Eur Biophys J (2011) 40:463–470
DOI 10.1007/s00249-010-0668-x

ORIGINAL PAPER

A kinked antimicrobial peptide from *Bombina maxima*. II. Behavior in phospholipid bilayers

Ralf Heinzmann · Stephan L. Grage · Constantin Schalck · Jochen Bürck · Zoltán Bánóczi · Orsolya Toke · Anne S. Ulrich

Received: 29 October 2010 / Revised: 3 December 2010 / Accepted: 23 December 2010 / Published online: 11 February 2011

Abstract The preceding contribution by Toke et al. has studied the structure of the cationic antimicrobial peptide maximin-4 in detergent micelles and in organic solvent, revealing a different kink angle and side-chain interactions in the two different environments. Here, we have examined the same peptide in lipid bilayers using oriented circular dichroism (OCD) and solid-state ^{15}N nuclear magnetic resonance (NMR) in aligned samples. OCD showed that maximin-4 is helical and adopts an oblique alignment in the membrane, and lacks the characteristic realignment response that is often observed for amphipathic α-helical peptides at a peptide:lipid ratio between 1:100 and 1:20. Solid-state ^{15}N-NMR experiments suggest that maximin-4 also remains unaffected by lipid charge and temperature. Analyzing ^{15}N labels in positions Ala12, Ala13, and Leu14, an oblique tilt angle of the N-terminal helix of ~130° relative to the membrane normal was found, in good agreement with the amphiphilic profile of this segment. An additional constraint at Ala22 in the C-terminal segment is found to be compatible with a continuous α-helix, but unfavorable side-chain interactions make this solution unlikely. Instead, a kink at Gly16 seems fully compatible with all known constraints and with the biophysical expectations in the membrane-bound state, given the liquid-state NMR structures. It thus seems that the flexible kink in maximin-4 allows the two helical segments to adjust to the local environment. The irregular amphiphilic profile and the resulting versatility in shape might explain why maximin-4 lacks the realignment response that has been characteristically observed for many related frog peptides forming straight amphipathic α-helices.

Keywords Cationic amphipathic antimicrobial peptide · Solid-state NMR · Maximin · Oriented circular dichroism (OCD) · Lipid bilayer membrane · *Bombina maxima*

Membrane-active peptides: 455th WE–Heraeus-Seminar and AMP 2010 Workshop.

R. Heinzmann · S. L. Grage · C. Schalck · J. Bürck · A. S. Ulrich
Karlsruhe Institute of Technology (KIT), Institute of Biological Interfaces (IBG-2), P.O. Box 3640, 76021 Karlsruhe, Germany

S. L. Grage · A. S. Ulrich (✉)
KIT and DFG-Center for Functional Nanostructures (CFN), Institute of Organic Chemistry, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany
e-mail: anne.ulrich@kit.edu

Z. Bánóczi
Research Group of Peptide Chemistry, Eötvös Loránd University, P.O. Box 32, 1518 Budapest 112, Hungary

O. Toke
Institute of Structural Chemistry, Chemical Research Center of the Hungarian Academy of Sciences, 59-67 Pusztszeri út, 1025 Budapest, Hungary

Present Address:
R. Heinzmann
Karlsruhe Institute of Technology, Institute of Nanotechnology, P.O. Box 3640, 76021 Karlsruhe, Germany

Introduction

Antimicrobial peptides (AMPs) play a major role in the defense of many organisms. In particular, AMPs acting against the cell membrane of the invading microorganism have been discussed as potent antibiotics not subject to bacterial resistance, and their function has been studied intensively in recent years (Toke 2005; Grage et al. 2010). Common to all is a pronounced amphipathic, often cationic,

character of the peptide, which leads to their localization at the lipid–water interface, where they can interfere with membrane integrity. Different models for interactions between peptides and membranes have been proposed, such as the barrel-stave, wormhole or carpet models (Matsuzaki 1999; Shai 1999; Zasloff 2002). However, the molecular mode of action often remains poorly understood and getting insight into the peptide structure and any realignment steps or oligomerization events in the membrane can give important clues on their antimicrobial mechanism. To elucidate the function of maximin-4, we thus addressed its structure in membrane-mimicking environments in the preceding publication (Toke et al. 2010), and here the molecular orientation in a lipid bilayer is investigated.

Maximin-4 is part of the innate immune system of the Chinese red-belly toad *Bombina maxima*, and has been described to target bacterial membranes (Lai et al. 2002a, 2002b). Possessing four positively charged residues and a negative one [GIGGVLLSAGKAALKGLAKVLAEK-YAN-NH_2], it bears many characteristics of the class of cationic amphipathic helical peptides such as PGLa and the magainin family found in frogs (Zasloff 2002; Yang et al. 2000; Toke et al. 2004a). They have been studied as prototypes of membrane-active AMPs, and were found to adopt three characteristic alignment states in the lipid bilayer, classified as S-, T-, and I-states (referring to "surface"-aligned, "tilted," and "inserted"). For many members of this group of peptides, a change in orientation has been observed when increasing the peptide:lipid molar ratio above a critical threshold value (Ludtke et al. 1994; Toke et al. 2004b; Glaser et al. 2005; Strandberg et al. 2006, 2008; Tremouilhac et al. 2006a; Buerck et al. 2008). Recently, also other factors, such as changes in temperature, have been reported to trigger realignment (Tremouilhac et al. 2006b; Afonin et al. 2008a; Strandberg et al. 2009a, b; Grage et al. 2010). Even some structurally unrelated antimicrobial peptides, such as the cyclic gramicidin S or the hydrophobic alamethicin, have been found to realign in lipid membranes over a range of biologically relevant peptide:lipid ratios between 1:100 and 1:20 (Afonin et al. 2008b; Maisch et al. 2009). It could thus be expected that also maximin-4 may follow a similar behavior in the membrane under comparable experimental conditions. However, unlike the well-defined amphiphilic molecular structures noted above, liquid-state NMR of maximin-4 has revealed not a straight α-helix, but an unusual helix–kink–helix motif in which the helical conformation is interrupted at Gly16. The polar and charged residues were found to be distributed either on the convex face of the kinked structure, or clustered in the concave bend, depending on the solvent. In view of its membrane-perturbing activity, it is intriguing to find out in which conformation maximin-4 binds to a lipid bilayer. Thermodynamically it might be favorable to optimize hydrogen bonding in the low-dielectric environment by forming a continuous helix, but possibly at the expense of an optimized amphiphilic profile. Furthermore, if we observe any structural response to changes in concentration, temperature, and lipid composition, these factors could be examined with regard to molecular realignment or oligomerization. To elucidate these issues, important for the possible function of maximin-4 in terms of pore formation, we used oriented circular dichroism (OCD) and solid-state ^{15}N-NMR in oriented bilayers to study the conformation and alignment of the peptide in the membrane (Buerck et al. 2008; Strandberg and Ulrich 2004). We selected four positions for ^{15}N labeling such that (a) the four residues are optimally distributed around a helical wheel representation of maximin-4, (b) the NMR orientational parameters of three adjacent residues in the putative N-terminal helix of maximin-4 can be tested as to whether they really have an α-helical conformation in the membrane-bound state, and (c) a fourth position was selected in the putative C-terminal helix to test whether its alignment is correlated with the other three N-terminal labels or rather deviates from a straight α-helix.

Materials and methods

Peptide synthesis

Four maximin-4 analogues, amidated at the C-terminus, and labeled with ^{15}N in the backbone at either Ala12, Ala13, Leu14 or Ala22 were synthesized as described in the accompanying paper (Toke et al. 2010).

Sample preparation

OCD samples were prepared by codissolving dimyristoyl-phosphatidylcholine (DMPC) and dimyristoyl-phosphatidylglycerol (DMPG) (Avanti Polar Lipids, Alabaster, AL, USA) in the desired ratio in methanol and dissolving ~5–25 μg peptide in an aliquot of this solution to achieve the desired peptide:lipid ratio. After solvent evaporation and drying under vacuum overnight, 260 μl water was added to the dry sample. The mixture was ultrasonified for 5 min and homogenized by 10 freeze–thaw cycles to yield an opalescent suspension, of which 100 μl was spotted onto a quartz glass plate forming the window of the OCD chamber. The sample was dried and then hydrated over the gas phase in the OCD chamber containing a saturated K_2SO_4 solution for 15 h at 30°C, and measured immediately after preparation. Solid-state NMR samples were prepared by codissolving DMPC, DMPG, and the peptide in the desired ratio in methanol, and spotting 20–30 μl

drops, each containing ~0.8–1 mg sample, onto 16–18 thin glass plates of 7 mm × 9 mm size (Marienfeld, Germany). After drying under vacuum overnight, the glass plates were stacked and placed into a custom-made glass sample container. After rehydration under humid atmosphere (saturated K_2SO_4) at 48°C overnight, wet papers were placed in both ends of the sample container, and the container sealed with parafilm. The solid-state NMR measurements were performed immediately after sample preparation.

OCD experiments

A Jasco J-810 spectropolarimeter was used for the circular dichroism measurements. For OCD spectroscopy, the peptide was reconstituted into macroscopically aligned phospholipid bilayers and subsequently hydrated in an OCD cell. The OCD cell, developed and built in-house, is computer controlled and can be integrated in the J-810 spectropolarimeter as an accessory (for details, see Buerck et al. 2008). To avoid linear dichroism artifacts, spectra were recorded at eight different sample rotation angles and averaged.

Solid-state NMR experiments

Solid-state ^{15}N-NMR was performed on an Avance II wide-bore ultra-shield Bruker spectrometer operating at ^{1}H and ^{15}N resonance frequency of 600 and 60 MHz, respectively. A ^{15}N/^{1}H double-tuned probe (home-built in collaboration with Bruker, Rheinstetten, Germany, and with NHMFL, Tallahassee, USA) equipped with a lowE resonator was used for ^{15}N cross-polarization experiments. We employed a MOIST cross-polarization sequence (1 ms contact time, 50 kHz radiofrequency field strength) to enhance the ^{15}N signals, and SPINAL64 decoupling during acquisition (10 ms acquisition length, 30 kHz field strength) (Levitt et al. 1986; Fung et al. 2000). SAMMY and MREV8-decoupled spectra were also acquired, but not used due to very low signal-to-noise ratio (S/N). Approximately 10,000 scans were acquired with a recycle time of 6 s for samples containing ~1 mg of a singly labeled peptide, and spectra were processed with ~500 Hz line broadening.

Solid-state NMR data analysis

The measured ^{15}N chemical shift values obtained from four different labeled positions were compared with the respective values predicted for any one structural model. The chemical shift values were calculated for that model in different orientations with respect to the bilayer normal, where the averaged orientation ensemble was represented by two angles τ and ρ, relating a molecule fixed frame to the bilayer normal, and the zz-component of the order parameter matrix, S. A complete description by an order Saupe matrix would require more labeled positions, and hence was outside the scope of this study. The molecule-fixed frame of the liquid-state NMR structures was defined by the principal axes of the tensor of inertia of the backbone atoms, with the z-axis pointing along the most elongated axis of the molecule (smallest moment of inertia), with its direction from N- to C-terminus, and the y-axis pointing along the axis of the largest moment of inertia. The angle τ measures the angle from the bilayer normal to the z-axis. The angle ρ defines how far the molecule-fixed x-axis is turned away from the direction pointing into the membrane, when turning the molecule around its z-axis. For the ideal α-helix model, τ is defined as the angle from the bilayer normal to the helix axis (from N- to C-terminus), and ρ as the angle by which Cα of the 12th residue is turned away from the direction defined by turning τ (see Strandberg et al. 2006). The best-fit solution (in τ, ρ, and S) was determined by scanning through τ and ρ in steps of 2° over the range 0–180°, and S in steps of 0.1 over the range 0.1–1.0. For each point on this three-dimensional (3D) grid the root-mean-square deviation (RMSD) of the N measured chemical shift values $y_{\mathrm{exp},k}$ was calculated as the difference between the experimental value σ_k from the predicted value $y_{\mathrm{calc},k}$

$$\mathrm{RMSD} = \left[\sum_k \left((y_{\mathrm{exp},k} - y_{\mathrm{calc},k})/\sigma_k \right)^2 \Big/ N \right]^{1/2}$$

We note at this point that each data point is weighed according to its respective experimental error. The RMSD defined in this way is dimensionless, and values below ~1 indicate nominal agreement between the model and the experiment within average experimental error.

Results and discussion

OCD experiments

OCD measurements on oriented peptide samples allow qualitative analysis of the alignment of an α-helical peptide in the membrane. In particular, the band at 210 nm exhibits a pronounced dependence on the orientation of an α-helix with respect to the direction of the incident beam, i.e., the membrane normal, and can serve as a fingerprint for the alignment of the helix axis. A negative intensity at this frequency exceeding that of the band at 223 nm is indicative of an alignment parallel to the bilayer plane, whereas zero intensity at 210 nm indicates an upright transmembrane insertion (Buerck et al. 2008). The high sensitivity and low time requirement of this OCD method compared

with solid-state NMR allowed us in particular to compare the orientation of maximin-4 in different lipid systems and for different peptide:lipid ratios. The general α-helical conformation of this peptide in the presence of unilamellar lipid vesicles (DMPC:DMPG, 1:1) has been demonstrated by standard CD in the accompanying paper (Toke et al. 2010). When maximin-4 was reconstituted in oriented bilayers of DMPC:DMPG (1:1) at different peptide:lipid ratios (Fig. 1a), we noted a pronounced positive band near 194 nm, and two negative bands at 210 and 223 nm, confirming the α-helical character. The negative intensity at 210 nm, staying below the intensity of the band at 223 nm, suggests that the helix is tilted with an oblique angle in DMPC/DMPG membranes. Changing the peptide:lipid ratio from 1:20 to 1:100 (mol/mol, all ratios are given as molar ratios in the following) had no effect on the intensities at 210 and 223 nm. For this range of biologically relevant peptide concentrations the molecules thus maintain the same orientation in the membrane. This behavior of maximin-4 is notable and different from that of numerous other amphipathic helical peptides such as PGLa and the magainin family, for which a characteristic realignment had been reported to occur at a limiting threshold concentration (Tremouilhac et al. 2006a; Buerck et al. 2008; Glaser et al. 2005; Ludtke et al. 1994; Strandberg et al. 2008; Toke et al. 2004b). Comparison of the spectra in DMPC, and in 1:3 mixtures of DMPC:DMPG (Fig. 1b) shows that the absence or increased presence of lipid charge has only a marginal effect on the conformation or orientation of maximin-4 in membranes.

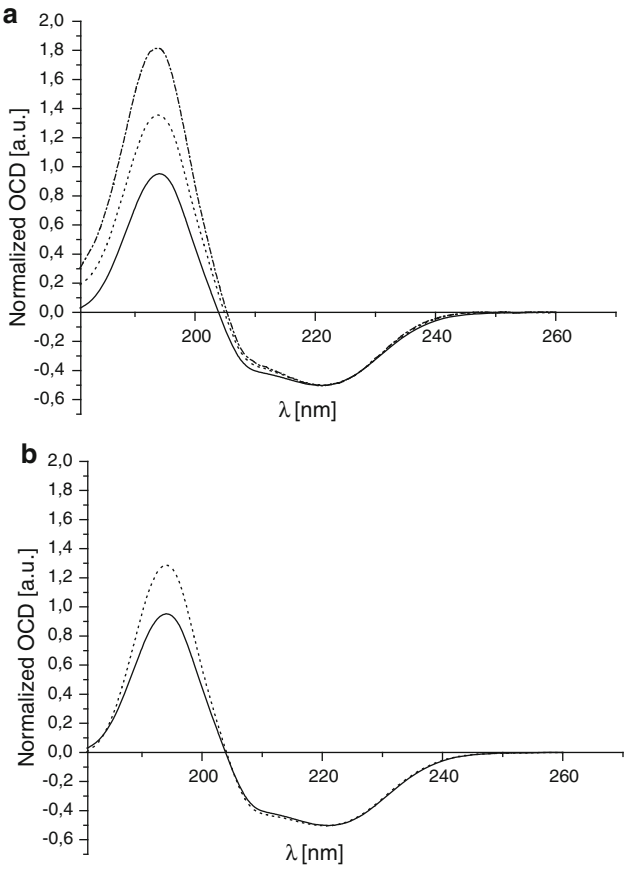

Fig. 1 Oriented CD (OCD) measurements of maximin-4 in oriented DMPC/DMPG bilayers show that it is α-helical and on average obliquely tilted in the lipid bilayer. When the three different peptide:lipid ratios of 1:20 (*solid line*), 1:50 (*dotted line*), and 1:100 (*dashed line*) are compared in DMPC:DMPG 1:1 bilayers (**a**), the intensities at 210 nm are indicative of a constant tilt independent of the peptide:lipid ratio. Spectra obtained with a peptide:lipid ratio of 1:20 in DMPC:DMPG 3:1 (*dotted line*) and DMPC:DMPG 1:1 (*solid line*) exhibit no significant variation with the percentage of charged lipids for the relevant intensity at 210 nm (**b**). Intensities were normalized to display the same value at 223 nm

Solid-state ^{15}N-NMR experiments

To extend the qualitative picture obtained above by OCD, which only yielded an average helix alignment, solid-state ^{15}N-NMR was used to describe the behavior of maximin-4 in lipid membranes in more detail, with special attention to the two putative helical regions (Toke et al. 2010). The ^{15}N chemical shift is a direct structural reporter on the orientation of the labeled peptide segment, and constraints obtained from several labels can be combined to determine the orientation of a given three-dimensional molecular model (Strandberg and Ulrich 2004). To this aim, the ^{15}N chemical shifts were measured for four analogues of maximin-4, carrying a single ^{15}N label at position Ala12, Ala13, Leu14 or Ala22. These peptides were studied in two lipid systems, to obtain two independent sets of data and to examine any influence of lipid charge in detail. The ^{15}N-NMR spectra of the four maximin-4 analogues, reconstituted in oriented DMPC or DMPC:DMPG (1:1) bilayers, are shown in Fig. 2, and the ^{15}N chemical shifts are summarized in Table 1. All measurements were performed at 35°C to characterize the peptide structure in the liquid-crystalline phase of the membrane, which is considered the biologically relevant environment. The peptide:lipid ratio in the samples was chosen as 1:50, high enough to obtain ^{15}N-NMR spectra with sufficient signal-to-noise ratio, but low enough to prevent peptide aggregation (Wadhwani et al. 2008). The temperature dependence of the alignment was monitored for the two labels Ala13 and Ala22 reconstituted in DMPC:DMPG (1:1) (Fig. 3) to detect any realignment that may be induced by the lipid phase state or temperature (Afonin et al. 2008b; Grage et al. 2010). However, neither the lipid composition nor the temperature was found to exhibit any significant influence on the observed chemical shifts. Upon varying the temperature, Ala13 stayed constant within experimental error, and Ala22 experienced changes of only 5–10 ppm. Since this slight shift occurs at the lipid phase-transition temperature

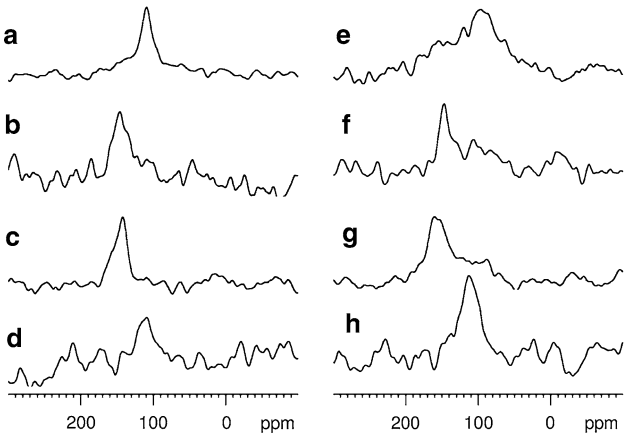

Fig. 2 Solid-state ^{15}N-NMR spectra of maximin-4, selectively labeled at Ala12 (*a*, *e*), Ala13 (*b*, *f*), Leu14 (*c*, *g*), and Ala22 (*d*, *h*). The peptides were reconstituted into oriented bilayers of either DMPC (*a–d*) or DMPC:DMPG 1:1 (*e–h*) at a peptide:lipid ratio of 1:50, and measured at 35°C

Table 1 ^{15}N chemical shift values and error estimates obtained from the solid-state ^{15}N-NMR spectra of Fig. 2, as used in the RMSD analysis of the peptide alignment

Label position	DMPC		DMPC:DMPG 1:1	
	^{15}N chemical shift/ppm	Error/ppm	^{15}N chemical shift/ppm	Error/ppm
Ala12	109	3	100	15
Ala13	145	10	149	10
Leu14	140	4	158	8
Ala22	111	15	110	10

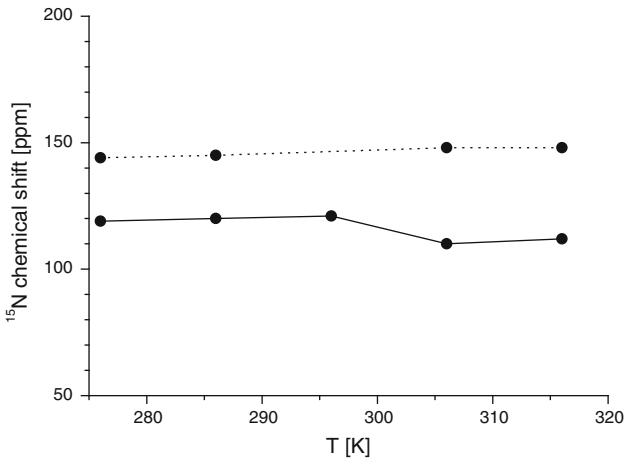

Fig. 3 The temperature dependence of the ^{15}N chemical shift monitored for maximin-4 labeled at position Ala13 (*dotted line*) and Ala22 (*solid line*), reconstituted into oriented bilayers of DMPC:DMPG 1:1 at a peptide:lipid ratio of 1:50

around ~23°C, it may reflect a minor difference in peptide conformation or alignment in the two phase states of the lipid bilayer, but the data clearly do not support a proper temperature-dependent realignment as has been reported for PGLa, MSI-103, MAP, gramicidin S or alamethicin (Afonin et al. 2008a, b; Grage et al. 2010). A different mobility of the peptide in the gel and fluid phase of the membrane does not seem to be compatible with the observed chemical shifts either, as in this case the values should shift towards the isotropic position near 120 ppm above the phase transition, which is not the case.

Liquid-state NMR has revealed a kink at Gly16 in the helical conformation of maximin-4 (Toke et al. 2010). The two helical segments of the peptide must be connected in a flexible way, as the interhelical angle and the respective side-chain interactions differ significantly in sodium dodecyl sulfate (SDS) micelles and in methanol/water. In detergent micelles the charged residues are distributed over the convex face of the kinked structure, whereas in organic solvent they are found to cluster in the concave bend. In the present solid-state ^{15}N-NMR study we therefore labeled three consecutive positions in the N-terminal segment of maximin-4 (Ala12, Ala13, Leu14), and a fourth label was placed near the C-terminus at Ala22 [GIGGVLLSAGK***AAL***K-G_{16}-LAKVL**A**E KYAN-NH_2]. In this way, we intend to characterize on the one hand the N-terminal region using three constraints, while on the other hand the remote label can be included to examine whether the full-length peptide structure is consistent with a continuous α-helix. We thus examined and compared the membrane alignment of an α-helix over the length of $N = 3$ and $N = 4$ constraints. As explained in the "Materials and methods" section, the ^{15}N chemical shifts were calculated for a helix orientation defined by the angle τ of the helix long axis, the angle ρ (defined for a selected residue), and an order parameter S (*zz*-component of the Saupe matrix). We used an ideal α-helix ($\varphi = -61°$, $\psi = -45°$), given that many membrane-active antimicrobial peptides can be readily fitted by such regular conformation (Glaser et al. 2005; Strandberg et al. 2006, 2008, 2009b; Tremouilhac et al. 2006a, b; Maisch et al. 2009). The best-fit solution was found by varying τ, ρ, and S, and evaluating the RMSD between the experimental and calculated chemical shifts (Fig. 4a). A single RMSD minimum was found in all calculations, as summarized in Table 2. The peptide structure is found to be essentially the same in DMPC and DMPC/DMPG, as suggested by the OCD analysis and by the raw ^{15}N-NMR data.

To assess the calculated alignments of maximin-4 and in an attempt to answer the question of whether it forms a straight helix or a kinked one, it is instructive to compare the results for $N = 3$ and $N = 4$. The fit to the first three labels (Ala12/Ala13/Leu14) gives a good RMSD value (<1.0) for the ideal helix model, which supports the finding

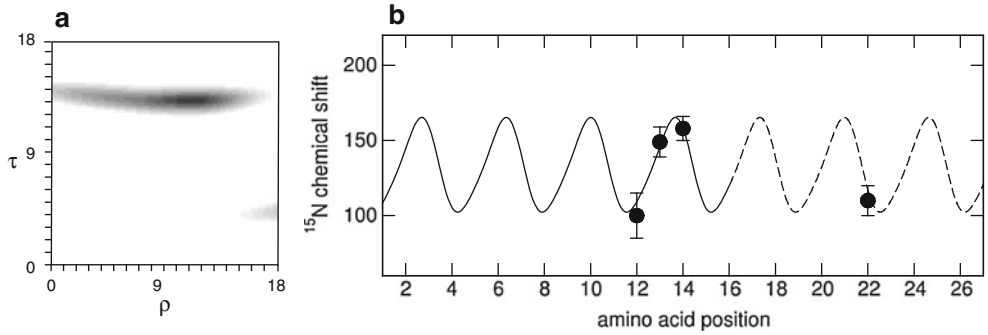

Fig. 4 RMSD analysis of the ^{15}N-NMR data for maximin-4, calculated by scanning through combinations of τ, ρ, and S, assuming an ideal α-helix. **a** Best-fit solution for the data obtained in DMPC:DMPG bilayers (see also Table 2), and **b** chemical shift wave plot. The chemical shift wave of the N-terminal half was fitted to the three positions Ala12, Ala13, and Leu14 (*solid line*). Residue Ala22 in the C-terminal half fits to the same curve (*dashed line*)

Table 2 Alignment of maximin-4 determined by fitting the experimental ^{15}N-NMR data of $N = 3$ or $N = 4$ label positions to an ideal α-helix

Lipid	N^a	τ	ρ	S	RMSD
DMPC	3	126	126	0.9	0.52
DMPC/DMPG	3	130	110	1.0	0.40
DMPC	4	128	124	0.8	0.49
DMPC/DMPG	4	130	114	1.0	0.44

The alignment is characterized by the tilt angle τ, the azimuthal angle ρ, and the order parameter S

[a] $N = 3$ labels (Ala12, Ala13, Leu14) or $N = 4$ labels (Ala12, Ala13, Leu14, Ala22) were used for fitting

from CD that this region is indeed α-helical. The N-terminal segment of maximin-4 is thus found to be oriented with an oblique tilt angle of $\tau \approx 130°$ in the membrane, and with an azimuthal rotation of $\rho \approx 110°$. This alignment is illustrated in Fig. 5a and agrees well with the amphiphilic character of the N-terminal helix. The charged NH_3^+-terminus is exposed at the membrane surface, and the two Lys residues can readily "snorkel" upwards. However, it is clear that in this straight α-helical representation of Fig. 5a the charged residues in the C-terminal segment of maximin-4 (explicit side-chains drawn in gray) would be unfavorably immersed into the hydrophobic bilayer core. The single ^{15}N label at Ala22 is clearly not enough to analyze the conformation and alignment of the C-terminal segment, neither on its own, nor as part of the $N = 4$ fit. Nevertheless, we can try to prove that the corresponding data point is not consistent with a straight α-helix continuing all the way from the N- towards the C-terminus, as recently done in the case of alamethicin (Maisch et al. 2009). If maximin-4 was to form a straight helix, then the ^{15}N chemical shift of Ala22 must lie on the so-called wave plot that is defined by the best-fit solution obtained for Ala12, Ala13, and Leu14. Figure 4b displays this periodic oscillation of the CSA values as a function of the primary peptide sequence. Contrary to our expectations, it turns out that Ala22 does not deviate significantly from the best fit for $N = 3$, and $N = 4$ produces a virtually identical best-fit solution (Table 2). Nevertheless, this seemingly good fit is most likely a coincidence, because in the present situation of a flexible kink any attempted $N = 4$ RMSD analysis can at best exclude possible conformations, but it cannot prove the validity of any one solution such as a continuous straight helix. From the present ^{15}N-NMR data alone it thus happens to be impossible to prove or to exclude a continuous straight α-helical structure for maximin-4.

Altogether, it is highly likely that the kink at Gly16, which was observed in SDS micelles and methanol/water (Toke et al. 2010), is also present in the membrane-bound state of maximin-4. This kink would allow the C-terminus to engage in favorable interactions with the lipids according to its amphiphilic profile, as illustrated in Fig. 5b. The orientation of the N-terminal helix is drawn according to our solid-state ^{15}N-NMR analysis, whereas the alignment of the C-terminal helix is more speculatively based on its amphiphilic character, on the overall helix content observed by CD, and on the average tilt angle measured by OCD. Note that the C-terminal half, as depicted in Fig. 5b, would still result in the same chemical shifts as the extension of a straight helix, as both the tilt angle τ and the rotation angle ρ are changed accordingly. The presence of a kink makes a full structure analysis rather demanding and would require many more labels, as previously noted in the case of alamethicin (Maisch et al. 2009). Nevertheless, many other antimicrobial peptides form uninterrupted α-helices in membranes, such as PGLa with a regular amphiphilic profile, or typical transmembrane peptides from the WALP family. It was demonstrated by careful

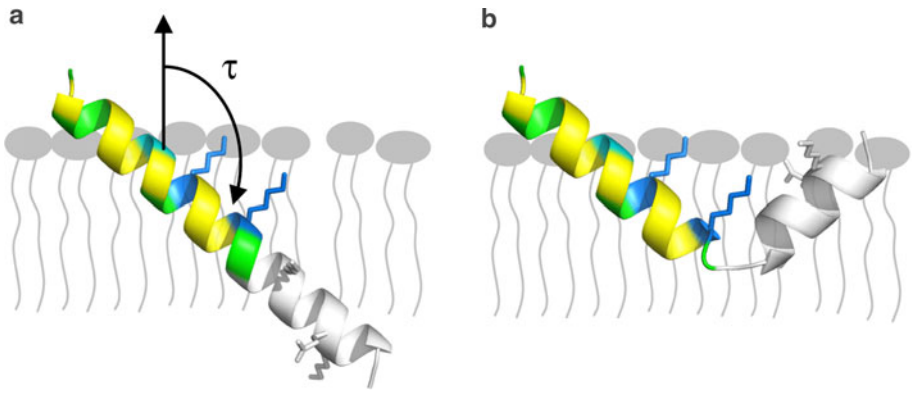

Fig. 5 Alignment of maximin-4 in the membrane, according to our combined analysis by liquid-state ^{1}H-NMR, solid-state ^{15}N-NMR, circular dichroism, and oriented CD. **a** The peptide is positioned into the membrane as a continuous α-helix, which is constrained by the three ^{15}N labels (Ala12/Ala13/Ile14) in the N-terminal segment. **b** Given the kink at Gly16, the C-terminal helix is drawn such that it can engage in favorable amphiphilic interactions with the lipids, while still giving the same ^{15}N chemical shift. *Colors* are used to indicate the type of residue: *yellow*—hydrophobic (Ala, Ile, Leu, Tyr, Val); *cyan*—polar (Ser, Asn); *blue*—cationic (Lys); *red*—anionic (Glu); *green*—glycine. The C-terminal segment is depicted in *gray*, with the charged residues explicitly drawn

^{2}H-NMR measurements that these structural elements can be comprehensively analyzed from three to four independent orientational NMR constraints (Glaser et al. 2005; Strandberg et al. 2006, 2009b), unless dynamic effects are to be included (Strandberg et al. 2009a; Esteban-Martin et al. 2009; Esteban-Martin et al. 2010). Unlike PGLa and its relatives, however, maximin-4 does not form a well-defined amphiphilic helical wheel. Instead, the less regular distribution of polar residues appears to be compensated for by the flexible kink, which allows the two helical segments to adjust independently to the local environment they are exposed to, be this an SDS micelle, an isotropic solvent, or a planar lipid bilayer.

In terms of its lipid–peptide interactions, maximin-4 shows an unusual behavior when compared with other antimicrobial peptides such as PGLa, MSI-103, MAP, gramicidin S, or alamethicin, which have been studied under comparable conditions (Ludtke et al. 1994; Toke et al. 2004b; Glaser et al. 2005; Strandberg et al. 2006, 2008, 2009a,b; Tremouilhac et al. 2006a, b; Buerck et al. 2008; Afonin et al. 2008a, b; Grage et al. 2010; Maisch et al. 2009). While all these peptides interconvert between several distinct orientational states in the membrane upon varying the peptide concentration, temperature or lipid phase state, for maximin-4 we detected no such effect. It thus appears that this kinked structure does not respond much to the lipid environment compared with more rigidly defined, long amphiphilic entities. Instead, the kink in maximin-4 seems to provide this peptide with a flexible joint by which it can adapt to its local environment. The molecular mechanism of membrane perturbation and pore formation may thus differ from that of the other examples named above.

References

Afonin S, Dürr UHN, Wadhwani P, Salgado JB, Ulrich AS (2008a) Solid state NMR structure analysis of the antimicrobial peptide Gramicidin S in lipid membranes: concentration-dependent realignment and self-assembly as a β-barrel. Topics Curr Chem 273:139–154

Afonin S, Grage SL, Ieronimo M, Wadhwani P, Ulrich AS (2008b) Temperature dependent transmembrane insertion of the amphiphilic peptide PGLa in lipid bilayers observed by solid state ^{19}F NMR spectroscopy. J Am Chem Soc 130:16512–16514

Buerck J, Roth S, Wadhwani P, Afonin S, Kanithasen N, Strandberg E, Ulrich AS (2008) Conformation and membrane orientation of amphiphilic helical peptides by oriented circular dichroism. Biophys J 95:3872–3881

Esteban-Martin S, Strandberg E, Fuertes G, Ulrich AS, Salgado J (2009) Influence of whole-body dynamics on ^{15}N PISEMA NMR spectra of membrane proteins: a theoretical analysis. Biophys J 96:3233–3241

Esteban-Martin S, Strandberg E, Salgado J, Ulrich AS (2010) Solid state NMR analysis of peptides in membranes: influence of dynamics and labelling scheme. Biochim Biophys Acta Biomembr 1798:252–257

Fung BM, Khitrin AK, Ermolaev K (2000) An improved broadband decoupling sequence for liquid crystals and solids. J Magn Reson 142:97–101

Glaser RW, Sachse C, Duerr UHN, Wadhwani P, Afonin S, Strandberg E, Ulrich AS (2005) Concentration dependent realignment of the peptide PGLa in lipid membranes observed by solid state ^{19}F-NMR. Biophys J 88:3392–3397

Grage SL, Afonin S, Ulrich AS (2010) Dynamic transitions of membrane-active peptides. Dynamic transitions of membrane active peptides. Meth Mol Biol 618:183–207

Lai R, Liu H, Hui Lee W, Zhang Y (2002a) An anionic antimicrobial peptide from toad *Bombina maxima*. Biochem Biophys Res Commun 295:796–799

Lai R, Zheng YT, Shen JH, Liu GJ, Liu H, Lee WH, Tang SZ, Zhang Y (2002b) Antimicrobial peptides from skin secretions of Chinese red belly toad *Bombina maxima*. Peptides 23:427–435

Levitt MH, Suter D, Ernst RR (1986) Spin dynamics and thermodynamics in solid-state NMR cross polarization. J Chem Phys 84:4243

Ludtke SJ, He K, Wu Y, Huang HW (1994) Cooperative membrane insertion of magainin correlated with its cytolytic activity. Biochim Biophys Acta 1190:181–184

Maisch D, Wadhwani P, Afonin S, Böttcher C, Koksch B, Ulrich AS (2009) Chemical labeling strategy with (R)- and (S)-trifluoromethylalanine for solid state ^{19}F-NMR analysis of peptaibols in membranes. J Am Chem Soc 131:15596–15597

Matsuzaki K (1999) Why and how are peptide-lipid interactions utilized for self defense? Magainins and tachyplesins as archetypes. Biochim Biophys Acta 1462:1–10

Shai Y (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membranelytic peptides. Biochim Biophys Acta 1462:55–70

Strandberg E, Ulrich AS (2004) NMR methods for studying membrane-active antimicrobial peptides. Concepts Magn Res 23A:89–120

Strandberg E, Wadhwani P, Tremouilhac P, Dürr UHN, Ulrich AS (2006) Solid state NMR analysis of the PGLa peptide orientation in DMPC bilayers: structural fidelity of ^{2}H- versus high sensitivity of ^{19}F-NMR. Biophys J 90:1676–1686

Strandberg E, Kanithasen N, Tiltak D, Bürck J, Wadhwani P, Zwernemann O, Ulrich AS (2008) Solid-state NMR analysis comparing the designer-made antibiotic MSI-103 with its parent peptide PGLa in lipid bilayers. Biochemistry 47:2601–2616

Strandberg E, Esteban-Martin S, Salgado J, Ulrich AS (2009a) Orientation and dynamics of peptides in membranes calculated from ^{2}H-NMR data. Biophys J 96:3223–3232

Strandberg E, Tremouilhac P, Wadhwani P, Ulrich AS (2009b) Synergistic transmembrane insertion of the heterodimer PGLa/magainin 2 complex studied by solid-state NMR. Biochim Biophys Acta 1788:1667–1679

Toke O (2005) Antimicrobial peptides: new candidates in the fight against bacterial infections. Biopolymers 80:717–735

Toke O, Maloy WL, Kim SJ, Blazyk J, Schaefer J (2004a) Secondary structure and lipid contact of a peptide antibiotic in phospholipid bilayers by REDOR. Biophys J 87:662–674

Toke O, O'Connor RD, Weldeghiorghis TK, Maloy WL, Glaser RW, Ulrich AS, Schaefer J (2004b) Structure of $(KIAGKIA)_3$ aggregates in phospholipid bilayers by solid-state NMR. Biophys J 87:675–687

Toke O, Bánóczi Z, Király P, Heinzmann R, Bürck J, Ulrich AS, Hudecz F (2011) A kinked antimicrobial peptide from *Bombina maxima*. I. Three-dimensional structure determined by NMR experiments in membrane-mimicking environments. Eur Biophys J. doi:10.1007/s00249-010-0657-0

Tremouilhac P, Strandberg E, Wadwhani P, Ulrich AS (2006a) Conditions affecting the re-alignment of the antimicrobial peptide PGLa in membranes as monitored by solid state ^{2}H-NMR. Biochim Biophys Acta 1758:1330–1342

Tremouilhac P, Strandberg E, Wadhwani P, Ulrich AS (2006b) Synergistic transmembrane alignment of the antimicrobial heterodimer PGLa/Magainin. J Biol Chem 281:32089–32094

Wadhwani P, Buerck J, Strandberg E, Mink C, Afonin S, Ulrich AS (2008) Using a sterically restrictive amino acid as a ^{19}F-NMR label to monitor and to control peptide aggregation in membranes. J Am Chem Soc 130:16515–16517

Yang L, Weiss TM, Lehrer RI, Huang HW (2000) Crystallization of antimicrobial pores in membranes: magainin and protegrin. Biophys J 79:2002–2009

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395