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Structure and Alignment of the Membrane-Associated Antimicrobial Peptide Arenicin by Oriented Solid-State NMR Spectroscopy

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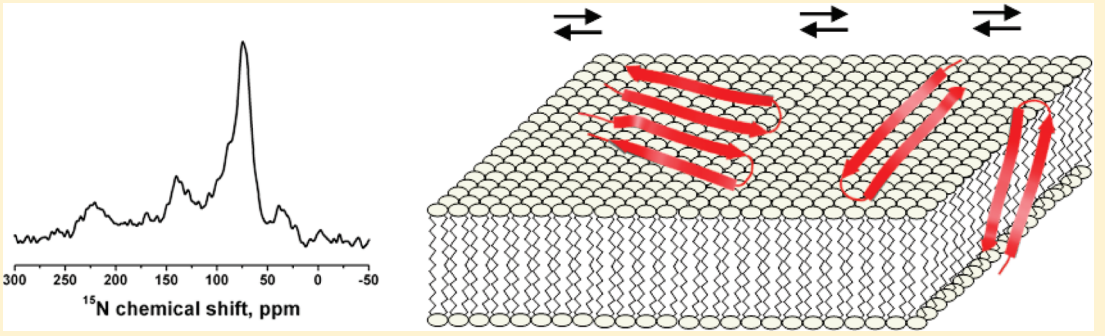
Supporting Information

ABSTRACT:



The antimicrobial arenicin peptides are cationic amphipathic sequences that strongly interact with membranes. Through a cystine ring closure a cyclic β-sheet structure is formed in aqueous solution, which persists when interacting with model membranes. In order to investigate the conformation, interactions, dynamics, and topology of their bilayer-associated states, arenicin 1 and 2 were prepared by chemical solid-phase peptide synthesis or by bacterial overexpression, labeled selectively or uniformly with ^{15}N, reconstituted into oriented membranes, and investigated by proton-decoupled ^{31}P and ^{15}N solid-state NMR spectroscopy. Whereas the ^{31}P NMR spectra indicate that the peptide induces orientational disorder at the level of the phospholipid head groups, the ^{15}N chemical shift spectra agree well with a regular β-sheet conformation such as the one observed in micellar environments. In contrast, the data do not fit the twisted β-sheet structure found in aqueous buffer. Furthermore, the chemical shift distribution is indicative of considerable conformational and/or topological heterogeneity when at the same time the ^{15}N NMR spectra exclude alignments of the peptide where the β-sheet lies side ways on the membrane surface. The ensemble of experimental constraints, the amphipathic character of the peptide, and in particular the distribution of the six arginine residues are in agreement with a boatlike dimer structure, similar or related to the one observed in micellar solution, that floats on the membrane surface with the possibility to oligomerize into higher order structures and/or to insert in a transmembrane fashion.

Arenicins are 21-residue cationic peptides isolated from marine polychaeta *Arenicola marina* with a potent broad-spectrum antimicrobial activity.[1] The arenicin isoforms, termed arenicin-1 and -2, have the amino acid sequence RWCVYAYVRVRGVLV-RYRRCW and a V to I replacement at position 10, respectively. Both peptides have a single disulfide bridge (Cys3−Cys20) forming a 18-residue ring. In aqueous solution arenicin-2 adopts a β-hairpin conformation stabilized by one disulfide and nine hydrogen bonds, which exhibits a significant right-handed twist.[2,3] The peptide forms asymmetric dimers upon incorporation into dodecylphosphocholine micelles.[4] In contrast, little structural information exists from lipid bilayers, and the mechanisms of the peptides' selective interactions with target cell membranes remain a matter of debate.

Solid-state NMR spectroscopy provides a powerful tool for the structural investigation of membrane polypeptides in their native lipid environment.[5−8] Two fundamentally different approaches are used in order to extract structural information from solid-state NMR spectroscopy. First, polypeptides reconstituted in membranes are studied by magic angle spinning (MAS) solid-state NMR spectroscopy. The resulting spectra resemble those obtained in solution and have been analyzed to extract chemical shift, secondary structure, and distance information. The method was also applied to β-sheet antimicrobial peptides such as

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protegrin-1 (PG-1) or retrocyclin-2[9,10] and has provided valuable data on penetration depth, oligomeric structure, dynamics, and interatomic distances. All these parameters have helped to better understand the mechanisms of action of these peptides.

Second, static solid-state NMR approaches have been developed for oriented[11−15] or nonoriented samples[16,17] from which angular constraints are obtained for structural analysis.[18] Using this approach, deuterium- and proton-decoupled ^{15}N solid-state NMR spectroscopy have proven particularly useful to study the conformation, topology, and dynamics of membrane-associated peptides.[18−20] For helical sequences the ^{15}N chemical shift provides a direct indicator of the approximate tilt angle, which can be refined by measuring this parameter for several residues and/or by adding to the analysis angular restraints from ^{15}N−^{1}H dipolar couplings, the deuterium quadrupole splittings,[19,20] or other alignment-dependent NMR parameters.

Biophysical studies on β-sheet antimicrobial peptides in lipid bilayers are less abundant when compared to their α-helical congeners. Therefore, the investigation of only few β-sheet peptides in oriented membrane samples has been reported including some inspiring papers on protegrin-1 and retrocyclin-2.[9,10,21,22] As a consequence, the investigation of additional molecular details of the interactions with, disruption of, and/or translocation of these and other β-sheet antimicrobial peptides across membranes remains of particular interest. Clearly, the information obtained from such β-hairpin peptides is less straightforward to analyze and requires the combination of well-resolved solid-state NMR spectra, molecular modeling, and spectral simulations. Here we reconstituted the 21-residue arenicin peptides into oriented bilayers in order to study the topology of their β-sheet structures by proton-decoupled ^{15}N and ^{31}P solid-state NMR spectroscopy.

MATERIALS AND METHODS

The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (C16:0, 18:1-PC, POPC), 1-palmitoyl(d31)-2-oleoyl-*sn*-glycero-3-phosphocholine (C16:0(d31), 18:1-PC, POPC-d31), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (C16:0, 18:1-PE, POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (C16:0, 18:1-PG, POPG) were from Avanti Polar Lipids (Alabaster, AL). $^{15}NH_4Cl$ (99,5% ^{15}N) was from Cambrige Isotope Laboratories, Andover, MA. All commercial material was used without further purification.

Heterologous Expression of Arenicin-2 in *E. coli*. The recombinant arenicin-2 (RWCVYAYVRIRGVLVRYRRCW) was expressed in *E. coli* and purified as described previously.[2] Briefly, the expression cassette was composed of a T7 promoter, a ribosome binding site, and a sequence encoding the recombinant protein that included an octahistidine tag, the TrxL carrier protein (*E. coli* thioredoxin A with M37L mutation), a methionin residue, and the mature arenicin-2. This sequence was ligated to the *Bgl*II/XhoI fragment of the pET-20b(+) vector, containing the pBR322 origin of replication, a β-lactamase gene, and the T7 terminator, resulting in the pET-His8-TrxL-Ar2 expression plasmid. The *E. coli* strain DH-10B (Life Technologies, Carlsbad, CA) was used for plasmid preparation, and *E. coli* BL-21 (DE3) (Merck KGaA, Darmstadt, Germany) was used as an expression host. The cells were induced by IPTG at 25−30 °C and incubated at this temperature for 4−6 h. Following the harvest of the cells, sonication and preparative centrifugation of the cell lysate the insoluble fraction was subjected to consecutive washing steps, solubilization, and Ni-NTA affinity purification. Arenicin-2 was obtained by CNBr cleavage of the fusion protein in 80% TFA under standard conditions. The lyophilized products of the cleavage reaction were dissolved in 10% acetonitrile containing 0.1% TFA and loaded onto a RP-HPLC semipreparative column C4 (Waters, Milford, MA). RP-HPLC was performed in a linear gradient of acetonitrile in water containing 0.1% TFA: 5−35% for 60 min, 35−80% for 15 min, and 80−5% for 15 min. The experimentally measured molecular mass of the purified recombinant arenicin-2 matched well with the calculated value (2772.3 Da).

Preparation of Uniformly ^{15}N-Labeled Recombinant Arenicin-2. A protocol for the production of uniformly ^{15}N-labeled recombinant arenicin-2, designated hereafter as [U-^{15}N]-arenicin-2, was developed. Expression of [U-^{15}N]-arenicin-2 was conducted using *E. coli* BL21(DE3) cells transformed with the pET-His8-TrxL-Ar2 plasmid and grown on a M9 minimal medium containing ^{15}N ammonium chloride as the only nitrogen source. The fusion protein consisting of the peptide of interest and the His8-tagged, M37L-modified *E. coli* thioredoxin A was isolated from inclusion bodies using Ni-NTA affinity chromatography and then cleaved by CNBr at the methionine residue. [U-^{15}N]-arenicin-2 was further purified using RP-HPLC following the protocol given above. The purified recombinant [U-^{15}N]-arenicin-2 was analyzed by MALDI-TOF MS. The experimentally measured molecular mass of [U-^{15}N]-arenicin-2 matched well with the calculated value (2813.5 Da).

Chemical Synthesis of [^{15}N-Ala^{6}]-arenicin-1. [^{15}N-Ala^{6}]-arenicin-1 was obtained by solid phase peptide synthesis as described in ref 2. The strategy of the synthesis was based upon the standard Fmoc-protocol with the TBTU/DIEA activation using the Wang resin as the solid phase. The Fmoc derivative of [^{15}N]-L-Ala-OH was obtained as described in ref 23. The cysteine side chains were protected with the Trt group. Deprotection and cleavage were performed in a TFA:anisole:DMS:EDT mixture (94:2:2:2). The synthetic peptide was purified by HPLC on a Reprosil-Pur C18 column (5 μm, 10 × 250 mm) using a linear gradient of acetonitrile (10−50% for 40 min) and oxidized with an isopropanol−water mixture (1:1) at pH 8.5. The experimentally measured molecular mass of [^{15}N-Ala^{6}]-arenicin-1 matched well the calculated value (2757.5 Da).

Characterization of Recombinant and Synthetic Arenicins. The homogeneity of the recombinant and synthetic peptides was monitored by SDS-PAGE, MALDI-TOF MS using the Reflex III mass spectrometer (Bruker Daltonics, Billerica, MA), automated microsequencing using the Procise cLC 491 protein sequencing system (Applied Biosystems Inc., Foster City, CA), and an antimicrobial assay based on microspectrophotometry using 96-well microplates as described previously.[2] The amino acid sequences and antimicrobial activities of the recombinant and synthetic arenicins were identical to the natural peptides.

Sample Preparation for Solid-State NMR Spectroscopy. For solid-state NMR measurements 0.5−8 mg of arenicin (uniformly or selectively labeled with ^{15}N) was dissolved in methanol (for POPC) or methanol/chloroform 1:1 by volume (for POPE/POPG) and mixed with the appropriate amount of lipid.[24] The solution was then spread onto 30 ultrathin cover glasses (9 × 22 mm, Marienfeld, Lauda-Königshofen, Germany), and the samples were first dried in air and thereafter in high vacuum overnight to remove all organic solvent. Subsequently, the membranes were equilibrated at 93% relative humidity. For NMR measurements the samples were tightly sealed, inserted

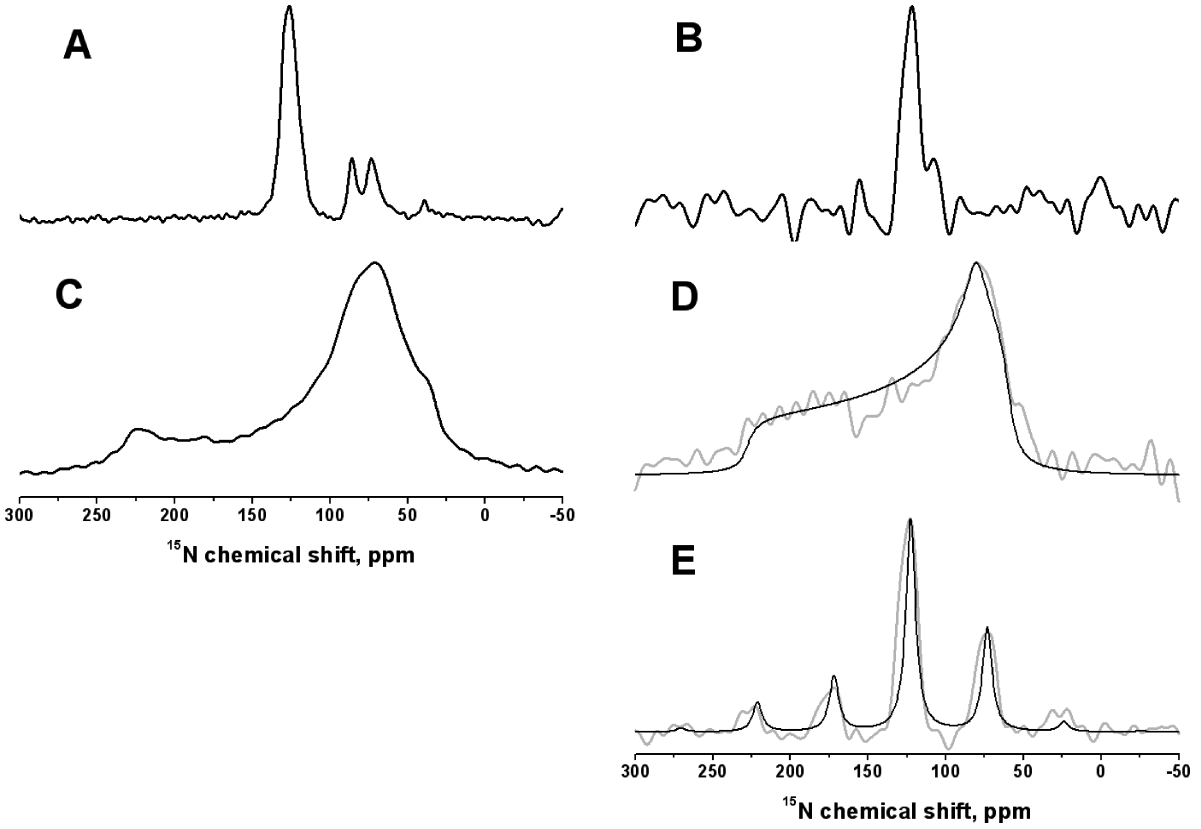

Figure 1. Proton-decoupled ^{15}N solid-state NMR spectra of pure arenicin-2 labeled uniformly with ^{15}N (A, C) and of pure arenicin-1 selectively labeled with ^{15}N at the Ala^{6} position (B, D, E). The spectra were acquired in a static arrangement (C, D) or under magic angle sample spinning with speeds of 7 kHz (A), 5 kHz (B), and 2 kHz (E). The spectra of the peptide carrying a single ^{15}N-labeled amide bond were simulated using σ_{11} = 60 ppm, σ_{22} = 80 ppm, σ_{33} = 227 ppm, and the result is shown in panels D and E by black lines superimposed on the experimental spectra (gray lines).

into a double resonance flat-coil probe head,[25] and, if not indicated otherwise, introduced into the magnet with the bilayer normal aligned parallel to the magnetic field direction.

Solid-State NMR Spectroscopy. The proton-decoupled ^{15}N cross-polarization (CP) spectra of static aligned samples were acquired at 40.54 MHz on a Bruker Avance wide bore NMR spectrometer operating at 9.4 T. An adiabatic cross-polarization pulse sequence[26] was used with a spectral width, acquisition time, CP contact time, and recycle delay of 75 kHz, 3.5 ms, 800 μs, and 3 s, respectively. The ^{1}H $\pi/2$ pulse and spinal64 heteronuclear decoupling field strengths B_1 corresponded to a nutation frequency of 42 kHz. About 40 000 scans were accumulated and the spectra were zero-filled to 4096 data points. An exponential line-broadening of 100 Hz was applied before Fourier transformation. Spectra were externally referenced to $^{15}NH_4Cl$ at 40 ppm. Samples were cooled with a stream of air at a temperature of 22 °C. For the POPE/POPG sample the temperature of the air was set to 37 °C.

The two-dimensional PISEMA experiment[27] was used to correlate the ^{15}N−^{1}H dipolar coupling with the ^{15}N chemical shift of the same nitrogen. The effective ^{1}H B_1 field of the spin-lock amplitude was 45 kHz. During the spin exchange period the amplitude of the ^{1}H B_1 field was decreased to 36.8 kHz in order to maintain the Hartmann− Hahn match condition with an effective field along the magic angle of 45 kHz. For the experimental setup a fully ^{15}N,^{13}C-labeled *N*-acetylleucine single crystal was used.

Proton-decoupled ^{31}P solid-state NMR spectra were acquired at 161.953 MHz using a double resonance flat-coil probe[25] and a Hahn-echo pulse sequence[28] with a $\pi/2$ pulse of 2.5 μs. The spectral width was 75 kHz, and the echo and recycle delays were 40 μs and 3 s, respectively. The samples were cooled with a stream of air at a temperature of 22 °C. Spectra were referenced externally to 85% H_3PO_4 at 0 ppm.

Spectral Simulations. The spectral simulations of the one-dimensional solid-state NMR spectra were performed on a personal computer using the MATHEMATICA 3.0 software (Wolfram Research, Champaign, IL) and followed the protocols described in refs 29 and 30. The *z*-axis was defined to correspond to the long axis of the β-sheet, and the orthogonal *y*-axis lies in the plane of the β-sheet. This coordinate system was chosen in analogy to that of previous simulations of oriented solid-state NMR spectra arising from α-helical peptides (e.g., ref 20). Correspondingly, the angles τ and ρ correspond to deviations from perfect transmembrane alignments as defined by the tilt and pitch angles (cf. Figure 5A). It also represents the shape of the β-sheet with the longest dimension of the polypeptide being associated with the *z*-axis and the shortest with the *x*-axis.

Furthermore, the starting configuration can be defined in such a manner that the *z*-axis is collinear with the magnetic field direction, thereby matching the coordinate system of the laboratory frame used in NMR spectroscopy. At sample orientations where the normal is parallel to the magnetic field direction the latter corresponds to a "transmembrane" β-sheet (or α-helix).

In order to screen all possible alignments the peptide was rotated in a stepwise manner (5°−15° intervals) first around the *z*-axis and thereafter around the *y*-axis. For each orientation the

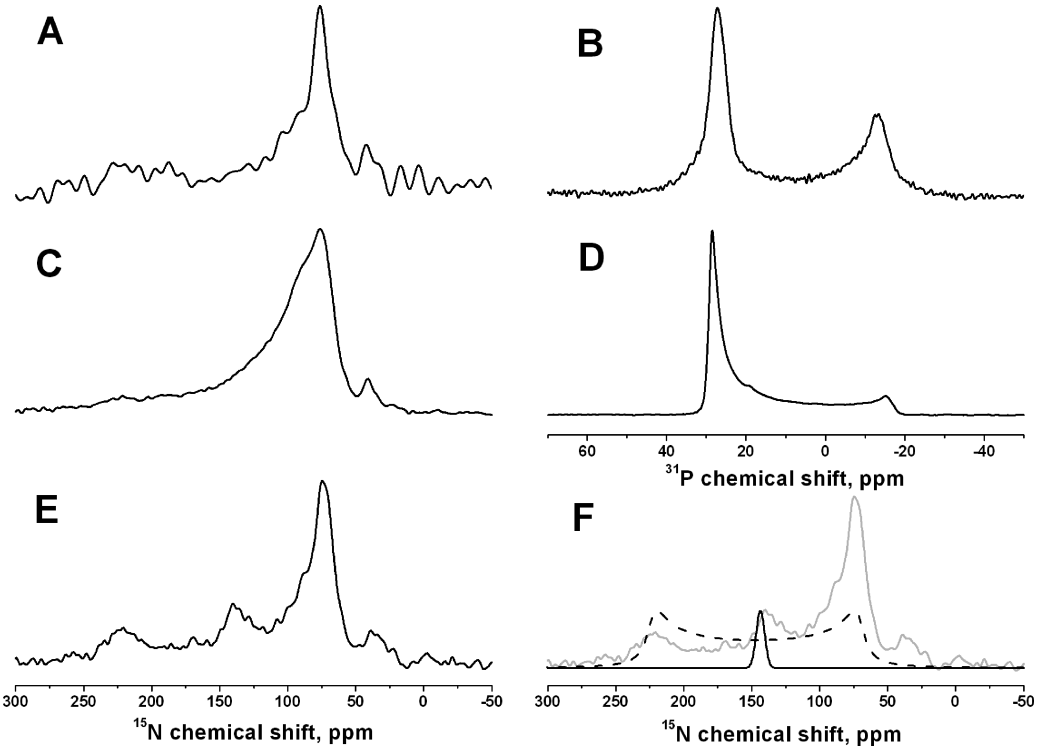

Figure 2. Proton-decoupled ^{15}N (A, C, E) and ^{31}P (B, D) solid-state NMR spectra of uniformly ^{15}N-labeled arenicin-2 reconstituted into POPE/POPG 3/1 mol/mol at a P/L molar ratio of 1/30 recorded at 37 °C (A, B) or in POPC membranes at P/L molar ratios of 1/100 at room temperature (C−E). The samples were macroscopically oriented between microscope cover glasses. For panels A−D the glass plate normal is parallel to the external magnetic field. For panel E the glass plate normal is oriented perpendicular to the external magnetic field. Panel F shows how the spectrum shown in panel E can be deconvoluted into two components: Whereas the intensity at 140 ppm arises from a peptide population undergoing fast reorientational diffusion around the membrane normal the majority of the signal intensity appears static on the time scale of the ^{15}N chemical shift (10^{-4} s; ref 35). The seeming asymmetry of the experimental line shape associated with this later population is probably due to the side chain resonances that accumulate in the 70−90 ppm region.[15]

^{15}N chemical shift values of each of the 20 individual backbone amides were calculated and added to yield the final spectrum.

The simulations of the two-dimensional spectra were accomplished on a 3.4 GHz Pentium(R) D workstation operating under Windows XP Professional using the SIMPSON/SIMMOL software package.[31] As it is difficult to estimate the spectral intensities of side-chain nitrogens in cross-polarization solid-state NMR spectra,[15] only backbone amide ^{15}N were taken into consideration during the simulations. The calculated PISEMA spectra were visualized using the GSim software, version 0.12.0. The ^{15}N−^{1}H dipolar coupling was 9.9 kHz corresponding to a 1.07 Å interspin distance and the amide ^{1}H CSA tensor σ_{11} = 2.95, σ_{22} = 7.95, and σ_{33} = 17 (low RF field limit). The ^{15}N amide chemical shift tensor was represented by σ_{11} = 60 ppm, σ_{22} = 80 ppm, and σ_{33} = 227 ppm. These values were found by fitting the powder spectra of [^{15}N-Ala^{6}]-arenicin (see Figure 1) and agree well with previous analyses of the ^{15}N chemical shift tensor.[32] When the spectra are acquired with the external magnetic field direction perpendicular to the bilayer plane, this tensor accounts for the anisotropic chemical shift or dipolar interactions regardless of motional averaging around the bilayer normal.[11,33] In order to better represent the experimental spectra, a Lorentzian line broadening of 300 Hz was applied in the direct dimension and of 1 kHz in the indirect dimension.

RESULTS

In order to investigate the membrane interactions and topology of arenicins with phospholipid bilayers, arenicin-1 and -2 were prepared by solid-phase peptide synthesis and by heterologous expression in *E. coli*, respectively. Whereas the latter technique is well suited to produce uniformly labeled samples, in the former case a single ^{15}N-labeled L-Ala was introduced at position 6.

In a first step proton-decoupled ^{15}N solid-state NMR spectra of the pure peptide powders were recorded in order to analyze in some detail the chemical shift interactions of the labeled sites with the external magnetic field of the NMR spectrometer (B_0). Figures 1A and C show the spectra of the uniformly labeled compound. When the sample was spun around the magic angle at 7 kHz, only the isotropic chemical shift contributions remain, and the spectrum exhibits a predominant intensity arising from 20 amides of the peptide bonds (125 ± 7 ppm) as well as two tryptophan side chains (tabulated value:[34] 129 ppm). Minor intensities are centered around 86, 73, and 39 ppm (Figure 1A), which correspond to the side chains of the six arginines (76 and 90 ppm[34]) and the N-terminal amine of the sequence.[15] When the static spectrum of the same sample is recorded (Figure 1C), a powder pattern line shape is obtained where the orientational dependence of the chemical shift relative to the magnetic field direction becomes obvious in particular for the rigid backbone amides, which are known to cover an anisotropy of about 175 ppm.[11,33] The powder pattern line shape of the uniformly labeled peptide is somewhat distorted when compared to a pure amide powder pattern due to the additional intensities of the side chains and the N-terminus[15] as well as cross-polarization artifacts.[16] Similar experiments were also performed with the arenicin-1

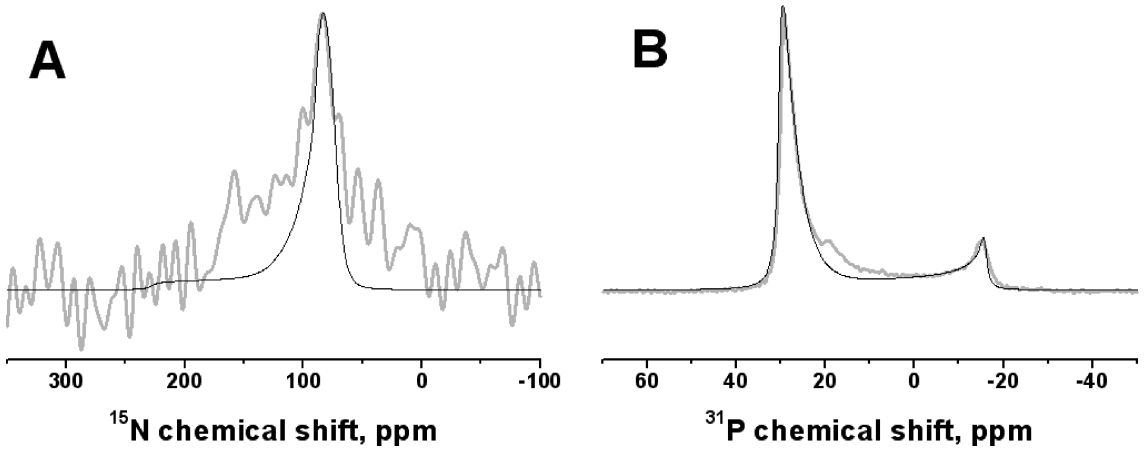

Figure 3. Proton-decoupled ^{15}N (A) and ^{31}P (B) spectra of arenicin-1 labeled selectively with ^{15}N at the Ala^6 position and reconstituted into POPC membrane at a P/L molar ratio of 1/100 macroscopically oriented between glass plates (light gray lines). The glass plate normal is parallel to the external magnetic field of the NMR spectrometer. The black lines correspond to spectral simulations assuming that 70% of the membrane is aligned with a 12° Gaussian distribution and 30% of the signal intensities arise from nonoriented components. In the experimental ^{15}N solid-state NMR spectrum (A) supplementary signal intensities appear in the range 150 ± 30 ppm, suggesting an additional peptide population.

analogue labeled only at the alanine-6 position (Figure 1B,D,E). The proton-decoupled ^{15}N cross-polarization MAS spectrum (5 kHz) of the peptide powder exhibits an isotropic chemical shift intensity at 122 ± 8 ppm (Figure 1B), in agreement with previous investigations.[32] The spectrum of the static sample and at an MAS spinning speed of 2 kHz is shown in panels D and E of Figure 1, respectively, together with the corresponding spectral simulations. The latter were obtained using the ^{15}N chemical shift tensor elements σ_{11} = 60 ppm, σ_{22} = 80 ppm, and σ_{33} = 227 ppm.

In a next step the [U-^{15}N]-arenicin-2 peptide was reconstituted into POPE/POPG or POPC phospholipid bilayers at peptide-to lipid ratios of 1/30 and 1/100, respectively (Figure 2). In the presence of arenicin-2 the proton-decoupled ^{31}P solid-state NMR spectrum of POPC shows a predominant signal intensity at about 30 ppm characteristic of phosphatidylcholine membranes in the fluid liquid crystalline bilayer phase and an alignment of the lipids with their long axis parallel to the magnetic field direction (Figure 2D). Additional intensities are observed that reach into the −15 ppm region indicative of an orientational and/or conformational heterogeneity at the level of the phosphatidylcholine headgroup. In contrast, the alignments of the ^{31}P sites of the POPE/POPG sample are more heterogeneous and indicate considerable conformational and/or orientational disordering (Figure 2B) probably due to the 3 times higher peptide concentration.

The proton-decoupled ^{15}N solid-state NMR spectra of [U-^{15}N]-arenicin-2 reconstituted into supported POPC or POPE/POPG bilayers exhibit predominant intensities at about 75 ppm, showing that the NH vectors are aligned approximately perpendicular to the membrane normal (Figure 2A,C). Furthermore, contributions from 18 nitrogens of the arginine side chains contribute at ^{15}N chemical shifts <100 ppm (cf. supra). However, additional intensities spread into the 200 ppm region indicative of some N−H vectors oriented approximately parallel to the magnetic field direction.

When the sample encompassing arenicin-2 reconstituted into the POPC membrane is titled by 90°, the spectrum shown in Figure 2E is obtained. As discussed in earlier publications,[35] this sample alignment allows for the investigation of the rotational averaging around the membrane normal. The tilted spectrum ($n \perp B_0$) is composed of contributions that, due to reorientation around the membrane normal (n), arise from peptides being localized along the surface of a cone (circle), thereby exhibiting a wide variety of alignments relative to the magnetic field direction (B_0). Two major contributions can be distinguished in Figure 2E, and this is illustrated by the simulations shown in Figure 2F. First, a contribution from a conical distribution of peptides that rotate slowly around the membrane normal when compared to the time scale of the ^{15}N chemical shift anisotropy (7000 Hz) and that thereby give rise to spectral contributions between 60 and 230 ppm. Second, a population that undergoes fast motional averaging around this axis and exhibits spectral intensities of 140 ± 13 ppm. Thereby the spectra shown in Figure 2C,E are indicative of at least two amide populations undergoing different dynamic averaging within the oriented membrane sample. Notably, intensities from the labeled side chains superimpose within the 70−130 ppm region on the line shape of the circular distribution and thereby hamper a more quantitative analysis.

Reconstitution of the ^{15}N-Ala^6-arenicin-1 peptide into oriented POPC bilayers resulted in the proton-decoupled ^{15}N solid-state NMR spectra shown in Figure 3. The proton-decoupled ^{31}P NMR spectra can be simulated by a phosphatidylcholine membrane that is well-aligned with the normal parallel to the magnetic field direction (70% of the total intensity with a Gaussian distribution ±12°), and the remaining signal intensities corresponding to a powder pattern contribution (Figure 3B). Similar ^{31}P NMR line shapes have been simulated for "wormhole" type arrangements of phospholipid−peptide mixtures in the presence of β-hairpin peptides.[21] The proton-decoupled ^{15}N solid-state NMR spectra of the oriented sample encompassing Ala 6-arenicin-1 exhibits predominant signal intensities at 85 ppm and a second population (30 ± 10% of the total) extending in the range 110−180 ppm. This observation is suggestive that the peptide exhibits at least two different topological/conformational states when associated with POPC bilayers. In order to obtain additional alignment information, a two-dimensional solid-state NMR experiment correlating the ^{15}N chemical shift and the ^{1}H−^{15}N dipolar coupling of each labeled site was recorded from the uniformly labeled sample. The spectrum exhibits its main intensities in the 80−100 ppm/5−8 kHz range (Figure 4A).

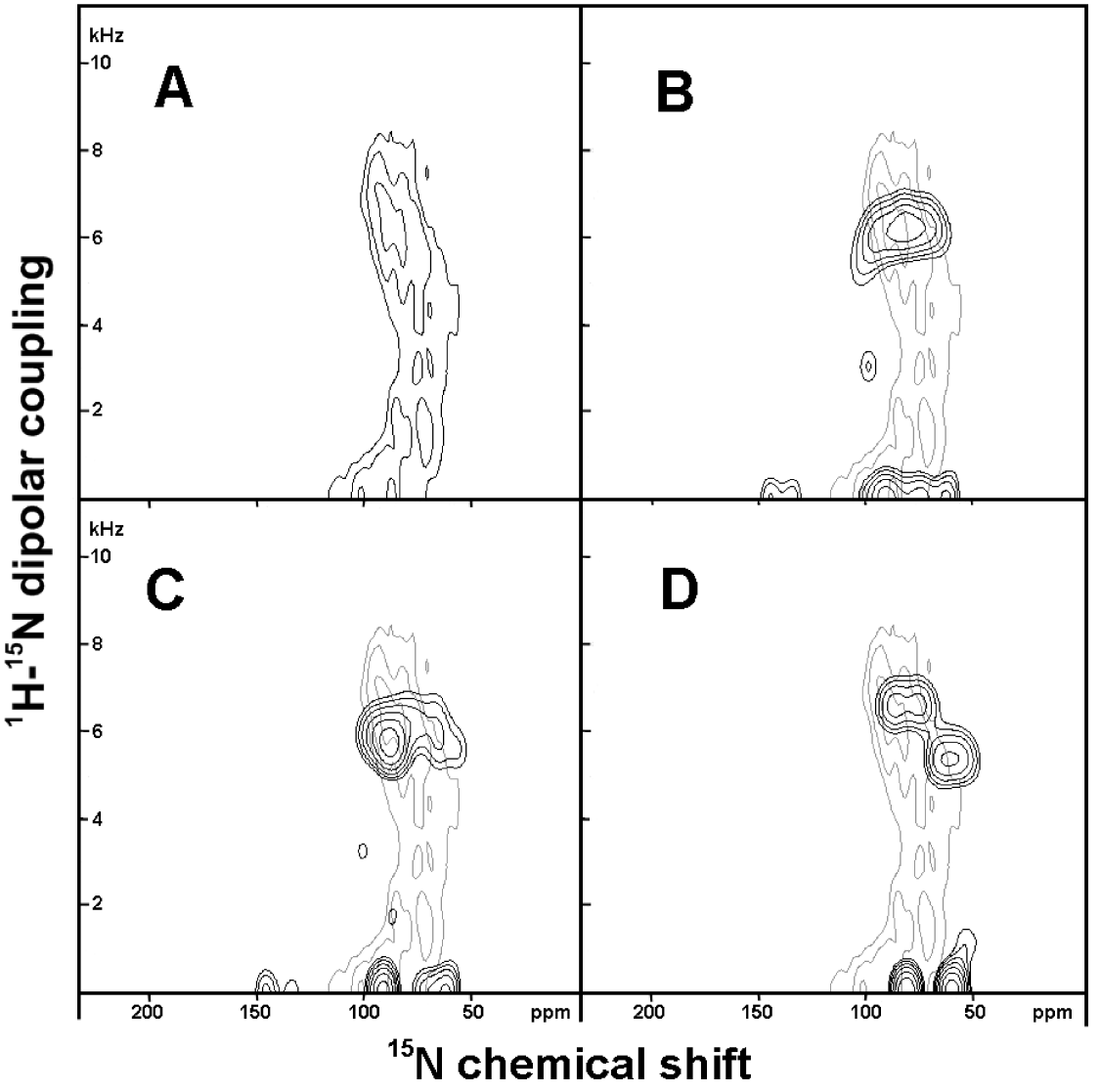

Figure 4. (A) Experimental separated local field spectrum of [U-^{15}N]-arenicin reconstituted into uniaxially aligned POPC membranes. Superimposed onto the experimental spectrum are simulations of the spectral intensities of the solution NMR structure in DPC micelles (panel B, monomer unit A of PDB 2L8X) or in aqueous aqueous solution (panel C, PDB 2JNI) both in a surface-associated state, or of an idealized β-strand ($\varphi = -180°$, $\psi = 180°$) with the tilt angle of 10° (panel D, "transmembrane" orientation).

In a next step we simulated the ^{15}N solid-state NMR spectra that arise from the backbone amides of uniformly labeled arenicin at different alignments relative to the membrane normal (magnetic field direction). Two known conformers of arenicin, namely the twisted β-sheet structure obtained in aqueous solution (PDB 2JNI; ref 2), a dimeric β-sheet structure observed in micellar solution (ref 4; PDB 2L8X), and an idealized β-strand formed the basis of the calculations.[29] The micellar β-sheet structure is shown in Figure 5A with the N−H bonds of the ^{15}N-labeled backbone sites highlighted and is characterized by a more planar conformation when compared to the twisted β-sheet in aqueous solution.

In the first simulation the arenicin structure observed in micellar environments (PDB 2L8X, Figure 5A) was initially positioned at an orientation of the peptide lying flat on the membrane surface. From the alignment of each amide bond the oriented ^{15}N solid-state NMR spectrum was calculated, and the resulting 20 resonances were added to provide the sum spectrum for the whole peptide. In this arrangement most of the spectral intensities add up to a predominant intensity with its maximum at 78 ppm (Figure 5C), thereby resulting in an intensity distribution that closely resembles that of the experimental spectrum (Figure 2C). As will be discussed later, this chemical shift value and the intensity distribution reflect an orientation of all the NH vectors approximately parallel to the membrane surface. However, it should also be noted that the experimental spectrum exhibits less resolution than the simulation, suggesting considerable conformational and topological heterogeneity of the peptide even in the oriented sample (cf. Figure 2).

Starting from this first alignment, the peptide was subsequently tilted around the *y*-axis being perpendicular to the β-sheet. As the alignment of the N−H vectors relative to the magnetic field undergoes only very little changes during such movements the spectra remain much confined to the region <120 ppm (Figure 5D). In contrast, rotations around an axis that is parallel to the sheet direction significantly change the alignment of the N−H vectors, and spectral intensities in the region

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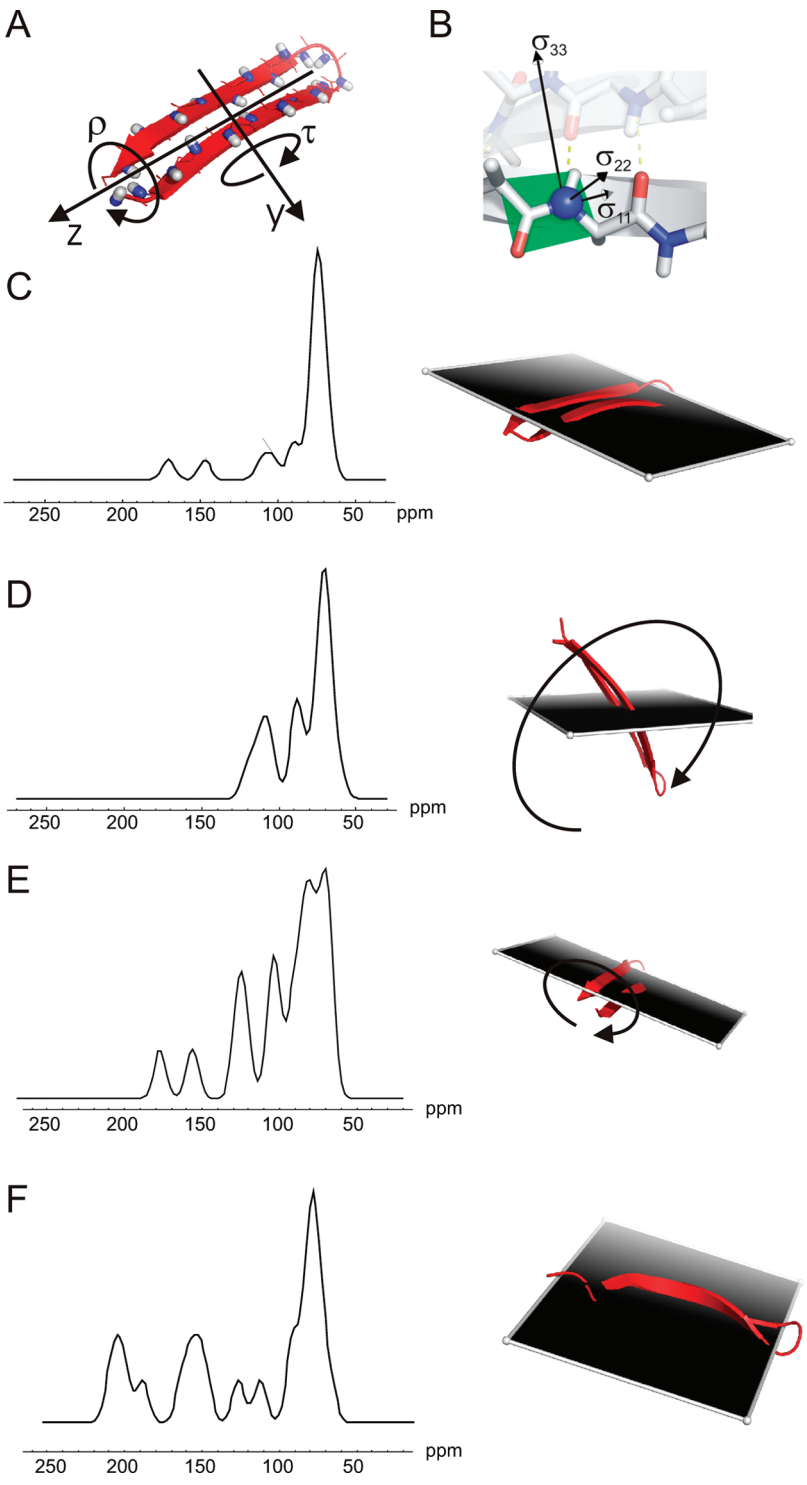

Figure 5. (A) Structure of an extended β-sheet of arenicin observed in micellar solution (extracted from PDB 2JNI, unit A). The N−H vectors of the peptide backbone are highlighted. (B) Alignment of the ^{15}N chemical shift tensor of the peptide bond within the molecular coordinate system. The main tensor elements used in this work are σ_{11} = 60 ppm, σ_{22} = 80 ppm, and σ_{33} = 227 ppm. (C) Simulation of the proton-decoupled ^{15}N solid-state NMR spectrum of uniformly labeled arenicin in an in-planar orientation. The alignment shown corresponds to a tilt angle of 90° (rotation around the *y*-axis). (D) Spectral simulation of a peptide alignment obtained after the extended β-sheet structure of arenicin is rotated by 60° around the *y*-axis relative to the situation shown in (C) (the resulting tilt angle is 30°) and (E) after rotation around the *z*-axis by 20° (tilt/pitch 90°/20°). (F) Spectral simulation of the arenicin structure in aqueous solution (twisted β-sheet structure) at an in-planar orientation (tilt/pitch 90°/15°).

>160 ppm are obtained (Figure 5E). A full set of ^{15}N chemical shift spectra where all possible alignments have been screened at 15° intervals is shown in the Supporting Information.

As the detailed conformation(s) of arenicin in phospholipid bilayers remains unknown, we also simulated spectra that result from different orientations of the peptide structure observed in

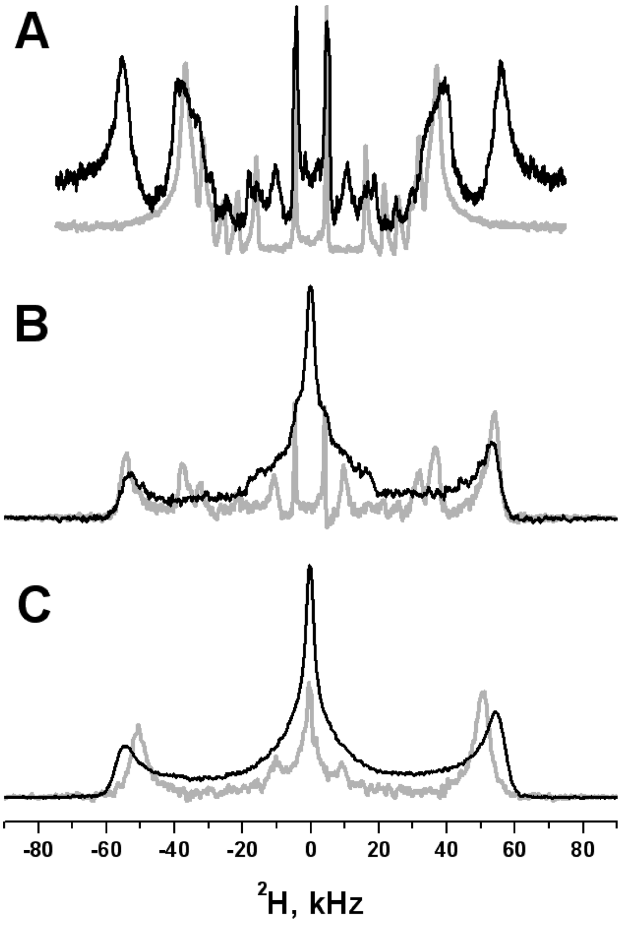

Figure 6. ^{2}H spectra of macroscopically oriented membranes in the presence (black line) and absence of arenicin-2 (gray line). (A) POPC/POPC-d31 6/1 mol/mol in the absence or presence of 1 mol % peptide. (B) POPE/POPG-d31 3/1 mol/mol in the absence or presence of 3.3 mol % arenicin-2. (C) POPE-d31/POPG 3/1 mol/mol in the absence or presence of 3.3 mol % arenicin-2. The ^{2}H NMR spectra shown in panels B and C were acquired at 37 °C.

aqueous solution (ref 2; PDB 2JNI). In this conformation the β-sheet structure is twisted along its long axis which causes a much wider distribution of the NH-vector alignments within the peptide. This is also reflected in the spectral simulations, which in most cases exhibit spectral distributions covering almost the full width of the ^{15}N chemical shift anisotropy (e.g., Figure 5F). Only a few angular distributions come close to but do not match the experimental spectrum. The best fits all represent transmembrane orientations (Supporting Information).

Finally, we also evaluated the data obtained by two-dimensional oriented solid-state NMR spectroscopy (Figure 4). These investigations reveal not only the ^{15}N chemical shift but also the alignment-dependent ^{1}H−^{15}N dipolar coupling. The latter are likely to be different for mobile side chains when compared to the immobile backbone amides. Indeed, at chemical shifts $\leq$100 ppm the intensities exhibit dipolar splittings in the full range between 10 and 0 kHz (Figure 4), suggesting that considerable signal contributions in this chemical shift range arise from side-chain nitrogens. The dipolar splitting could also be used to complement the chemical shift information and to further select among the structural models and alignments; however, the contour plots representing such spectra are less informative on the small signal intensities which tend to disappear in the noise (or below the cutoff level). In the case of the arenicin spectral simulations such intensities are particularly important to distinguish between the different models (cf. Figure 5 and Supporting Information). As a consequence, the maximal intensities apparent in the two-dimensional spectrum agree with both the arenicin structure aqueous solutions (PDB 2JNI) or in DPC micelles (PDB 2L8X) when aligned parallel to the surface (Figure 4B,C) or with an idealized β-strand that is aligned at a tilt angle of 10° (Figure 4D), although the quality of the fit of such a "transmembrane" orientation (Figure 4D) is below that of the in planar alignments shown in Figure 4B,C.

To study the peptide−lipid interactions in further detail, samples were prepared where arenicin was reconstituted into oriented phospholipid bilayers carrying deuterated fatty acyl chains (Figure 6). The deuterium quadrupolar splitting observed from such samples can be directly correlated to the deuterium order parameter which takes into account the alignment of the C−^{2}H bond relative to the membrane normal as well as averaging motions at the position of the label. Figure 6A compares the ^{2}H solid-state NMR spectrum of POPC-d_{31}, where all the protons of the palmitoyl chain have been exchanged by deuterons, in the presence or absence of 1 mol % arenicin. When the spectra are compared to each other, it is interesting to note that the peptide causes a significant augmentation in the line width of the ^{2}H resonances. Furthermore, the deuterium quadrupole splittings that are usually assigned to the plateau region (i.e., segments close to the glycerol backbone) are increased due to the presence of arenicin. At the same time a more modest decrease of some of the resonances from the membrane interior is recorded comparable to the changes that have been observed in the presence of amphipathic helical peptides residing in the interfacial region.[36] When 3.3 mol % of arenicin-2 is reconstituted into POPE/POPG bilayers, profound changes in the line width of the individual resonances are again observed (Figure 6B,C). Whereas the plateau region of the POPE palmitoyl chain exhibits an increase in the quadrupolar splittings (Figure 6C), the spectral changes associated with the POPG plateau region (i.e., the most intense and outermost quadrupolar splittings) remain minor (Figure 6B).

DISCUSSION

Arenicins have been investigated by CD- and multidimensional solution NMR spectroscopies in water, in micellar solutions, or in the presence of lipid bilayers.[2] Although the various studies focused on two different isoforms of the peptide, arenicin-1 and -2 distinguish themselves only at position 10 where valine is exchanged by isoleucine. This highly conservative amino acid replacement may cause differences in some structural detail close to the site but has probably no influence on the overall structural and biophysical properties of the peptide. Therefore, we present studies of both sequences in this paper.

In water a twisted helical hairpin structure has been observed for arenicin-2.[2,3] Upon insertion into DPC or SDS detergent micelles the CD spectra of the peptide exhibit pronounced changes. This observation in combination with multidimensional NMR spectroscopy is indicative of structural alterations upon membrane insertion when at the same time the overall β-sheet conformation is preserved.[2,4] Furthermore, these investigations indicate that arenicin-2 forms asymmetric dimers in DPC micelles.[4] Notably, a more regular β-hairpin structure is observed

for arenicin-1 in DMSO suggesting that the peptides exhibit a considerable degree of flexibility of their three-dimensional fold[3,37] when at the same time their secondary structure is stabilized by the cystine bond between residues 3 and 20.

When the membrane association of arenicin-2 is investigated by CD spectroscopy, a relatively low partitioning constant into zwitterionic membranes is observed; however, membrane association significantly increases in the presence of negative surface charge densities due to the presence of anionic phospholipids.[2] This agrees with similar observations made with other cationic antimicrobial peptides.[38,39] For example, whereas the hydrophobic partitioning of some linear cationic peptides (e.g., 10^3 M^{-1} for magainin) in dilute dispersion results in little interactions with PC vesicles, the electrostatic attraction to the surface of negatively charged liposomes causes an increase in the apparent membrane partitioning constants by 2−3 orders of magnitude.[38,39] Alternatively, the reduction in bulk water, as in the mechanically supported bilayers used for oriented solid-state NMR forces the peptides to be localized close to the membrane surface at high concentrations and thereby enhances their apparent interaction with the lipid bilayers.[40] The pronounced changes in the deuterium order parameter of labeled phospholipid fatty acyl chains due to the presence of arenicin is indeed indicative that the peptide interacts with zwitterionic as well as acidic membranes (Figure 6).

The formation of arenicin-2 dimers was observed in the presence of DPC micelles by solution NMR,[4] in agreement with a high propensity of structurally related β-sheet peptides to oligomerize when associated with membranes.[41] Indeed, the ^{15}N solid-state NMR spectra of arenicins support the formation of oligomeric structures as the spectra obtained from the tilted samples (Figure 2E) indicate that a population of the peptides exhibits long rotational correlation times. In order to abolish averaging by rotation around the membrane normal, the diameter of a corresponding circular object projected onto the membrane surface must correspond to at least the length of the β-hairpin lying flat in the membrane (∼3 nm).[35] Furthermore, the broad line observed when the proton-decoupled ^{15}N spectrum of arenicin-1 labeled with ^{15}N at a single site and reconstituted into POPC (Figure 3A) reveals a considerable degree of conformational and/or topological heterogeneity, which is in agreement with different oligomeric states and topologies. Notably the dynamics and penetration of protegrin-1 was also found to strongly depend on the lipid composition and the peptide/lipid ratio.[10]

This heterogeneity makes it impossible to define a single structure from the angular constraints, but some conclusions can nevertheless be drawn. A structural model of the arenicin-2 dimer was derived from solution NMR data obtained in DPC micelles (PDB 2L8X), and a monomeric unit is shown in Figure 5A with the N−H vectors of the amide bond being highlighted. Also shown is the alignment of the ^{15}N chemical shift tensor within the molecular coordinate system of the peptide bond (Figure 5B). This latter figure illustrates that the tensor element σ_{33} (227 ppm, Figure 1), which is unique when compared to σ_{11} and σ_{22} (60 and 80 ppm, respectively), is aligned within the peptide plane and deviates by about 18° from the NH orientation.[32] Therefore, to first approximation the ^{15}N chemical shift can be estimated from the NH-vector alignment relative to the magnetic field direction (B_0). Whereas ^{15}N chemical shifts in the 200 ppm range are observed when the NH vector is oriented parallel to the magnetic field direction values <100 ppm are indicative of orientations perpendicular to B_0.[11,29] When the ^{15}N−^{1}H dipolar coupling (D) is analyzed, the alignment of this vector relative to the magnetic field direction exhibits an angular dependence which follows the correlation $D \sim \langle 3\cos^2\Theta - 1\rangle$, with Θ being the angle between N−H and the magnetic field and the brackets indicating the time average.[11] The dipolar interaction thereby provides additional angular restraints for the NH vector. Whereas in the case of helical peptides the ^{15}N chemical shift can be directly correlated with the helix orientation,[11] this correlation is less straightforward for β-sheets but alignment information has nevertheless been obtained through spectral simulations.[22]

We, therefore, systematically calculated the one-dimensional solid-state NMR spectra of U-^{15}N-arenicins as a function of membrane topology (i.e., alignment relative to B_0). Both the tilt and rotational pitch angles were varied in a systematic manner, and the resulting spectra were compared to the experimental result (Figure 5). The experimental spectra are composed of signal intensities arising from 20 amide bonds as well as the N-terminus and 20 ^{15}N-labeled sites within the Trp and Arg side chains. The latter exhibit isotropic signal intensities around 129 ppm and in the 70−90 ppm range, respectively, but difficulties arise as to estimate their contribution as the line width and intensity both strongly depend on the local and global motions of these sites.[15] Therefore, only backbone ^{15}N were taking into account during the simulations. The closest agreement between the experimental spectrum and simulations that are based on the structure of arenicin-2 in aqueous solution (twisted β-sheet, PDB 2JNI) is obtained at ∼0° pitch angles and tilts in the 15°−45° range (Supporting Information). Better agreement between simulation and experiment is obtained when the flat β-sheet structure in micellar environments forms the basis of these calculations (Figures 4B and 5C and Supporting Information). The underlying conformational changes are in agreement with CD-spectroscopic investigations that indicate that arenicin-2 undergoes comparable alterations upon membrane association[2] as well as with solution NMR structural data obtained in micellar solution (Shenkarev et al., unpublished observations).

When the experimental (Figure 2C) and the simulated spectra (Figure 5 and Supporting Information) are compared to each other, it becomes apparent that a number of alignments can explain the measurements. Therefore, the experimental spectra do not allow us to define a unique model of the arenicin peptides when interacting with oriented POPC membranes, but the information can be used to exclude many possibilities and to test the validity of a number of reasonable models. For example, inserting the β-sheet into the membrane (by tilting the peptide around the y-axis) results in relatively little changes in the overall spectral appearance (Figure 5D). On the other hand, the experimental spectra only agree with small rotations around the long axis of the β-sheet (Figure 5E). Best agreement between the experimental and the simulated spectra is observed at tilt/pitch angles of 75°−90°/0°−15° (cf. Supporting Information). Furthermore, a limited set of combinations of rotations around these two axes result in borderline agreement between calculated and experimental spectra. These include other alignments with pitch angles of 0°, the angular combinations of 15°−30°/15°, or the transmembrane orientation (tilt angle 0°). However, most peptide alignments beyond these limits result in pronounced spectral intensities between 100 and 230 ppm that are not visible in the experimental spectra, and these topologies can therefore be excluded.

The structural analysis of arenicins in water or in detergent micelles indicates that its side chains are arranged in such a manner to form a largely amphipathic structure.[2] The arginines are positioned at the C-terminus, close to the loop region (R9 and R11) and grouped along the second β-strand of the peptide (positions 16, 18, and 19). The NMR data in DPC micelles suggest that the dimerization interface involves the first β-strand, which, due to its more hydrophobic composition, probably inserts somewhat deeper into the bilayer. Indeed, the topological analysis shown in Figure 5 agrees with the dimer being oriented along the membrane surface, and the spectra allow for small deviations from perfect in-plane (by at most 20°). On the basis of paramagnetic relaxation measurements, similar alignments have also been suggested for protegrin in POPC/cholesterol bilayers.[10] Together the data and energetic considerations suggest an arrangement of the dimer taking the shape of a boat body floating on a sea of lipids where the N-terminal strand is close to the keel and more deeply inserted than the C-terminal portion of the β-sheet forming the rim.

It should be noted that the ^{15}N solid-state NMR spectrum of the Ala-6-labeled arenicin-1 (Figure 3A) or the spectra of the tilted sample (Figure 2E) are indicative of significant topological and conformational heterogeneity. Indeed, the structural data obtained in different environments show considerable conformational plasticity even though the β-sheet character is preserved in the presence of the cystine bridge.[2,4,37] Furthermore, the possibility exists that the peptide inserts into the membrane to adopt a "transmembrane" alignment by rotating around an axis (y-axis) that is perpendicular to the molecular long axis (z-axis). In a related manner lipid-dependent topologies and oligomerization have been observed for protegrin-1 and tilted alignments have been observed for retrocyclin-2 in DLPC membranes.[9,22]

The high abundance of arginines in the arenicin sequences is a striking characteristic of this class of peptides, which they share with other β-sheet antimicrobials[9,22] as well as cell penetrating peptides.[42,43] The guanidinium group of this amino acid has been shown to be able to form a bidentate complex with the phosphate groups of the lipids, thereby anchoring this rather hydrophilic side chain within the membrane interface.[44] For PG-1 this interaction has been suggested to promote lipid and peptide realignment in the membrane concomitant with the formation of torroidal pores.[45]

The ^{31}P NMR spectra of the arenicin phospholipid mixtures agree with such a model as the peptide strongly affects the orientational order of the lipid headgroups. This effect is most pronounced in the case of the POPE/POPG sample where a large fraction of the lipid occurs at strongly tilted alignments. The spectra are in agreement with the formation of "wormholes" or torroidal pores[21] although other membrane morphologies may be found to also explain such intensity distributions. The effects are less pronounced for the POPC sample where considerably less peptide has been added (Figure 2D). Nevertheless, signal intensities that deviate from the ideal headgroup conformation in the absence of peptide are also observed. Therefore, these data are suggestive that the antimicrobial activities of arenicins are related to pores formed by supramolecular rearrangements of the lipids and the peptides. Similar structures have also been suggested to form in the presence of α-helical polypeptides although the detailed mechanism how this occurs remains a matter of debate.[46−49] Lipid membranes are soft and flexible and can adopt a wide variety of supramolecular arrangements depending on a multitude of factors including peptide sequence and concentration, membrane lipid composition, and other environmental factors.[49] The data presented here and previously seem to indicate that the arginine-rich antimicrobial β-sheet peptides seem to have a particular high potential to interact with and reshape the lipid membrane.

■ ASSOCIATED CONTENT

Supporting Information. Simulations of static oriented ^{15}N solid-state NMR spectra of the arenicin structures found in aqueous solution (PDB 2JNI) and in micellar environments (PDB 2L8X) are shown as a function of the peptide topology. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

CD, circular dichroism; CP, cross-polarization; DPC, dodecylphosphocholine; NMR, nuclear magnetic resonance; MAS, magic angle spinning; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PG-1, protegrin 1; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol).

■ REFERENCES

(1) Ovchinnikova, T. V., Aleshina, G. M., Balandin, S. V., Krasnosdembskaya, A. D., Markelov, M. L., Frolova, E. I., Leonova, Y. F., Tagaev, A. A., Krasnodembsky, E. G., and Kokryakov, V. N. (2004)Purification and primary structure of two isoforms of arenicin, a novel antimicrobial peptide from marine polychaeta Arenicola marina. *FEBS Lett. 577*, 209–214.

(2) Ovchinnikova, T. V., Shenkarev, Z. O., Nadezhdin, K. D., Balandin, S. V., Zhmak, M. N., Kudelina, I. A., Finkina, E. I., Kokryakov, V. N., and Arseniev, A. S. (2007)Recombinant expression, synthesis, purification, and solution structure of arenicin. *Biochem. Biophys. Res. Commun. 360*, 156–162.

(3) Stavrakoudis, A., Tsoulos, I. G., Shenkarev, Z. O., and Ovchinnikova, T. V. (2009)Molecular dynamics simulation of antimicrobial peptide arenicin-2: beta-hairpin stabilization by noncovalent interactions. *Biopolymers 92*, 143–155.

(4) Ovchinnikova, T. V., Shenkarev, Z. O., Balandin, S. V., Nadezhdin, K. D., Paramonov, A. S., Kokryakov, V. N., and Arseniev, A. S. (2008)Molecular insight into mechanism of antimicrobial action of the

beta-hairpin peptide arenicin: specific oligomerization in detergent micelles. *Biopolymers 89*, 455–464.

(5) Frericks, H. L., Zhou, D. H., Yap, L. L., Gennis, R. B., and Rienstra, C. M. (2006)Magic-angle spinning solid-state NMR of a 144 kDa membrane protein complex: E. coli cytochrome bo3 oxidase. *J. Biomol. NMR 36*, 55–71.

(6) Castellani, F., van Rossum, B., Diehl, A., Schubert, M., Rehbein, K., and Oschkinat, H. (2002)Structure of a protein determined by solid-state magic-angle-spinning NMR spectroscopy. *Nature 420*, 98–102.

(7) Bockmann, A. (2007)High-resolution solid-state MAS NMR of proteins-Crh as an example. *Magn. Reson. Chem. 45*, S24–S31.

(8) Lange, A., Becker, S., Seidel, K., Giller, K., Pongs, O., and Baldus, M. (2005)A concept for rapid protein-structure determination by solid-state NMR spectroscopy. *Angew. Chem., Int. Ed. 44*, 2089–2092.

(9) Hong, M. (2006)Solid-state NMR studies of the structure, dynamics, and assembly of beta-sheet membrane peptides and alpha-helical membrane proteins with antibiotic activities. *Acc. Chem. Res. 39*, 176–183.

(10) Tang, M., and Hong, M. (2009)Structure and mechanism of beta-hairpin antimicrobial peptides in lipid bilayers from solid-state NMR spectroscopy. *Mol. Biosyst. 5*, 317–322.

(11) Bechinger, B., and Sizun, C. (2003)Alignment and structural analysis of membrane polypeptides by 15N and 31P solid-state NMR spectroscopy. *Concepts Magn. Reson., Part A 18A*, 130–145.

(12) Davis, J. H., and Auger, M. (1999)Static and magic angle spinning NMR of membrane peptides and proteins. *Prog. NMR Spectrosc. 35*, 1–84.

(13) Gottler, L. M., and Ramamoorthy, A. (2009)Structure, membrane orientation, mechanism, and function of pexiganan--a highly potent antimicrobial peptide designed from magainin. *Biochim. Biophys. Acta 1788*, 1680–1686.

(14) Traaseth, N. J., Buffy, J. J., Zamoon, J., and Veglia, G. (2006) Structural dynamics and topology of phospholamban in oriented lipid bilayers using multidimensional solid-state NMR. *Biochemistry 45*, 13827–13834.

(15) Aisenbrey, C., Prongidi-Fix, L., Chenal, A., Gillet, D., and Bechinger, B. (2009)Side chain resonances in static oriented proton-decoupled 15N solid-state NMR spectra of membrane proteins. *J. Am. Chem. Soc. 131*, 6340–6341.

(16) Prongidi-Fix, L., Bertani, P., and Bechinger, B. (2007)The membrane alignment of helical peptides from non-oriented 15 N chemical shift solid-state NMR spectroscopy. *J. Am. Chem. Soc. 129*, 8430–8431.

(17) Cady, S. D., Goodman, C., Tatko, C. D., DeGrado, W. F., and Hong, M. (2007)Determining the orientation of uniaxially rotating membrane proteins using unoriented samples: a 2 H, 13 C, and 15 N solid-state NMR investigation of the dynamics and orientation of a transmembrane helical bundle. *J. Am. Chem. Soc. 129*, 5719–5729.

(18) Bechinger, B., Resende, J. M., and Aisenbrey, C. (2011)The structural and topological analysis of membrane-associated polypeptides by oriented solid-state NMR spectroscopy: Established concepts and novel developments. *Biophys. Chem. 153*, 115–125.

(19) Resende, J. M., Mendonca Moraes, C., Munhoz, V. H. D. O., Aisenbrey, C., Verly, R. M., Bertani, P., Cesar, A., Pilo-Veloso, D., and Bechinger, B. (2009)Membrane structure and conformational changes during bilayer-association of the antibiotic heterodimeric peptide distinctin by oriented solid-state NMR spectroscopy. *Proc. Natl. Acad. Sci. U.S.A. 106*, 16639–16644.

(20) Aisenbrey, C., and Bechinger, B. (2004)Tilt and rotational pitch angles of membrane-inserted polypeptides from combined 15N and 2H solid-state NMR spectroscopy. *Biochemistry 43*, 10502–10512.

(21) Wi, S., and Kim, C. (2008)Pore structure, thinning effect, and lateral diffusive dynamics of oriented lipid membranes interacting with antimicrobial peptide protegrin-1: P-31 and H-2 solid-state NMR study. *J. Phys. Chem. B 112*, 11402–11414.

(22) Tang, M., Waring, A. J., Lehrer, R. I., and Hong, M. (2006) Orientation of a beta-hairpin antimicrobial peptide in lipid bilayers from two-dimensional dipolar chemical-shift correlation NMR. *Biophys. J. 90*, 3616–3624.

(23) Carpino, L. A., and Han, G. Y. (1972)The 9-fluorenylmethoxycarbonylamino-protecting group. *J. Org. Chem. 37*, 3404.

(24) Bechinger, B., Bertani, P., Werten, S., Mendonca de Moraes, C., Aisenbrey, C., Mason, A. J., Perrone, B., Prudhon, M., Sudheendra, U. S., Vidovic, V., Miguel, C. (2009) The structural and topological analysis of membrane polypeptides by oriented solid-state NMR spectroscopy: Sample preparation and theory in *Membrane-Active Peptides: Methods and Results on Structure and Function*, International University Line, La Jolla, CA, in press.

(25) Bechinger, B., and Opella, S. J. (1991)Flat-Coil Probe for NMR Spectroscopy of Oriented Membrane Samples. *J. Magn. Reson. 95*, 585–588.

(26) Hediger, S., Meier, B. H., and Ernst, R. R. (1995)Adiabatic passage Hartmann-Hahn cross polarization in NMR under magic angle sample spinning. *Chem. Phys. Lett. 240*, 449–456.

(27) Ramamoorthy, A., Wei, Y., and Lee, D. (2004)PISEMA Solid-State NMR Spectroscopy. *Annu. Rep. NMR Spectrosc. 52*, 1–52.

(28) Rance, M., and Byrd, R. A. (1983)Obtaining High-Fidelity Spin-1/2 Powder Spectra in Anisotropic Media: Phase-Cycled Hahn Echo Spectroscopy. *J. Magn. Reson. 52*, 221–240.

(29) Bechinger, B., Aisenbrey, C., and Bertani, P. (2004)The alignment, structure and dynamics of membrane-associated peptides by solid-state NMR spectroscopy. *Biochim. Biophys. Acta 1666*, 190–204.

(30) Aisenbrey, C., Sizun, C., Koch, J., Herget, M., Abele, U., Bechinger, B., and Tampe, R. (2006)Structure and dynamics of membrane-associated ICP47, a viral inhibitor of the MHC I antigen-processing machinery. *J. Biol. Chem. 281*, 30365–30372.

(31) Bak, M., Rasmussen, J. T., and Nielsen, N. C. (2000)SIMPSON: A general simulation program for solid-state NMR spectroscopy. *J. Magn. Reson. 147*, 296–330.

(32) Salnikov, E., Bertani, P., Raap, J., and Bechinger, B. (2009) Analysis of the amide (15)N chemical shift tensor of the C(alpha) tetrasubstituted constituent of membrane-active peptaibols, the alpha-aminoisobutyric acid residue, compared to those of di- and tri-substituted proteinogenic amino acid residues. *J. Biomol. NMR 45*, 373–387.

(33) Salnikov, E., Aisenbrey, C., Vidovic, V., and Bechinger, B. (2010)Solid-state NMR approaches to measure topological equilibria and dynamics of membrane polypeptides. *Biochim. Biophys. Acta 1798*, 258–265.

(34) Eldon, L. U., Akutsu, H., Doreleijers, J. F., Harano, Y., Ionnidis, Y. E., Lin, J., Livny, M., Mading, S., Maziuk, D., Miller, Z., Nakatani, E., Schulte, C. F., Tolmie, D. E., Wenger, R. K., Hongyang, Y., and Markley, J. L. (2007) *Nucleic Acids Res. 36*, D402–D408 (410.1093/nar/gkm1957).

(35) Aisenbrey, C., and Bechinger, B. (2004)Investigations of peptide rotational diffusion in aligned membranes by 2H and 15N solid-state NMR spectroscopy. *J. Am. Chem. Soc. 126*, 16676–16683.

(36) Salnikov, E. S., Mason, A. J., and Bechinger, B. (2009)Membrane order perturbation in the presence of antimicrobial peptides by 2 H solid-state NMR spectroscopy. *Biochimie 91*, 743.

(37) Lee, J. U., Kang, D. I., Zhu, W. L., Shin, S. Y., Hahm, K. S., and Kim, Y. (2007)Solution structures and biological functions of the antimicrobial peptide, arenicin-1, and its linear derivative. *Biopolymers 88*, 208–216.

(38) Wenk, M., and Seelig, J. (1998)Magainin 2 amide interaction with lipid membranes: Calorimetric detection of peptide binding and pore formation. *Biochemistry 37*, 3909–3916.

(39) Wieprecht, T., Apostolov, O., and Seelig, J. (2000)Binding of the antibacterial peptide magainin 2 amide to small and large unilamellar vesicles. *Biophys. Chem. 85*, 187–198.

(40) Bechinger, B. (2004)Membrane-lytic peptides. *Crit. Rev. Plant Sci. 23*, 271–292.

(41) Mani, R., Cady, S. D., Tang, M., Waring, A. J., Lehrer, R. I., and Hong, M. (2006)Membrane-dependent oligomeric structure and pore formation of a beta-hairpin antimicrobial peptide in lipid bilayers from solid-state NMR. *Proc. Natl. Acad. Sci. U.S.A. 103*, 16242–16247.

(42) Ziegler, A. (2008)Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans. *Adv. Drug Delivery Rev. 60*, 580–597.

(43) Su, Y., Mani, R., Doherty, T., Waring, A. J., and Hong, M. (2008)Reversible sheet-turn conformational change of a cell-penetrating peptide in lipid bilayers studied by solid-state NMR. *J. Mol. Biol. 381*, 1133–1144.

(44) Tang, M., Waring, A. J., Lehrer, R. I., and Hong, M. (2008) Effects of guanidinium-phosphate hydrogen bonding on the membrane-bound structure and activity of an arginine-rich membrane peptide from solid-state NMR spectroscopy. *Angew. Chem., Int. Ed. 47*, 3202–3205.

(45) Tang, M., Waring, A. J., and Hong, M. (2007)Phosphate-mediated arginine insertion into lipid membranes and pore formation by a cationic membrane peptide from solid-state NMR. *J. Am. Chem. Soc. 129*, 11438–11446.

(46) Shai, Y. (2002)Mode of action of membrane active antimicrobial peptides. *Biopolymers 66*, 236–248.

(47) He, K., Ludtke, S. J., Huang, H. W., and Worcester, D. L. (1995) Antimicrobial peptide pores in membranes detected by neutron in- plane scattering. *Biochemistry 34*, 15614–15618.

(48) Matsuzaki, K. (1998)Magainins as paradigm for the mode of action of pore forming polypeptides. *Biochim. Biophys. Acta 1376*, 391–400.

(49) Bechinger, B., and Lohner, K. (2006)Detergent-like action of linear cationic membrane-active antibiotic peptides. *Biochim. Biophys. Acta 1758*, 1529–1539.