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Detergent-like properties of magainin antibiotic peptides: A ^{31}P solid-state NMR spectroscopy study

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Abstract

^{31}P solid-state NMR spectroscopy has been used to investigate the macroscopic phase behavior of phospholipid bilayers in the presence of increasing amounts of magainin antibiotic peptides. Addition of >1 mol% magainin 2 to gel-phase DMPC or liquid crystalline POPC membranes respectively, results in ^{31}P NMR spectra that are characterized by the coexistence of isotropic signals and line shapes typical for phospholipid bilayers. The isotropic signal intensity is a function of temperature and peptide concentration. At peptide concentrations >4 mol% of the resulting phospholipid ^{31}P NMR spectra are characteristic of magnetically oriented POPC bilayers suggesting the formation of small disk-like micelles or perforated sheets. In contrast, addition of magainin to acidic phospholipids results in homogenous bilayer-type ^{31}P NMR spectra with reduced chemical shift anisotropies. The results presented are in good agreement with the interfacial insertion of magainin helices with an alignment parallel to the surface of the phospholipid bilayers. The resulting curvature strain results in detergent-like properties of the amphipathic helical peptides.

Keywords: Magainin; Lipid membrane; Polypeptide–lipid interactions; ^{31}P solid-state NMR; Phase transition; Phosphatidylcholine; Phase diagram; Detergent; Peptidergent

1. Introduction

Magainin antibiotics are a family of immunogenic peptides which were first found in the skin of frogs [1–3]. These peptides exhibit bacteriocidal, fungicidal and virocidal activities and thereby provide an immediate immunogenic response to infections [4,5]. In a related manner, defense mechanisms are established in insects by the presence of cecropins or in humans by defensins [6–8]. Magainins were also shown to selectively lyse tumor cells without killing healthy vertebrate cells [9–11].

Magainin polypeptides consist of 21 to 26 amino acid residues and are strongly basic in character. Although they dissolve well in aqueous solution, they also show a strong affinity for phospholipid membranes. Electrophysiological experiments indicate that, when added to phospholipid bilayers, magainins have membrane-disruptive properties and in some instances cause pore-like openings [12,13]. Such effects are responsible for the breakdown of transmembrane ionic gradients across lipid bilayers. Their ability to decouple the respiratory chains of bacterial, mitochondrial, as well as spermatozoal membranes, was therefore suggested to be related to their antibiotic activity [14].

CD [15–17], Raman [18] and FTIR [19,20], as well as multidimensional solution [21,22] and solid-state NMR spectroscopies [23,24], all indicate that magainins and related peptides adopt right-handed α-helical conformations in model membranes. In addition, ^{15}N solid-state NMR spectroscopy of magainins, which have been incorporated into oriented phospholipid membranes, shows that the helix axis of the peptide is oriented parallel to the bilayer surface [23]. The α-helical conformation of magainins and related polypeptides results in the separation of polar and hydro-

Abbreviations: NMR, nuclear magnetic resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine; PI, diacyl-phosphatidylinositol; CD, circular dichroism; FTIR, Fourier transform infrared

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phobic residues along opposite faces of the helix. Interestingly, all-D-magainins as well cause channel formation and cell lysis of the same organisms sensitive to the L-enantiomer [25]. These results, together with the lack of primary sequence homology within the magainin family, indicate that the structural and physicochemical properties of magainins rather than specific receptor–ligand interactions are responsible for their biological activity.

Although the secondary structure and the functional properties of magainins have been characterized by a multitude of techniques, the mechanism of how magainins cause an increase in electrical conductivity across the lipid bilayer is still a matter of debate. Different models have been suggested to describe the magainin–lipid interactions [26]. Initially, the peptides were thought to form transmembrane helical bundles in analogy to channels formed by peptiabol antibiotics including alamethicin [27,28]. This model was later revised to include acidic phospholipids in the pore lumen, whereby so called 'wormholes' are created [29]. Experiments which provide evidence for such structures were performed at high peptide-to-lipid ratios. Furthermore, the 'carpet model' proposes that amphipathic peptides that align parallel to the membrane surface disrupt the bilayer integrity at high local peptide concentrations [30]. Finally, a unifying model was proposed to explain the experimental observations at low concentrations of magainins and other amphipathic helices [26]. This model takes into consideration the detergent-like properties of magainins and its various interaction models should therefore be represented in a phase diagram where a wide range of parameters affect the macroscopic phase properties of the peptide–lipid mixtures [8]. These characteristics include peptide concentration, lipid composition, temperature, pH and hydration. Interestingly, the antibiotic properties of specially designed amphipathic model peptides have been correlated to experimental conditions where the peptides adopt in-plane alignments [17,24]. It should be possible to provide evidence of the detergent-like action of amphipathic peptide antibiotics by monitoring the macroscopic phase properties of peptide–lipid mixtures, albeit small local changes, e.g., at low peptide concentrations, might not easily be detected using structural methods.

^{31}P solid-state NMR spectroscopy is particularly well suited for the study of phospholipid–protein interactions since the naturally abundant ^{31}P atom provides a sensitive indicator for the structure and dynamics of the phospholipid headgroup as well as the macroscopic phase behavior of phospholipid membranes [31]. This technique has been used to investigate the phase transitions in phospholipid membranes as well as in phospholipid–peptide or phospholipid–detergent mixtures. In this paper, the modulations of the macroscopic phase transitions due to the presence of magainins has been investigated by solid-state ^{31}P NMR spectroscopy. The possible role of the observed phase alterations during the magainin-induced permeability increases, and thus antibiotic action, will be discussed.

2. Materials and methods

2.1. Peptide synthesis

Magainin 2 with the sequence GIGKFLHSAKKFGKAFVGEIMNSamide was prepared by solid-phase peptide synthesis using Fmoc chemistry. After cleavage off the resin, the purity of the synthetic peptide was analyzed by reversed phase HPLC and mass spectroscopy. Two-dimensional ^{1}H–^{1}H high-resolution NMR spectroscopy further confirmed the purity and composition of the synthetic product as well as its formation of α-helical secondary structures in membrane mimetic environments [21].

2.2. Sample preparation

Solid-state NMR-samples were prepared by dissolving 20–25 mg of phospholipid (Avanti Polar Lipids, Birmingham, AL) and the appropriate amount of peptide in dichloromethane/trifluoroethanol/water mixtures. The solvent was removed with a stream of nitrogen and subsequent exposure to high vacuum overnight. 100 μl of 150 mM NaCl, 20 mM Tris, pH 7.4 were added to the resulting peptide–lipid films and the samples were thoroughly vortexed. Alternatively dry powders or films of pure lipid were hydrated by adding solutions of magainin 2 in saline buffer ($\leq$39 mg/ml). In order to allow access of solvents to all monolayers, the samples were submitted to several freeze-thaw-vortex-cycles. The resulting high concentrations of lipid and peptide ensures that $\geq$98% of the polypeptide is in the membrane-associated state (binding constant 2000 M^{-1}, [32]). Furthermore, the high abundance of ^{31}P nuclei in the sample volume ensures good sensitivity during ^{31}P NMR measurements.

2.3. NMR spectroscopy

^{31}P solid-state NMR spectra were recorded on a Bruker AMX400 NMR spectrometer equipped with a MSL high-power cabinet using a 7-mm CP-MAS probe without rotation. A Hahn echo pulse sequence with high power proton decoupling was employed [33]. The following spectral parameters were used: spectral width 40 kHz, acquisition time 6.4 ms, 512 time domain data points, 90° pulse width 6 μs, interpulse delay 40 μs, recycle delay 1.5 s, number of scans 500–2500. Before Fourier transformation zero filling to 2048 points and an exponential multiplication corresponding to a line broadening of 100 Hz were applied.

3. Results

In order to test for the macroscopic phase behavior of POPC membranes when increasing amounts of magainin 2 are added, solutions of peptide and phospholipid were prepared in dichloromethane/trifluoroethanol/water, thor-

oughly dried and hydrated. The initial use of organic solvents ensures that polypeptides and phospholipids are homogeneously mixed. All samples discussed in this paper were investigated by ^{31}P solid-state NMR spectroscopy covering a temperature range of at least 40 K (between $\leq$278 K and $\geq$313 K). The ^{31}P NMR line shape of pure POPC in saline buffer is characteristic of randomly dispersed phospholipid bilayers (Fig. 1A) and exhibits a chemical shift anisotropy of 45 ppm at 308 K in agreement with previous publications [34]. Addition of $\geq$3.6 mol% magainin 2 causes a decrease of the low field intensity of the phospholipid powder pattern (Fig. 1C–E) and results in ^{31}P NMR spectra which are indicative of phospholipid bilayers whose normal is (partially) oriented perpendicular to the magnetic field direction [35].

This magnetic orientation of lipid membranes is the result of the cooperative alignment of the diamagnetic moments of the individual molecules [36,37]. In the magnetic fields of modern NMR spectrometers (in this work 9.4 T) the interaction energy of the diamagnetic moment of single molecules is insufficient to overcome the orientational fluctuations caused by thermal energy. However, the molecular diamagnetic moments of the individual molecules composing lyotropic mesophases align in a cooperative manner and experimental conditions that allow for the magnetic alignment of phospholipid bilayers as well as bicellar structures have been presented [38,39].

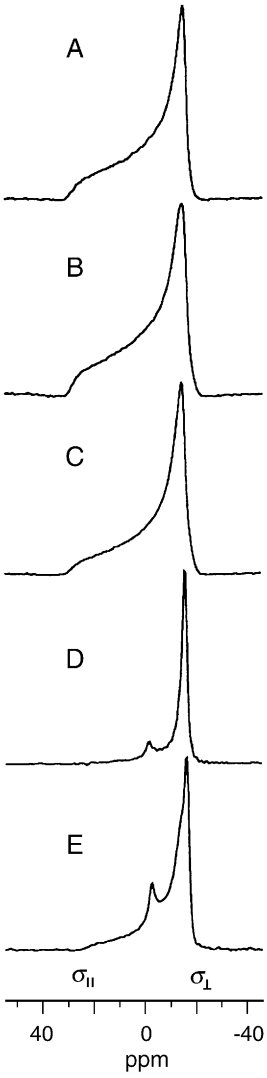

Fig. 1. ^{31}P solid state NMR spectra of magainin 2-POPC membranes premixed in organic solution. 150 mM NaCl, 20 mM Tris, pH 7.4, temperature 308 K. The peptide concentrations are: A: no peptide present, B: 1.6 mol%, C: 3.6 mol%, D: 6.3 mol%, E: 8.4 mol%.

At magainin 2 concentrations $\geq$6.3 mol%, the mixtures become optically transparent and the ^{31}P NMR spectra exhibit an additional signal at the isotropic chemical shift position (Fig. 1D, E). This is in good agreement with freeze fracture electron microscopy experiments which show the disruption of extended phospholipid vesicles into smaller structures in the presence of high magainin concentrations (K. Zierold and B. Bechinger, unpublished). The presence of two well resolved ^{31}P NMR signals indicates that the phospholipid populations are in slow exchange with respect to the ^{31}P NMR time scale (10^{-4} s).

In order to investigate how the composition and geometry of the phospholipid fatty acyl chains affect the magainin–phospholipid interactions additional experiments were performed using samples prepared from saturated fatty acyl chain phosphatidylcholines. ^{31}P NMR spectra of DMPC dispersions in saline buffer exhibit powder pattern line shapes characteristic of liquid crystalline bilayers at temperatures $\geq$298 K and of gel state bilayers at temperatures $\leq$288 K in agreement with a published phase transition temperature at 296 K [40]. The measured chemical shift dispersion of 61 ppm (278 K) and 46 ppm (308 K) agree well with those of previously published ^{31}P NMR spectra [41] (Fig. 2A and G).

With increasing magainin 2 concentrations, the DMPC gel phase is characterized by a concentration dependent appearance of strong isotropic signal intensities (Fig. 2B–F). In contrast, liquid crystalline DMPC membranes exhibit non-bilayer signals only at high magainin 2 concentrations (Fig. 2M). Upon addition of magainin 2, the ^{31}P anisotropy of DMPC liquid crystalline bilayers decreases (Table 1). This effect is most pronounced at temperatures close to the main phase transition temperature and agrees well with previously published ^{2}H-NMR experiments [42]. In these studies, the addition of magainin 2 causes a decrease in second moments of the deuterium signal of $^{2}H_{52}$-DMPC/POPC/POPG 3/3/2 phospholipid bilayers when investigated at 303 K.

In vivo magainin antibiotics are stored in the granules of the frog skin, from which they are released into the aqueous environment when infections occur [3]. The macroscopic phase behavior of POPC membranes was therefore also investigated when aqueous magainin solutions where added to preformed membranes. In order to ensure the accessibility of buffer, salt and polypeptide to all lipid monolayers, the preparations were thoroughly vortexed and submitted to

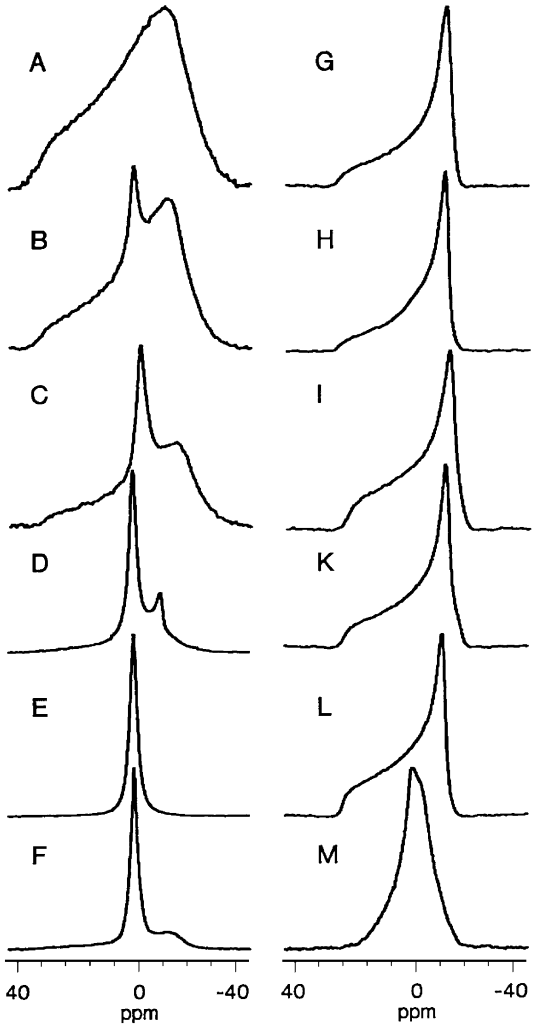

Fig. 2. ^{31}P NMR spectra of DMPC membranes after the addition of solutions of magainin 2 in 150 mM NaCl, 20 mM Tris, pH 7.4. Temperature: A–F: 278 K, G–M: 308 K. The magainin concentrations are: A, G: no peptide added, B, H: 0.8 mol%, C, I: 1.5 mol%, D, K: 2.3 mol%, E, L: 3.9 mol%, F, M: 7.9 mol%.

several freeze–thaw cycles. At physiological temperatures, the addition of 0.8 to 4 mol% magainin 2 results in the appearance of additional isotropic signal intensities (Figs. 3H, I and 4A) which disappear after cooling the sample to room temperature (Fig. 3B, C). These isotropic signals are indicative of phospholipid populations that reorient quickly with respect to the magnetic field direction, but which exchange slowly with the bilayer phase when compared to the ^{31}P NMR time scale. At high polypeptide concentrations, the ^{31}P NMR spectra are again indicative of oriented phospholipid bilayers (Fig. 3E, F, L and M).

In the presence of 3 mol% magainin 2, the intensity of this isotropic signal decreases significantly after the temperature has been cycled in a systematic manner between $\leq$298 K and $\geq$308 K (Fig. 4). During these experiments, the sample was prepared as described in Materials and methods and measured for a first time at 308 K (Fig. 4A). Thereafter, NMR spectra were obtained at temperatures alternating

Table 1
The experimentally measured difference between $\sigma_{\parallel}$ and $\sigma_{\perp}$ ($\Delta\sigma$) from proton-decoupled ^{31}P solid-state NMR spectra of magainin 2/phospholipids mixtures (±1 ppm)

Phospholipid	mol% Magainin 2	$\Delta\sigma$/ppm (288 K)	$\Delta\sigma$/ppm (308 K)
POPC	0	47	45
	2.0	48	46
	4.0	47	44
DMPC	0	58	46
	1.6	56	44
	4.1	42	41
POPS	0	58	56
	8.1	52	52
PI	0	65	62
	8.1	53	52

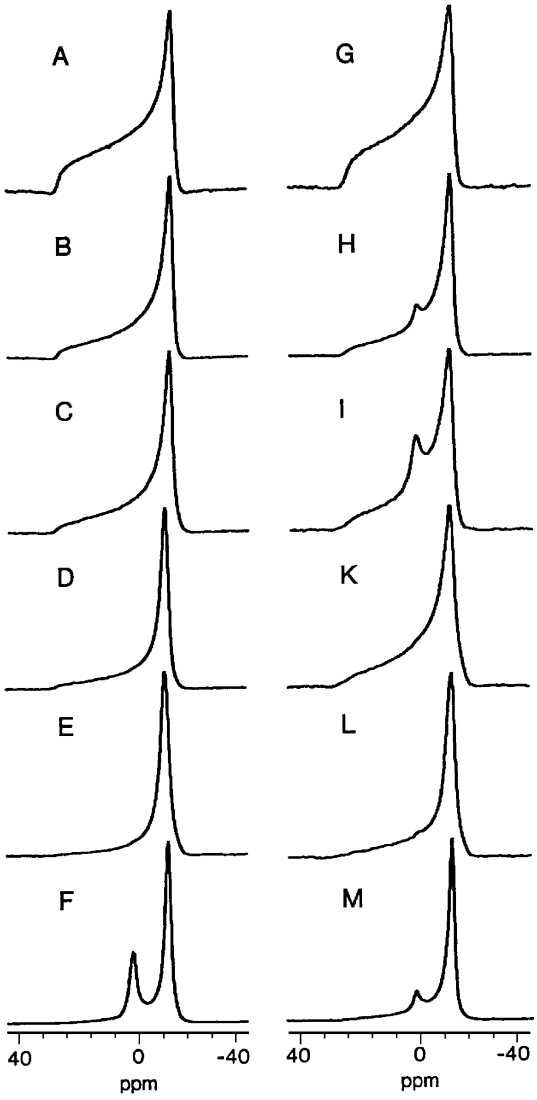

Fig. 3. ^{31}P NMR spectra of POPC membranes after the addition of solutions of magainin 2 in 150 mM NaCl, 20 mM Tris, pH 7.4 to dry phospholipid followed by several freeze/thaw/vortex as well as additional temperature cycles (see Fig. 4 and text for details). Temperature: A–F: 278 K, G–M: 308 K. The magainin concentrations are: A, G: no peptide added, B, H: 0.8 mol%, C, I: 1.5 mol%, D, K: 3 mol%, E, L: 5.7 mol%, F, M: 8 mol%.

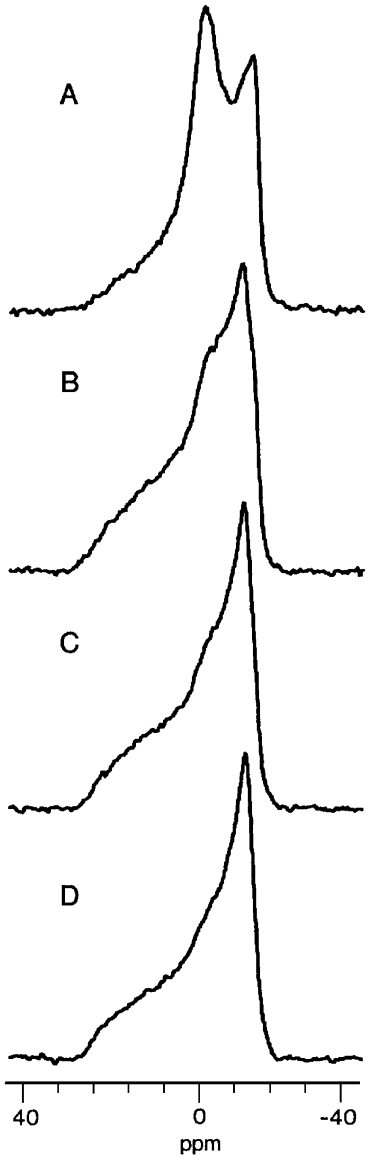

Fig. 4. The effect of cycling the temperature between 298 and 308 K on the ^{31}P NMR spectral line shape of a sample of 3 mol% magainin 2 in POPC is shown. The spectra were recorded at 308 K in 150 mM NaCl, 20 mM Tris, pH 7.4. A: before temperature cycling, B: after 1 cycle, C: after 2 cycles, D: after 4 cycles.

between ≤298 K and ≥308 K. Each renewed passage at 308 K completed a cycle and the NMR spectra obtained at this temperature are shown in Fig. 4B–D. After the samples were submitted to a few of these temperature cycles, the resulting NMR spectra closely resemble those obtained of magainin–phospholipid preparations first mixed in organic solutions (cf. Figs. 1C, 3K and 4D). When merely incubating the samples at constant temperatures of 298 K or 308 K the decrease in isotropic signal intensity is considerably less pronounced (not shown).

Previous measurements of bilayer-association constants using CD spectroscopy indicate that the association of magainins with vesicles composed of negatively charged phospholipids is 2 to 3 orders of magnitude higher when compared to dipolar phosphatidylcholines. The negative surface charge density of these bilayers results in an increase of the local concentration of positively charged molecules, including magainins, along the bilayer surface. The apparently high association constants measured in the bulk phase therefore reflect the high local concentrations of positively charged magainin in the Gibbs–Helmholtz double layer along the membrane surface [8]. The increased affinity of magainins to negatively charged membrane surfaces has been suggested as providing a basis for the antibiotic and tumoricidal selectivity of these peptides [43,44]. In the study presented in this paper, the bulk water phase was kept small, therefore ≥98% of polypeptide is associated with both, charged or uncharged membranes. The ^{31}P NMR spectra of POPS or PI membranes do not exhibit the significant distortions of the bilayer structure observed for POPC membranes. The chemical shift anisotropy of these acidic phospholipid bilayers exhibit, however, an up to 18 % decrease due to the presence of large amounts of polypeptides (Table 1).

4. Discussion

The addition of magainin 2 results in macroscopic phase changes of phospholipid membranes which are a function of the chemical composition of the lipids, the temperature of the sample and the concentration of the polypeptide. Whereas in the presence of magainins, a significant decrease in the chemical shift anisotropy of acidic phospholipid bilayers is observed, isotropic ^{31}P NMR signal intensities appear in the spectra of zwitterionic phosphatidylcholines. These signals are indicative for the formation of non-bilayer structures. At the same time, the optical density of the dispersions become clear. We, therefore, propose that small vesicles or micellar structures form when phosphatidylcholines and magainin 2 are mixed. The resulting short correlation times of these structures results in the efficient averaging of the ^{31}P chemical shift anisotropy and narrow resonances at the isotropic chemical shift position are observed (Figs. 1D, E and 2B–F, M).

Furthermore, the shift in ^{31}P resonance intensity to the $\sigma_{\perp}$ frequency indicates that POPC membranes (partially) orient in the magnetic field of the NMR spectrometer with the membrane normal perpendicular to the magnetic field direction (Figs. 1D, E, 3E, F, L and M). Such spectra have been related to the formation of disk-like bicelles, perforated membranes, or wormlike micelles, structures which are commonly observed when short-chain and long-chain phospholipids are mixed at the appropriate ratio [45–48].

The membrane disruptive properties of magainins are not unique, but have also been observed in the presence of other amphipathic helical peptides, such as apolipoproteins [49], myelin basic protein [50], glucagon [51], melittin [52,53], pardaxin [54], signal sequences [55,56], basic amphiphilic

model peptides [57] or upon addition of lysolipids [58] or detergents [39] to phospholipid bilayers.

In previous studies, magainins interfered with the free-energy transduction of whole cells, isolated mitochondria, or reconstituted cytochrome oxydase liposomes [14]. In the latter study 0.6 to 3 mol% magainin uncoupled the respiratory chain to half maximum values at lipid concentrations very similar to those used in the ^{31}P NMR experiments presented in this paper. The isotropic signal intensities are emphasized in the presence of gel phase lipids (Fig. 2B–F), or when magainin solutions are added to preformed membranes without equilibration (Fig. 4A). This latter situation mimics to some extent the physiological situation where the peptides are released from skin granules into the infected aqueous environment. Thereby, it is possible that high local peptide concentrations are reached on the bacterial membranes.

The spectral changes observed during the addition of magainin 2 polypeptides can be explained by studying the geometry of lipids and peptides. To this purpose, the molecular shape concept has been developed and tested extensively in order to systematically describe the polymorphism of lipid membranes and lipid–detergent mixtures [31]. In aqueous environments, the molecules composing biological membranes form tight structures of reduced permeability due to strong hydrophobic, van der Waals and electrostatic intermolecular interactions. As a result, the geometrical and interaction space of the molecule is an important factor that determines the shape of the resulting aggregate. In order to form lipid bilayers, the shape of the individual molecules has to be approximately cylindrical, whereas molecular geometries that resemble inverted cones result in micellar phases. The experimentally observed decrease in ^{31}P chemical shift anisotropy as well as isotropic phase properties of phospholipid membranes suggest that magainin 2 intercalates into the lipid interface without penetrating into the full depth of the hydrophobic bilayer region.

Previous multidimensional solid-state NMR spectroscopy as well as CD, Raman and FTIR spectroscopy indicate that magainin 2 indeed adopts a right-handed α-helical conformation extending at least from residues 2 to 20 in lipid bilayers [23]. Helical wheel projection shows that such a helix is amphipathic with the polar and hydrophobic amino acid side chains neatly separated on opposite faces of the helix (Fig. 5). An intercalation of this helix into the bilayer interface allows for both hydrophobic and electrostatic interactions to be satisfied. Proton-decoupled ^{15}N solid-state NMR spectroscopy of oriented lipid bilayers indicate an alignment of the magainin 2 helical axis parallel to the membrane surface at peptide concentrations of 0.8–3 mol% [23]. Furthermore, fluorescence-quenching experiments of magainin derivatives where the three phenylalanines of magainin 2 are independently replaced by tryptophanes were performed [59]. These experiments indicate that all three fluorescent

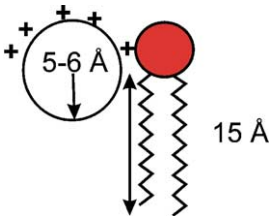

Fig. 5. The hydrophobic thickness of POPC membranes (15 Å) and the radius of α-helices (5–6 Å) are compared to each other. The distribution of positive charges (+) along one face of the peptide illustrates the amphipathic nature of the peptide helix.

probes are localized approximately 10 Å equidistant from the bilayer center (Fig. 5). A comparison of the molecular dimensions shows that the diameter of the magainin 2 α-helix is insufficient to completely fill the thickness of one POPC monolayer (Fig. 5).

Magainins share their capability to increase the conductivity across lipid bilayers with other amphipathic polypeptides such as melittin [60], helical fragments from channel proteins [61,62], designed amphipathic helical peptides [63,64] as well as detergents [65]. Although transmembrane electrical potentials increase the electrophysiological activity of magainin peptides [12,13], it is not a requirement for them to develop membrane-permeabilizing properties. In the absence of electric fields, a chemical gradient and the presence of magainin are sufficient to allow for the leakage of the large fluorescence dyes calcein (MW 623) or 6-carboxyfluorescein/Co^{++}, respectively [15,66]. The onset of leakage takes place at magainin concentrations of approximately 3 mol% which is equivalent to 81 g/mol lipid, a based-on-weight-value very similar to those observed for the permeability increases in the presence of the detergents Triton-X100 and octyl glucoside [65,66]. The use of magainins as 'peptidergents' for protein crystallography further emphasizes the close biophysical relationship between amphipathic peptides and detergents [67].

The proton-decoupled ^{31}P solid-state NMR spectroscopy together with the experimental results shown in this paper provide structural indications of the detergent-like macroscopic phase properties of amphipathic peptide antibiotics. Geometrical considerations (Fig. 5), the observation of isotropic phases in phosphatidylcholine/magainin 2 mixtures (Figs. 1–4) are suggestive that the peptide exerts a positive curvature strain. This is confirmed by the inhibitory action of phosphatidylethonalamines when the membrane-permeabilizing properties of magainin were tested in PG bilayers [68]. It is also evidenced by the increase in the bilayer to inverted hexagonal phase transition temperature due to related peptides [69]. Although in the liquid crystalline phase, the curvature strain is eased by the conformational adaptations of the fatty acyl chains, this becomes more difficult in gel-phase magainin 2–DMPC mixtures. Therefore, the macromolecular phase transitions are more pronounced in gel-phase membranes (Fig. 2B–F).

On the other hand, the cationic phospholipid membranes do not show the same pronounced phase transitions observed for phosphatidylcholine membranes, albeit a decrease of the chemical shift anisotropy has also been observed for PI and POPS membranes (Table 1). Interestingly, designed amphipathic peptide antibiotics have been shown to exhibit several orders of magnitude decreased activities in the presence of anionic vesicles when the amount of membrane association is taken into consideration [17]. It has been suggested that penetration of the bilayers of these peptides is reduced due to electrostatic interactions at the membrane surface.

Several models have been proposed in order to explain the pore-forming and antibiotic properties of cationic peptide antibiotics and reviewed recently in considerable detail [6–8,26,30]. The work presented here demonstrates that the macroscopic phase changes due to the presence of magainin 2 support views that consider their detergent-like properties.

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