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Two-dimensional ^{1}H NMR experiments show that the 23-residue magainin antibiotic peptide is an α-helix in dodecylphosphocholine micelles, sodium dodecylsulfate micelles, and trifluoroethanol/water solution

Jennifer Gesell[a], Michael Zasloff[b] and Stanley J. Opella[a,*]

[a]*Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.*
[b]*Magainin Pharmaceuticals Inc., 1550 Campus Drive, Plymouth Meeting, PA 19355, U.S.A.*

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Summary

Magainin2 is a 23-residue antibiotic peptide that disrupts the ionic gradient across certain cell membranes. Two-dimensional ^{1}H NMR spectroscopy was used to investigate the structure of the peptide in three of the membrane environments most commonly employed in biophysical studies. Sequence-specific resonance assignments were determined for the peptide in perdeuterated dodecylphosphocholine (DPC) and sodium dodecylsulfate micelles and confirmed for the peptide in 2,2,2-trifluoroethanol solution. The secondary structure is shown to be helical in all of the solvent systems. The NMR data were used as a set of restraints for a simulated annealing protocol that generated a family of three-dimensional structures of the peptide in DPC micelles, which superimposed best between residues 4 and 20. For these residues, the mean pairwise rms difference for the backbone atoms is 0.47 ± 0.10 Å from the average structure. The calculated peptide structures appear to be curved, with the bend centered at residues Phe^{12} and Gly^{13}.

Introduction

The magainin antibiotic peptides were originally identified in frog skin because of their ability to protect against wound infections (Zasloff, 1987). The amidated 23-residue magainin2 peptide, GIGKFLHSAKKFGKAFVGEIMNS-NH_2, a typical member of this family, and the subject of this article, has a broad range of antifungicidal, antibacterial, and tumericidal activities (Cruciani et al., 1991). Magainin2 is highly basic with a net charge of +4 at pH 7. It has similarities to other highly charged antibiotic peptides, such as cecropins, bombolitins, mastoparans and melittin, found in other organisms (Segrest et al., 1990,1994).

Magainins perform their defensive biological functions by selectively permeabilizing bacterial membranes, disrupting their ionic gradient (Cruciani et al., 1991). Since magainin synthesized with all D-amino acids has essentially the same biological activity as either isolated or synthetic magainin with all L-amino acids, the mechanism of action is presumed to involve interactions with membranes rather than protein receptors (Bessalle et al., 1990).

In general, amphipathic helical peptides that disrupt the ionic gradient of cells are thought to do so by forming ion channels assembled from 4–6 peptide molecules aligned to form a cylinder with their hydrophilic residues on the interior of the channel, forming a central pore for ions, and their hydrophobic residues on the outside to interact with the hydrocarbon chains of the lipids (Oiki et al., 1990). The key features of this model have been demonstrated for several channel-forming peptides in model membrane environments. For example, the peptide sequence corresponding to membrane spanning segment 2 of the δ subunit of the acetylcholine receptor was found to be helical and to span phospholipid bilayers (Bechinger et al., 1991). Various segments of the α subunit of the voltage gated sodium channel, including a possible chan-

*To whom correspondence should be addressed.

nel-lining segment, have been shown to be helical (Mulvey et al., 1989; Doak et al., 1996). Although magainin has also been shown to be an amphipathic α-helix in membrane environments (Marion et al., 1988; Bechinger et al., 1993; Opella et al., 1993), our earlier solid-state NMR results show that its helix axis lies in the plane of phospholipid bilayers, suggesting that magainin's mechanism for disrupting the ionic gradient may be fundamentally different from those amphipathic peptides generally recognized as channel forming (Bechinger et al., 1991, 1996; Opella et al., 1993). This unexpected finding provides substantial motivation for further studies of these peptides, especially in the light of recently presented evidence (Matsuzaki et al., 1995,1996; Ludtke et al., 1996) indicating that magainin may form trans-membrane pores as it translocates across membrane bilayers.

In contrast to nearly all membrane-associated peptides, magainins are soluble in aqueous solutions. However, they are unstructured without the presence of co-solvents or lipids to simulate a membrane environment. Initial NMR studies demonstrated that magainin2 is nearly fully helical in 25% 2,2,2-trifluoroethanol (TFE)/water solution (Marion et al., 1988). Because of its tendency to induce helices in polypeptides, the results obtained in TFE/water solutions have to be interpreted cautiously. Other techniques have also been used to characterize the secondary structure of magainin in aqueous solution with and without added TFE, and in the presence of various lipids, with a wide variety of results (Chen et al., 1988; Wade et al., 1990; Williams et al., 1990; Jackson et al., 1992). Our solid-state NMR experiments on oriented bilayer samples showed that magainin2 was helical throughout its length when associated with phospholipids (Bechinger et al., 1991,1993,1996; Ramamoorthy et al., 1995). Recent FTIR and solid-state NMR results on unoriented samples have been interpreted in terms of magainin existing as one-third β-sheet and two-thirds α-helix when associated with phospholipids (Hirsh et al., 1996). In order to reconcile some of the conflicting results on the secondary structure of magainin in various environments and to gain additional information about the influences of various model membrane environments on structure from both multidimensional solution NMR and high-resolution solid-state NMR experiments, we have characterized the structure of magainin2 in dodecylphosphocholine (DPC) micelles, sodium dodecylsulfate (SDS) micelles, and TFE/water solution by two-dimensional ^{1}H NMR spectroscopy. SDS and DPC are commonly used detergents for the solubilization of membrane peptides and proteins because the small uniform micelles reorient rapidly enough for solution NMR spectroscopy (McDonnell and Opella, 1993). The results of multidimensional solution NMR studies of amphipathic helices in micelles are complementary to those from solid-state NMR studies of these same peptides and proteins in phospholipid bilayers (Opella et al., 1993).

Materials and Methods

Sample preparation

Automated solid-phase peptide synthesis with Fmoc chemistry was used to prepare the amidated form of magainin2. The micelle samples used in the NMR experiments were prepared by dissolving 5 mg of the peptide and 80 mg of perdeuterated DPC or SDS (Cambridge Isotope Laboratories, Andover, MA, U.S.A.) in 0.5 ml of water (90% H_2O, 10% D_2O) with 20 mM NaCl and 5 mM deuterated citrate (Cambridge Isotope Laboratories). The pH of the samples was adjusted to 4.1. The peptide in TFE/water solution was prepared by dissolving 10 mg of the peptide in 325 μl of water containing 100 mM NaCl. The pH was adjusted to 4.1 and then 125 μl of TFE was added to the sample.

NMR experiments

The NMR experiments were performed at ^{1}H resonance frequencies of 500 and 600 MHz on Bruker AMX spectrometers. Experiments on micelle samples were carried out at 303 K and those in TFE/water solution at 291 K. In the two-dimensional (2D) experiments, quadrature detection in t_1 was accomplished with time-proportional phase incrementation (Bodenhausen et al., 1984). Two-dimensional double quantum filtered correlation (DQF-COSY) (Rance et al., 1983), nuclear Overhauser enhancement (NOESY) (Kumar et al., 1980; Macura and Ernst, 1980), and total correlation (TOCSY) (Bax and Davis, 1985) spectra were acquired using standard pulse sequences. Typically, the sweep width was 12 ppm, 2K real points were acquired in the t_2 dimension, and 64 transients were co-added for each of 512 t_1 points.

Data processing and analysis

NOESY and TOCSY data were Fourier transformed after applying phase-shifted squared sine-bell functions in both dimensions; t_1 data were zero filled to 1024 points to give a final matrix size of $2K \times 1K$ points. The data were processed with the programs UXNMR (Bruker Instruments, Rheinstetten, Germany) or FELIX (Biosym Technologies, San Diego, CA, U.S.A.) on Silicon Graphics computers and then imported into NMR Compass (Molecular Simulations Inc., Waltham, MA, U.S.A.) for data analysis. DQF-COSY data were transformed after applying an exponential function with a time constant corresponding to 2 Hz and zero filling the t_2 data to 8K points. Data sets processed with UXNMR were converted to the FELIX matrix format prior to the analysis of $^3J_{HN\alpha}$ coupling constants.

Determination of scalar coupling constants

$^3J_{HN\alpha}$ coupling constants were determined utilizing the method described by Titman and Keeler (1990) as modified by Caldwell et al. (1994). First, slices corresponding

to NH-$C^{\alpha}H$ correlations were taken from the 2D NOESY and DQF-COSY spectra. The NOESY peak was convoluted with an antiphase doublet while the DQF-COSY cross section was convoluted with an in-phase doublet of separation J_{trial}. This was repeated for a range of possible active couplings. When $J_{trial} = J_{active}$, the results of the two convolutions were identical and the difference was a null.

Identification of slowly exchanging amide hydrogens

To detect slowly exchanging amides, a series of 2D TOCSY experiments was acquired from a freshly prepared sample of magainin2/DPC in D_2O. The NH resonances detectable after 4 h were classified as slowly exchanging, as indicated in Fig. 1.

$C^{\alpha}H$ chemical shift analysis

Chemical shift analysis was performed using the approach described by Wishart et al. (1992). The frequencies of all resonances were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0.015 ppm and then compared to random coil chemical shifts. Those amino acids with α-hydrogen chemical shifts greater than the random coil values were assigned a value of 1. If the α-hydrogen chemical shift value was less than the random coil value, the amino acid residue was assigned a value of –1. If the experimentally determined chemical shift was in the allowed range for random coil values, the residue was assigned a value of 0.

Structure calculations

Cross peaks in NOESY spectra acquired with a 100 ms mixing period were divided into classes corresponding to strong, medium and weak intensities and assigned target inter-hydrogen distances of 1.9–2.5, 1.9–3.5 and 3.0–5 Å, respectively (Clore et al., 1986). Appropriate pseudoatom corrections were applied to methylene and methyl hydrogens that were not stereospecifically assigned (Wüthrich et al., 1983). In addition, 1.5 Å was added to the upper limits of distances involving methyl hydrogens. A total of 227 NOE-derived internuclear constraints were used in the calculations. These included 124 interresidue and 103 intraresidue constraints. In addition, eight torsional restraints derived from $^3J_{HN\alpha}$ coupling constants were used. Dihedral constraints were given a target angle of –60° and a range of ±25°. Two distance restraints for each hydrogen bond were included for those residues with slowly exchanging amide hydrogens. Three-dimensional (3D) structures were calculated using XPLOR which was interfaced through QUANTA (Molecular Simulations Inc.). A combination of simulated annealing, restrained molecular dynamics, and energy minimization was used to generate a set of 50 structures. First, an extended polypeptide was subjected to 10 ps of high-temperature molecular dynamics at 850 K. Next, the system was cooled to 300 K over 10 ps. The resulting 50 structures were subjected to 10 ps of NOE-restrained molecular dynamics steps at 300 K. This was immediately followed by 500 steps of energy minimization. The final structures were analyzed using QUANTA.

Results

Sequence-specific resonance assignments of the ^1H resonances of magainin2 in DPC micelles, SDS micelles, and TFE/water solution were obtained using standard

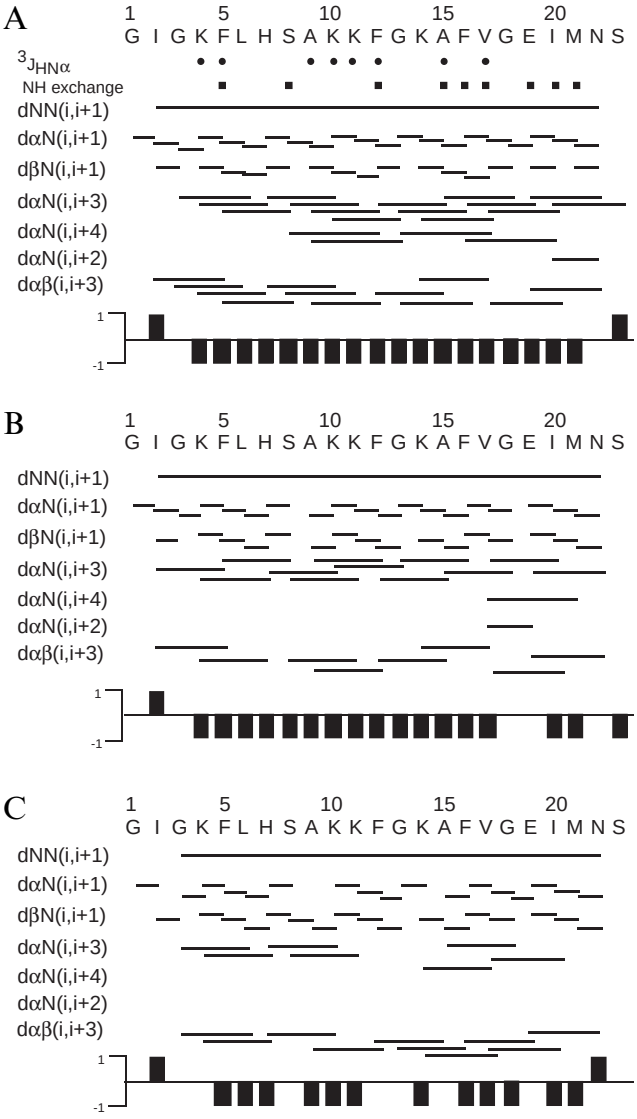

Fig. 1. Summary of the sequential and medium-range NOEs, $C^{\alpha}H$ chemical shift index, and $^3J_{HN\alpha}$ coupling constants for magainin2 in (A) DPC micelles, (B) SDS micelles, and (C) TFE/water solution. Ambiguous NOEs have been omitted from the maps. Filled circles indicate residues that have $^3J_{HN\alpha}$ coupling constants less than 5.5 Hz. Unmarked residues either have values of 5.5 Hz or were not unambiguously determined. Triangles mark residues which are still visible after 4 h in D_2O while unmarked residues exchange on an intermediate scale.

TABLE 1
^{1}H RESONANCE ASSIGNMENTS FOR MAGAININ2 IN DPC MICELLES, SDS MICELLES, AND TFE/WATER SOLUTION

Residue#	DPC micelles				SDS micelles				TFE/water solution			
	NH	$C^{\alpha}H$	$C^{\beta}H$	Other	NH	$C^{\alpha}H$	$C^{\beta}H$	Other	NH	$C^{\alpha}H$	$C^{\beta}H$	Other
Gly[1]												
Ile[2]	9.39	4.20	2.01	1.50, 1.35, 1.00, 0.93	8.67	4.16	1.94	1.57, 1.30, 0.97, 0.89	8.85	4.39	2.07	1.63, 1.41, 1.09, 1.02
Gly[3]	9.06	4.04, 3.70			8.53	4.00, 3.70			8.80	4.05, 3.93		
Lys[4]	8.01	4.14	1.81, 1.74	2.85, 1.58, 1.25, 1.16	7.85	4.08	1.79, 1.70	2.86, 1.55, 1.22, 1.15	8.19	4.26	1.94, 1.77	3.06, 1.45
Phe[5]	7.86	4.46	3.21	7.28	7.96	4.40	3.18		8.04	4.50	3.37, 3.27	7.30
Leu[6]	8.25	3.98	1.92	1.55, 0.95, 0.89	8.25	3.94	1.85	1.45, 0.83, 0.47	8.47	4.14	1.57	1.88, 1.00
His[7]	8.32	4.36	3.40	7.38	8.29	4.37	3.35	7.41	8.39	4.52	3.40	7.39
Ser[8]	8.15	4.25	4.00, 3.93		8.10	4.28	3.98, 3.88		8.07	4.42	3.88, 4.04	
Ala[9]	8.40	4.02	1.32		8.39	3.98	1.27		8.39	4.16	1.36	
Lys[10]	8.13	3.85	1.89	1.51, 1.37, 1.76, 0.80, 2.88	8.00	3.89	1.89, 1.81	2.94, 1.47, 1.22	8.11	4.17	1.92	3.05, 1.59, 1.51
Lys[11]	7.66	3.95	1.74, 1.55	2.85, 1.32, 1.13	7.63	3.98	1.71, 1.67	2.83, 1.52, 1.27	7.88	4.16	1.69	2.96, 1.26, 1.12
Phe[12]	8.21	4.42	3.11	7.23	8.19	4.41	3.11, 3.02	7.18	8.19	4.68	3.28, 3.05	7.25
Gly[13]	8.81	3.75			8.69	3.73			8.25	4.17, 4.00		
Lys[14]	8.44	3.93	1.88	2.93, 1.59, 1.39	8.36	3.94	1.85	2.96, 1.38	8.43	4.19	1.96	3.11, 1.81
Ala[15]	7.72	4.17	1.46		7.73	4.16	1.45		8.29	4.32	1.54	
Phe[16]	8.38	4.28	3.12, 3.00	7.14	8.39	4.23	3.11, 2.97	7.10	8.12	4.50	3.21, 3.16	7.19
Val[17]	8.23	3.45	2.15	1.10, 0.93	8.33	3.44	2.11	1.07, 0.92	8.01	3.70	2.19	1.14, 1.04
Gly[18]	8.10	3.88, 3.78			8.16	3.89			8.20	3.93, 3.87		
Glu[19]	7.78	4.18	2.09, 2.16	2.43	7.79	4.21	2.12	2.43	7.91	4.28	2.17	2.47
Ile[20]	7.70	3.80	1.83	0.78, 1.01, 1.33	7.74	3.76	1.78	1.30, 0.74, 0.55	8.00	3.88	1.96	1.41, 1.08, 0.89, 0.74
Met[21]	8.14	4.04	2.04	2.65, 2.52	8.20	4.26	1.99, 2.09	2.62, 2.49	8.44	4.41	2.19	2.75, 2.62
Asn[22]	7.88	?	2.87, 2.76	6.87, 7.61	7.88	4.71	2.84, 2.75	7.56, 7.15	8.07	4.87	2.99, 2.90	7.74, 6.89
Ser[23]	7.91	4.33	3.92	terminal NH_2 7.48, 7.21	7.87	4.33	3.92	terminal NH_2 6.97, 7.13	8.04	4.52	4.07	terminal NH_2 7.58, 7.13

methods of protein NMR spectroscopy (Wüthrich, 1986). First, the chemical shift frequencies of the scalar coupled backbone amide hydrogen resonance and the α-hydrogen (NH-$C^{\alpha}H$) resonance of each residue were determined from their correlation peak in 2D DQF-COSY spectra. Second, the spin system of each side chain was identified and correlated with its backbone resonances through the multiple peaks observed in 2D TOCSY spectra. After organizing all scalar coupled resonances into spin systems of possible amino acid types, NOE data were used to place the spin systems in sequential order. By using the chemical shift of each residue's $C^{\alpha}H$, as determined from a 2D DQF-COSY spectrum, and then looking for an NOE cross peak between the resonance from the α-hydrogen and the next sequential backbone NH, it was possible to specifically assign all spin systems. NOEs were used to confirm the correlations between resonances of the C^{β} and aromatic ring hydrogens of phenylalanine residues. Similarly, NOE cross peaks from the amide side chain of Asn[22] to its own $C^{\beta}H$ were also useful for confirming resonance assignments. Table 1 lists the chemical shift values of the assigned resonances in magainin2 amide in DPC micelles, SDS micelles, and TFE/water solution. The ^{1}H NMR resonances of magainin2 are very well resolved in DPC and SDS micelles, although there is some overlap in the $C^{\alpha}H$ resonance region of spectra obtained in TFE/water solution; in particular, residues 6, 9, 10, and 11 have nearly identical $C^{\alpha}H$ chemical shifts, which results in some of the medium and sequential NOE connectivities in the $C^{\alpha}H$-NH region of 2D spectra being unassigned.

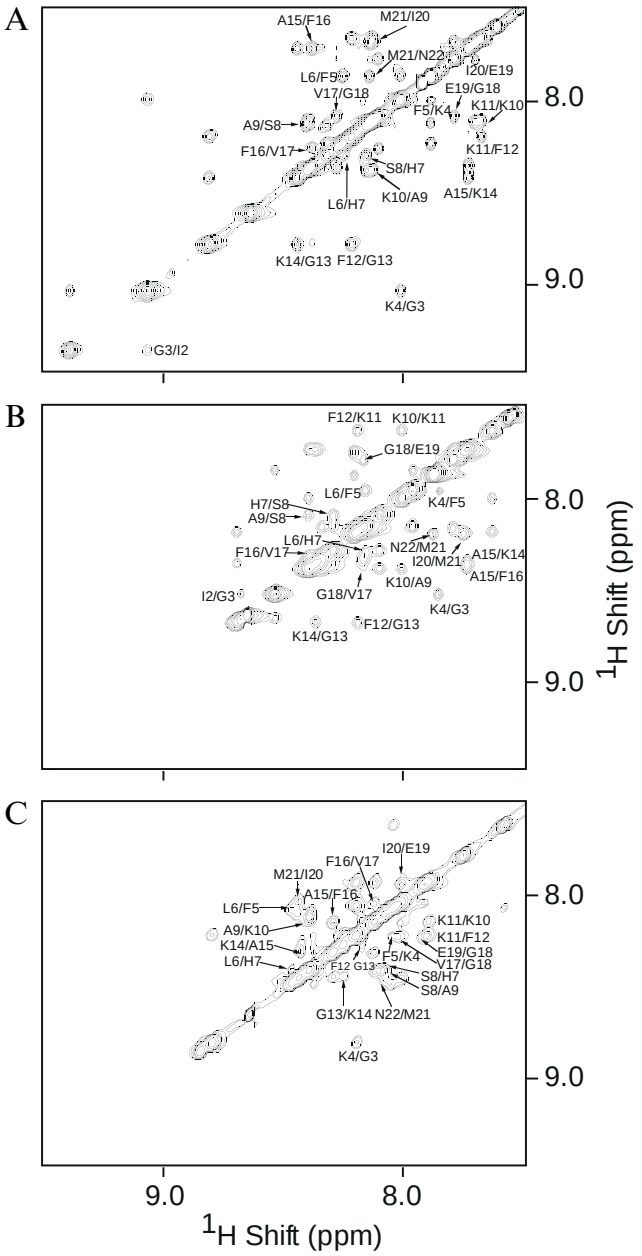

Fig. 2. Amide resonance regions of the experimental 2D NOE spectra for magainin2 in (A) DPC micelles at 600 MHz, (B) SDS micelles at 600 MHz, and (C) TFE/water solution at 500 MHz. The mix time for the NOE experiment was 100 ms in all the cases. Resonance assignments of the NOE cross peaks are indicated.

Since the chemical shifts of some hydrogens are strongly dependent on local secondary structure (Wishart et al., 1992), comparisons of the chemical shift frequencies of $C^{\alpha}H$ resonances to the values found for the same amino acids in random coil peptides make it possible to identify regions of regular secondary structure. $C^{\alpha}H$ resonance frequencies for every residue are determined and assigned a chemical shift index value of 1, 0, or −1 based on its chemical shift relative to random coil values. The secondary structure of the residues is then assigned to α-helix, β-sheet, or random coil based on the local density of the chemical shift indices. These results complement the secondary structure information derived from NOE-based methods. The reliability of these results depends on the choice of the reference chemical shifts. In the case of micelle samples, the random coil values were taken from Wishart et al. (1992). The data for magainin2 in TFE/water solution were obtained by comparing the observed chemical shifts with those from random coils in TFE/water solution tabulated by Merutka et al. (1995). Figure 1 contains chemical shift index plots of the $C^{\alpha}H$ resonances for magainin2 in DPC micelles, SDS micelles, and TFE/water solution. The results in SDS and DPC are strikingly similar and indicate that there is helical structure throughout the length of the peptide. The results in TFE/water are not as clear, but still suggest a helical secondary structure.

The nuclear Overhauser enhancement (NOE) provides the inter-hydrogen distance information required for structure determination by solution NMR since regular secondary structures in proteins and peptides have well-documented NOE patterns (Wüthrich, 1986). The periodicity of α-helical structure allows for NOE cross peaks between sequential backbone amide hydrogens, as well as cross peaks between the $C^{\alpha}H$ of a residue and amide hydrogens three and four residues toward the carboxy terminus to be observed. Also present in a typical α-helix

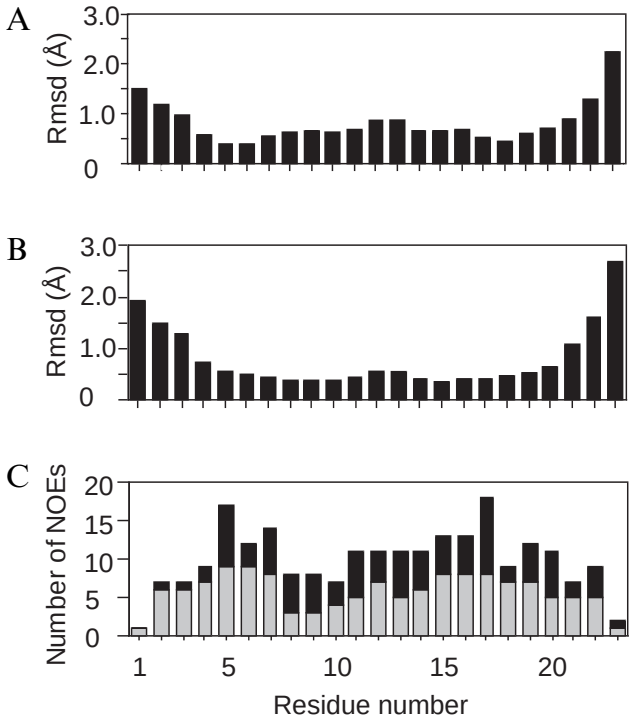

Fig. 3. (A) Rms difference of the backbone atoms to the mean structure after superimposing the entire backbone. (B) Rms difference of the backbone atoms to the mean structure after superimposing the well-defined region between residues Lys^{4} and Ile^{20}. (C) The distribution of NOEs used in the structure calculations with sequential NOEs in gray and medium-range NOEs in black.

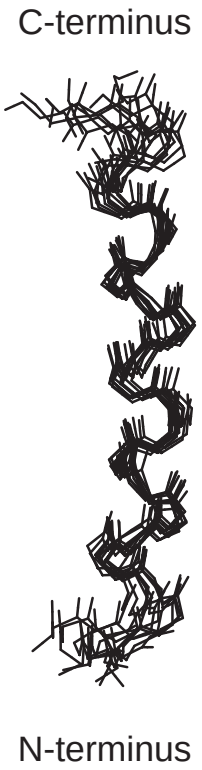

Fig. 4. Structure of magainin2 in DPC micelles shown as a superposition of the backbone heavy atoms of the 10 lowest energy structures calculated from the NMR data. The structures are aligned for residues 4–20.

would be cross peaks between the $C^{\alpha}H$ resonance of residue i and the $C^{\beta}H$ resonance of residue i+3. The amide resonance regions of the 2D NOE spectra of magainin2 peptide in DPC micelles, SDS micelles, and TFE/water solution are shown in Fig. 2. All three spectra show many strong NH-NH NOE cross peaks between adjacent residues, as is expected for a helical peptide in solution.

Medium-range NOEs between the $C^{\alpha}H$ of residue i and the backbone amide hydrogen of residue i+3 are present throughout the peptide in all samples, as shown in Fig. 1. These cross peaks are characteristic of α-helical secondary structure, as are the cross peaks between the $C^{\alpha}H$ of residue i and the backbone amide hydrogen of residue i+4. Significantly, the NOE cross peaks observed between the resonances of the $C^{\alpha}H$ of residue i and the backbone NH of residue i+4 are specific to α-helices and are not present in 3_{10}-helices. The data in Fig. 1 indicate that magainin2 is α-helical from residues 2 to 22 in DPC and SDS detergent micelles, and from residues 3 to 22 in TFE/water solution. These results are in agreement with the initial solution NMR studies of magainin (Marion et al., 1988) as well as the solid-state NMR studies in lipid bilayers (Bechinger et al., 1993).

The observed spin–spin coupling constant is equal to the average of the coupling constant of each available conformation multiplied by the statistical weight of that conformation. In an α-helix, the expected value for $^3J_{HN\alpha}$ is 4 Hz (Wüthrich, 1986). Extended conformations have values of $^3J_{HN\alpha}$ around 8 Hz. Figure 1 indicates amino acid positions where the $^3J_{HN\alpha}$ values have been determined to be less than 5.5 Hz for magainin2 in DPC micelles. The actual values observed are in the range of 4–5.5 Hz and are consistent with helical structure.

The 10 lowest energy structures calculated for magainin2 DPC micelles with the aid of simulated annealing, restrained molecular dynamics, and energy minimization have no dihedral restraint violations and no distance restraint violations greater than 0.2 Å. The mean pairwise rms differences for the backbone atoms when the structures are superimposed over the entire length of the peptide are shown in Fig. 3A. These data indicate that the structure of magainin2 is well defined between residues 4 and 20. The mean pairwise rms difference for all atoms to the average structure is 1.63 ± 0.2 Å. The mean pairwise backbone rms difference for the peptide is 0.81 ± 0.4 Å from the average structure. The 10 lowest energy structures are shown in Fig. 4. They have been superimposed over the well-defined region between Lys^4 and Ile^{20} and, for these residues, the mean pairwise rms difference for the backbone atoms is 0.47 ± 0.10 Å from the average structure. The mean pairwise rms differences for the backbone atoms of each residue are plotted in Fig. 3B.

Discussion and Conclusions

Magainin is a naturally occurring peptide whose antibiotic activity has been linked to its interactions with membranes. One of the main reasons why membrane peptides and proteins are difficult to study by NMR spectroscopy is because they are insoluble in water. Magainin is an exception since it is highly soluble, albeit unstructured, in water. In many cases organic solvents such as methanol, chloroform, and TFE have been used alone or in various combinations, including with water, to solubilize membrane peptides for NMR spectroscopy and other spectroscopic methods. Most membrane peptides yield much better resolved NMR spectra in these solvents than they do in even the most carefully prepared detergent micelles. However, these solvents have been shown to have the additional effect of inducing or stabilizing helical secondary structure in peptides, and this occurs with the water-soluble magainin as well. TFE/water solutions, in particular, are likely to induce helical structures in peptides (Doty et al., 1954; Conio et al., 1970; Liebes et al., 1975; Nelson and Kallenbach, 1989).

Detergent micelles are an attractive alternative to mixed organic solvents for studying membrane peptides and proteins by solution NMR spectroscopy. These lipids are amphipathic molecules, and the peptide-containing micelles formed emulate phospholipid bilayers in ways that are generally regarded as more reliable for structure determinations than the mixed organic solvents. In our experi-

ence, magainin is an exceptional peptide in several ways. The structure appears to be very similar in mixed organic solvents and detergent micelles, perhaps because it is water soluble and interacts primarily with the head groups of lipids. Most membrane peptides that we have examined, including some with properties similar to magainin, give quite different spectroscopic and structural results in mixed organic solvents and detergent micelles.

Magainin2 provides an example of a membrane-associated peptide which has been studied by NMR spectroscopy not only in organic solvents, and now in detergent micelles, but also by solid-state NMR spectroscopy in phospholipid bilayers. Solid-state NMR experiments have shown that magainin2 is fully structured between residues 2 and 20 with its helix axis parallel to the plane of the lipid bilayer (Bechinger et al., 1991,1996; Opella et al., 1993; Ramamoorthy et al., 1995). All of the solution NMR data, including $C^{\alpha}H$ chemical shifts, NOEs, and $^{3}J_{HN^{\alpha}}$ coupling constants, indicate that the peptide is essentially fully helical in DPC micelles and SDS micelles. As is often the case, the ends of the helix are difficult to define precisely. The secondary structure maps in Figs. 1A and B show that there are NOE cross peaks characteristic of helical structure present from residues 2 to 23 in DPC micelles and from residues 2 to 22 in SDS micelles. The 3D structures generated from these data are shown in Fig. 4. The backbone atoms of calculated structures for the peptide in DPC micelles align best from residues 4 to 20. The apparent disorder at the termini may reflect the presence of large-scale internal motions in this region. Alternatively, by virtue of their position along the linear peptide chain, residues at the termini have fewer neighbors and thus fewer detectable NOEs to convert to constraints to limit the allowed conformations during structure calculations. An inspection of Fig. 3C reveals the paucity of NOEs in the region of Gly^{1}–Gly^{3} and at the C-terminus, Ser^{23}. The results in TFE/water are an extension of the earlier work by Marion et al. (1988), and there is complete agreement with their findings. Although the data in Fig. 1C indicate that the chemical shift index is not as regular as it is in the detergent systems, the tabulated NOE data indicate helical structure for residues 3–22. The structures generated for magainin2 in DPC micelles are based on an average of 11 constraints per residue. Given the number of constraints, the convergence of the calculated structures is similar to that predicted for globular proteins (Clore and Gronenborn, 1994), which is a backbone rms difference of between 0.4 and 0.8 Å to the mean structure.

Figures 3A and B show that there is a region in the middle of the structure, centering around residues 12 and 13, that is less defined than neighboring regions. This is not a reflection of a lack of NOEs for these residues, as seen in Fig. 3C. A careful examination of the structures indicates that they are not perfectly linear but, instead, are curved or kinked around these residues. The extent of curvature varies slightly from structure to structure; however, in all cases the hydrophobic face of the peptide is concave, as seen in previous studies of amphipathic helices (Barlow and Thornton, 1988; Zhou et al., 1992; McLeish et al., 1994). In addition, comparison of the observed NH chemical shift with random coil values (Wishart et al., 1991) reveals a periodic oscillation consistent with a bent helix (Zhou et al., 1992). In the mean structure, the bend results in a 16° angle between the two regions Lys^{4}–Phe^{12} and Phe^{12}–Ile^{20}.

NMR spectroscopy is a highly reliable approach for determining the secondary structure of individual residues in peptides. It has major advantages over other measurements that reflect averages over the entire peptide rather than multiple parameters for each residue. Our NMR studies of magainin in a variety of model membrane environments are consistent in showing the peptide to be completely α-helical except for a few terminal residues. The results presented in this article show magainin to be fully helical in DPC micelles, SDS micelles, and TFE/water solution, with the latter being in complete agreement with the earlier results of Marion et al. (1988). Our solid-state NMR experiments on oriented samples of magainin associated with phospholipid bilayers also show that the peptide is helical throughout its length and that the helix axis is in the plane of the bilayer (Bechinger et al., 1991, 1993,1996; Ramamoorthy et al., 1995). In particular, residues 16 and 17 were fully characterized and shown to be helical with no evidence of other conformations with 3D solid-state NMR experiments on an oriented sample of magainin in phospholipid bilayers. These results are in contrast to the recent report based on REDOR solid-state NMR and FTIR experiments which suggest that residues 15–19 of magainin associated with phospholipids have one-third β-sheet and two-thirds α-helix secondary structure (Hirsh et al., 1996).

It has been proposed that magainin2 may initially form antiparallel dimers on the surface of the bilayer (Matsuzaki et al., 1994). We have not detected any long-range NOEs between N- and C-terminal residues that would indicate the presence of antiparallel packing of helices. This would be an unlikely situation in the micelle samples studied here.

The amphipathic α-helix is a critical component of the pore lining of ion channels. Some of the best characterized channel proteins are ionotropic neurotransmitter receptors. Images from electron micrograph reconstruction of intact acetylcholine receptors show that a transmembrane pore is formed by a bundle of α-helices (Unwin, 1993). Synthetic amphipathic peptides corresponding to transmembrane segments of a variety of receptors have been shown to form functional ion channels of heterogeneous lifetimes and conductances in artificial planar lipid bilayers (Oiki et al., 1988,1990; Grove et al., 1991). One of these peptides, membrane spanning segment 2 (M2) of the

δ subunit of the acetylcholine receptor, has been shown to be helical by solution NMR spectroscopy and to be oriented transmembrane with its helix axis perpendicular to the plane of the lipid bilayer (Bechinger et al., 1991). Information from electron micrograph reconstruction, NMR spectroscopy, and molecular cloning and mutagenesis used in conjunction with computer modeling techniques have provided a variety of ion channel models, most of which are variations of bundles of amphipathic helices oriented with their hydrophilic residues facing inward towards a solvent-accessible pore and their hydrophobic residues directed away from the pore and towards the acyl chains of the lipids or hydrophobic surfaces of neighboring protein structures (Oiki et al., 1990; Montal et al., 1993; Kerr et al., 1994).

Other members of the family of cytotoxic amphipathic α-helical peptides form channels in bilayers, including alamethicin, melittin, δ-hemolysin, cecropins and pardaxin (Tosteson and Tosteson, 1981; Christensen et al., 1988; Shai et al., 1990; Molle et al., 1991). These naturally occurring channel-forming peptides, except for alamethicin, have highly charged primary sequences. They have all been shown to be amphipathic helices by NMR and/or X-ray crystallography (Brown et al., 1982; Fox and Richards, 1982; Terwilliger and Eisenberg, 1982; Lee et al., 1987; Holak et al., 1988; Tappin et al., 1988; Inagaki et al., 1989; Zagorski et al., 1991; Yee and Oneil, 1992; Franklin et al., 1994). Structural details like helix axis bend and even the number of helices present are variable. Their most striking similarity is the amphipathic nature of the helices formed by these peptides. In contrast to the channel peptides derived from ionotropic neurotransmitter receptors, the equilibrium helix axis orientations of magainins and melittin have been shown to be in the plane of the bilayer (Stanislawski and Rüterjans, 1987; Bechinger et al., 1991). Until recently it had been difficult to reconcile the classic model of a transmembrane ion channel with the equilibrium in-plane orientation of the magainin helix axis. However, Matsuzaki et al. (1995, 1996) have proposed a mechanism whereby transient pore formation is coupled with the translocation of the peptide across the bilayer. This is supported by physical measurements indicating that, at high concentrations, magainin peptide can form pores perpendicular to the membrane bilayer (Ludtke et al., 1996). This mechanism allows for the peptide's helix axis to lie in the plane of the bilayer under equilibrium conditions and then transiently span the lipid bilayer to form transmembrane pores similar to those formed by peptides derived from ionotropic receptors.

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