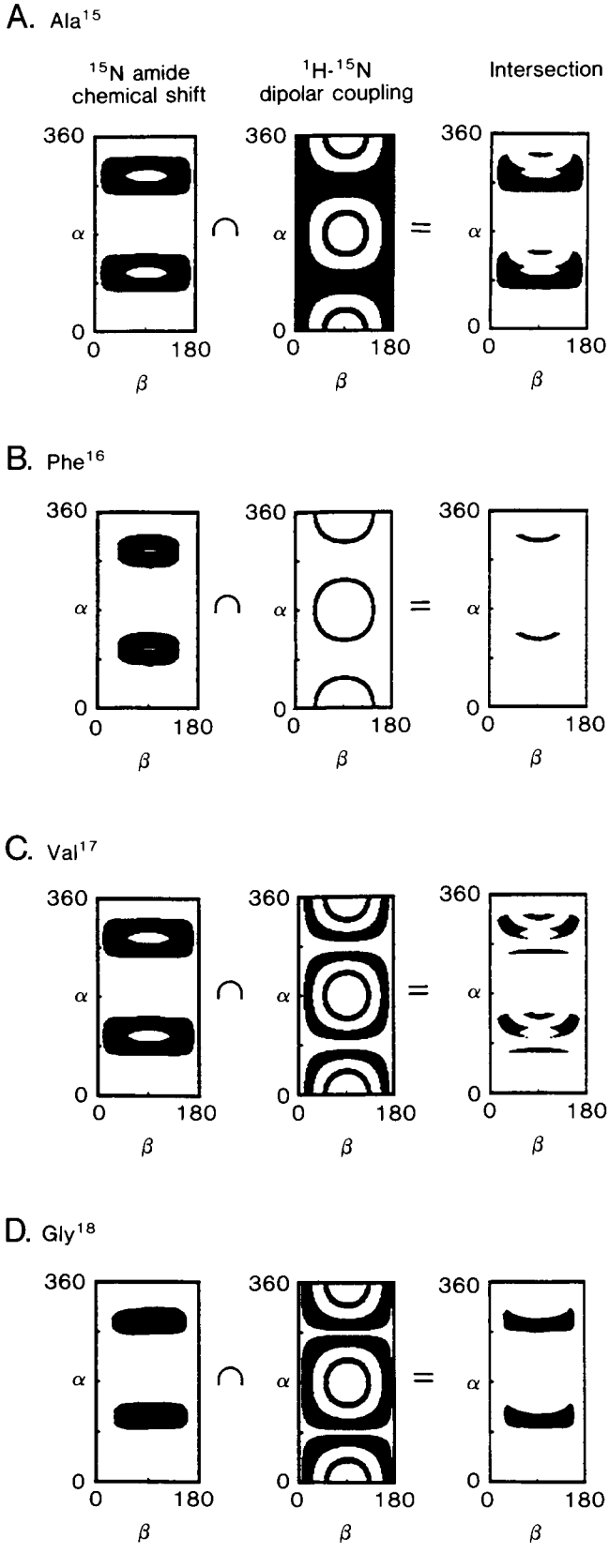

Fig. 4. Restriction plots obtained from the experimental measurements of the ^{15}N chemical-shift interactions and the ^{1}H–^{15}N dipolar interaction of specifically labeled Ala 15, Phe 16, Val 17, or Gly 18 magainin 2 in oriented lipid bilayers.

of the chemical-shift data for a single site (Bechinger et al., 1991).

Discussion

Magainin 2 is a 23-residue linear peptide that kills some types of cells but spares others (Zasloff, 1987; Cruciani et al., 1991). The mechanism for the antibiotic activities of this peptide is not known, and a major impetus for structural studies is to gain insights into both its selectivity and its toxicity. In addition, because this peptide has all of the characteristics of an amphipathic helix, it can serve as a model system for structural studies of broad classes of peptides and proteins that interact with membranes.

The peptide is water soluble; however, it is unstructured and highly flexible in aqueous solution. The peptide adopts a stable structure in the presence of membranes, and there is strong circumstantial evidence that its receptor is membrane rather than protein, especially the full biological activity of all-D-magainin. In general, 20–25-residue peptides that disrupt the ionic gradient of cells are thought to form channels in membranes by assembling as molecular aggregates with four to six amphipathic helices in a trans-bilayer configuration such that the hydrophilic residues are on the interior of the channel, constituting a central pore for ions, and the hydrophobic residues are on the outside of the channel, interacting with the hydrocarbon chains of the lipids (Lear et al., 1988; Oiki et al., 1988). Key features of this motif have been demonstrated by NMR spectroscopy. Candidate peptides show evidence of helical structures in model membrane environments (Mulvey et al., 1989; Reid et al., 1992; Opella et al., 1993). In addition, the 23-residue magainin 2 peptide with the sequence from the δ subunit of the acetylcholine receptor was found to span phospholipid bilayers (Bechinger et al., 1991).

There has been considerable discussion of the mechanism of magainins and other antibiotic peptides in terms of this general type of membrane-channel model, although often much larger aggregates have been invoked to explain the large range of single-channel conductances and relative lack of ion selectivity (Christensen et al., 1988; Guy & Raghunathan, 1988; Marion et al., 1988; Duclohier et al., 1989; Urrutia et al., 1989; Durell et al., 1992). However, the solid-state NMR experiments summarized with the structure in Figure 5 show that the magainin helices are oriented in the plane of the bilayer. These results indicate either that magainins allow ions to cross membranes by some other mechanism or that only a very small portion of the peptides associated with the membrane actually form transmembrane channels. Interestingly, calculations that combine Monte Carlo simulations with a hydropathy scale are in agreement with the solid-state NMR experimental results showing that magainin 2 is oriented in the plane of the membrane (Milik & Skolnick, 1993).

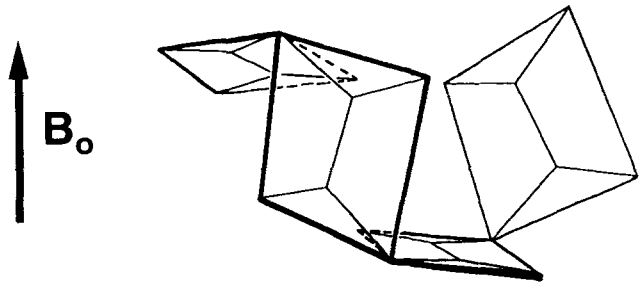

Fig. 5. Structure and orientations of the residues Ala 15, Phe 16, Val 17, or Gly 18 magainin 2 in phospholipid bilayers oriented with the bilayer normal parallel to the direction of the applied magnetic field.

The solid-state NMR data presented in this paper provide considerably more information than the overall orientation of the helical magainin peptides. In particular, the data in Figure 3 and Table 1, and their structural analysis summarized in Figure 5, demonstrate that four residues in the middle of the peptide participate in a stable α-helix in the presence of phospholipid bilayers. This structure determination is independent of the finding from multidimensional solution NMR experiments on samples of magainin 2 in various model membrane environments because entirely different samples, experiments, and, most importantly, nuclear-spin interactions are used in the analysis. The data in Figure 1 show that, at a minimum, residues 2–20 are immobilized by their interactions with the phospholipids and participate in a stable helix for time periods longer than 10^4 s; therefore, the secondary structure of the peptide is >90% α-helix in the presence of phospholipids. When the sequence of magainin 2 is displayed on a helical wheel (Schiffer & Edmundson, 1967), it is clearly an amphipathic α-helix. The analysis in Figure 2 shows that all of the residues in the helix are oriented with their N–H bonds in the plane of the lipid bilayer.

The solid-state NMR data show that the magainin helix is strongly associated with the membrane in an orientation parallel to the bilayer surface. The peptide is most likely positioned at the surface to accommodate its amphipathic character (Kaiser & Kezdy, 1987), thermodynamic requirements (Guy & Raghunathan, 1988), as well as experimental evidence in the form of decreased order parameters observed for the lipid acyl chains (Bechinger et al., 1992). Channel activity and antibiotic activity require high peptide concentrations (Matsuzaki et al., 1991), similar to those used in this investigation, and the tendency of the peptide to aggregate (Urrutia et al., 1989) suggests that oligomerization may occur in the membrane.

A mechanism of membrane perturbation has been proposed for peptides that aggregate on the surface of membranes. Helical peptides could organize into higher order "raft"-like structures that interact end to end and then insert through the membrane to form channels (Guy & Raghunathan, 1988). This is an elaboration of a conventional channel model, with the amphipathic helices in a transmembrane arrangement, at least transiently. On the other hand, an amphipathic helix oriented with its helix axis in the plane of the bilayer is most likely to be arranged so that its hydrophobic side is within the hydrocarbon chains of the lipids and its hydrophilic side interacts with the polar head groups and the aqueous solution. This may result in thinning of the lipid monolayer to accommodate the peptide (Durell et al., 1992), in turn causing sufficient destabilization of the bilayer structure to allow ions through. In this model, magainins selectively alter the organization of bacterial membranes (but not animal membranes) by their strong interactions as an amphipathic α-helix in the plane of the lipid bilayer, accounting for both their disruption of ionic gradients and antibacterial action.

All of the NMR results and mechanistic discussions agree that magainin 2 is an amphipathic helical peptide in membrane environments. The alignment of peptide helices parallel to the plane of the membrane, as well as their striking biochemical and cell-biological properties, suggest that these helical peptides disrupt membranes by an unusual mechanism. Whether the in-plane orientation of these helices is a prelude to the formation of transmembrane channels, or provides the mechanism for altering the membrane for the passage of ions, it is almost certain to have an important functional role in the disruption of ionic gradients as well as antibiotic and other activities of the peptide.

Materials and methods

The 23-residue magainin 2 peptide, Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-amide, was prepared by solid-phase peptide synthesis (*t*-Boc chemistry) with ^{15}N-labeled amino acids incorporated at specific sites. The labeled peptides (3 mol %) were buffered to neutral pH and incorporated into bilayers with a phospholipid composition approximating that of bacterial membranes (3POPE:POPG) (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine:1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol), hydrated at 93% relative humidity, and oriented between glass plates. A flat coil double-resonance probe (Bechinger & Opella, 1991) enabled ^{15}N NMR spectra to be obtained from samples oriented between 11 × 22-mm glass plates using cross-polarization and ^{1}H decoupling. The spectra were obtained using the MOIST (Levitt et al., 1986) version of cross-polarization (Pines et al., 1973). Typical experimental parameters include: 1.6 ms mix time, 6.4 ms acquisition time for 256 data points, ≥1.1 mT ^{1}H-decoupling field, 3 s recycle delay, 20,000 transients (one-dimensional spectroscopy). Separated local field spectroscopy without ^{1}H irradiation during t_1 (Waugh, 1976) was performed on selected samples with 16 t_1 valves, each acquired with 4,000 transients. The ^{1}H–^{15}N interac-

tions evolved for up to 300 ms. During data acquisition, the samples were maintained at room temperature in a stream of humidified air.

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