



Probing membrane topology of the antimicrobial peptide distinctin by solid-state NMR spectroscopy in zwitterionic and charged lipid bilayers

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ARTICLE INFO

Article history:

Received 1 December 2009

Received in revised form 26 July 2010

Accepted 9 August 2010

Available online 16 August 2010

Keywords:

Distinctin

Antimicrobial Peptides

Oriented Solid-State NMR

Mechanically Aligned Bilayer

Membrane Protein Topology

ABSTRACT

Distinctin is a 47-residue antimicrobial peptide, which interacts with negatively charged membranes and is active against Gram-positive and Gram-negative bacteria. Its primary sequence comprises two linear chains of 22 (chain 1) and 25 (chain 2) residues, linked by a disulfide bridge between Cys19 of chain 1 and Cys23 of chain 2. Unlike other antimicrobial peptides, distinctin in the absence of the lipid membrane has a well-defined three-dimensional structure, which protects it from protease degradation. Here, we used static solid-state NMR spectroscopy in mechanically aligned lipid bilayers (charged or zwitterionic) to study the topology of distinctin in lipid bilayers. We found that this heterodimeric peptide adopts an ordered conformation absorbed on the surface of the membrane, with the long helix (chain 2), approximately parallel to the lipid bilayer (~5° from the membrane plane) and the short helix (chain 1) forming a ~24° angle with respect to the bilayer plane. Since the peptide does not disrupt the macroscopic alignment of charged or zwitterionic lipid bilayers at lipid-to-protein molar ratio of 50:1, it is possible that higher peptide concentrations might be needed for pore formation, or alternatively, distinctin elicits its cell disruption action by another mechanism.

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1. Introduction

Naturally occurring antimicrobial peptides (AMPs) are becoming a new weapon in the fight against bacterial drug resistance [1–7]. Traditional approaches for the treatment of bacterial infections rely on the use of compounds that become quickly ineffective due to the genetic plasticity of pathogenic microorganisms. AMPs represent a promising alternative to common antibiotics because of their ability to directly interact with the bacterial membrane causing cell lysis [1–3]. Resistance to AMPs is an unlikely event since it would imply severe modification of the lipid composition and architecture of the cell membrane.

Multicellular organisms produce AMPs as a defense against microbial pathogens. AMPs have been isolated from fungi, insects, amphibians, mammals, and plants [4], with their expression triggered as a response to external stress or alternatively secreted from storage by

superficial glands [8]. Due to their impact on pathogenic cells, they have also been shown to be effective against tumors, HIV infection, and pulmonary infections, as well as having a role in modulating the immune system [4]. Over the past few decades, there have been many studies aimed at isolating and characterizing novel AMPs with the potential to be used as new therapeutic agents against bacterial infections [5]. These studies have shown that AMPs do not possess any common amino acid motif, but do share some common structural features, including the presence of an amphipathic helix [9]. Several models have been proposed to explain how antimicrobial peptides interact with bacterial membranes to cause cell lysis, although the molecular details on the mechanism of action have proved extremely challenging to elucidate [4,10]. This is largely due to difficulty in studying these peptides in the presence of lipid membranes with conventional techniques such as X-ray crystallography.

Because of the potential therapeutic use of AMPs it is fundamental to understand the structural details of peptide/membrane interactions to design more specific and potent variants [11]. Solution NMR spectroscopy has been applied to a myriad of small and medium size antimicrobial peptides [12–17], giving important insights into the mechanism of insertion into micelles. However, micellar systems are only a coarse approximation of membrane

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bilayers; synthetic lipid bilayers (vesicles or planar bilayers) are the preferable system to test lipid/peptide interactions. The large size of the lipid/peptide complexes requires solid-state NMR (ssNMR) techniques. Specifically, ssNMR spectroscopy of peptide and proteins in mechanically and magnetically aligned lipid bilayers is able to provide structural and dynamic information [18–20]. First, it is possible to measure the degree of lipid bilayer alignment using ^{31}P NMR spectroscopy [21]. Second, the orientation of peptide planes using selective or uniformly ^{15}N - or ^{13}C -labeled proteins can be determined [18,22]. As a result, it is possible to interpret the changes in topology of these proteins by following the perturbations to chemical shifts. Recent reports summarize the application of these methods to small membrane proteins as well as antimicrobial peptides [22–27].

In this work, we analyzed the topology of distinctin in both zwitterionic and charged oriented lipid bilayers by ssNMR spectroscopy. Distinctin is an antimicrobial peptide extracted from *Phyllomedusa distincta*, a tree frog that lives in the forests of Brazil [28]. It is active against Gram-positive and Gram-negative bacteria, but shows very little hemolytic activity against human erythrocytes [28,29]. In a recent study [30,31], distinctin was shown to be effective in the treatment of a severe staphylococcal infection (sepsis) in a murine model, underlying the potential role of this novel peptide for therapeutic purposes. These researchers found that distinctin can be associated with other antibiotic compounds, reducing the number of infections drastically [30].

Compared to other well-studied AMPs, distinctin presents some interesting peculiarities. It is a heterodimer composed of two polypeptide chains: chain 1 (22 residues) and chain 2 (25 residues) linked by a disulfide bridge between Cys-19 of chain 1 and Cys-23 of chain 2 (Fig. 1A). Solution NMR studies in aqueous environment revealed that both chains are amphipathic α -helices (Fig. 1B). It was also shown that distinctin, unlike other antimicrobial peptides, adopts a well-folded conformation in aqueous buffer, with a parallel four-helical bundle (dimer of dimers) (Fig. 1C) [29,31]. This conformation confers an increased stability and a marked resistance to proteolytic degradation [29].

When incorporated into phosphatidylcholine/phosphatidylethanolamine planar bilayers, distinctin gave rise to voltage-dependent behavior typical of ion channel formation [29]. Addition of negatively charged phosphatidylserine to the lipid preparations did not change this behavior, suggesting that the membrane composition does not play a major role in distinctin's function. Based on these data, different structural models were built to describe the pore formation in membranes. Molecular dynamics calculations based on structural and conductivity data suggested that distinctin could form pentameric pores in membranes with at least one of the helices crossing the lipid bilayer [31].

Recently, the topological preferences of distinctin in zwitterionic lipid bilayers were investigated by Bechinger et al. [32]. Using a combination of site specific labels (^{15}N and ^2H) and ssNMR spectroscopy, these researchers showed that distinctin in POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) lipid bilayers adopts an orientation with the two helical domains approximately parallel to the surface of the bilayers. This orientation is persistent both at lipid–protein ratios of 100:1 and 250:1. Although acquired in the absence of charged lipids and lipid–protein ratios higher than the limits observed for the formation of putative ion channels, the data from Bechinger et al. [32] do not support the mechanism of action proposed by Dalla Serra et al. [31].

To shed light on the topology of distinctin in charged and zwitterionic lipid membranes and its mechanism of action, we used ^{31}P and ^{15}N ssNMR spectroscopy in mechanically aligned lipid bilayers. We found that distinctin is absorbed on the surface of the lipid bilayers and that the charge of the lipid head groups does not perturb its topology.

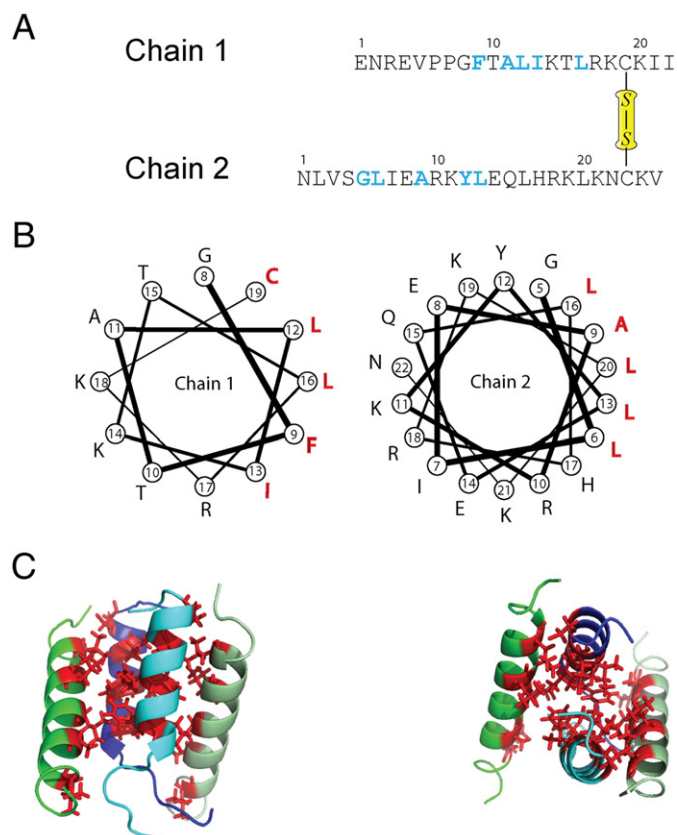


Fig. 1. (A) Amino acid sequence of distinctin heterodimer. ^{15}N -labeled amino acids are highlighted in blue. Residue Cys-19 of chain 1 and Cys-23 of chain 2 are involved in a disulfide bridge. (B) Helical wheel plots showing the amphipathic nature of each chain. Hydrophobic residues are in red. (C) Three-dimensional structure of distinctin in aqueous buffer [29]. The four-helical bundle is stabilized by hydrophobic interactions of the residues, forming a hydrophobic core in the interior of the structure.

2. Materials and methods

2.1. Peptide synthesis

Distinctin peptide chains were independently synthesized by 9-fluorenyl-methoxycarbonyl solid-phase chemistry as previously described [29]. Four different samples of distinctin labeled with ^{15}N at the amide nitrogen were prepared: a) Phe-9 of chain 1, b) Ala-9 of chain 2, c) Ala-11-Leu-12-Ile-13-Leu-16 of chain 1, and d) Gly-5-Leu-6-Tyr-12-Leu-13 of chain 2. For the ^{15}N isotopic enrichment, Fmoc- ^{15}N -Phe-OH, Fmoc- ^{15}N -Ala-OH, Fmoc- ^{15}N -Leu-OH, Fmoc- ^{15}N -Ile-OH, Fmoc- ^{15}N -Gly-OH, and Fmoc- ^{15}N -Tyr-OH (Sigma-Aldrich, St. Louis, MO) were used. To form the disulfide bond, the two peptide chains, dissolved in basic aqueous solution, were slowly mixed and dried under air flow [29]. All peptides were purified by reverse-phase HPLC (98% purity) with the molecular masses confirmed by MALDI-TOF mass spectrometry (m/z 5482).

2.2. Solid-state NMR sample preparation

For zwitterionic lipid bilayers, we used 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (POPC/DOPE); while for charged bilayers, we used 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/1,2-dioleoyl-*sn*-glycero-3-phosphate (POPC/DOPA) lipid mixtures. Briefly, a stock solution of 1 mg/mL in doubly distilled H_2O (ddH_2O) of each distinctin sample was prepared and kept at -20°C . For mechanically aligned samples, a mixture (4:1 mol/mol) of POPC/DOPE or a mixture (1:1) of

POPC/DOPA from Avanti Polar Lipids (Alabaster, AL) was dried from chloroform in a glass vial to a thin film; then ddH₂O (0.2% wt./vol.) was added to the dried lipids and the resulting suspension was extruded ten times through a polycarbonate filter (Millipore, Billerica, MA) with 0.05 μ m pore diameter using a Lipex extruder (Northern Lipids, Inc., Vancouver, BC, Canada). Ten passages were necessary to ensure that small unilamellar vesicles (SUVs) of homogeneous size were obtained. The suspension of SUVs was concentrated to 2 mL using an Amicon ultraconcentrator with a 10-kDa cutoff membrane. Distinctin was added to the lipids, and the mixture was stirred for 2 hours at 25 °C. The mixture was then transferred onto 25 glass plates (5.7 mm \times 12 mm \times 0.030 mm; Marienfeld GmbH, Lauda-Königshofen, Germany) with water slowly evaporated by incubation for 4 hours at 40 °C. The plates were stacked, rehydrated for 96 hours in a humidity chamber to reach the liquid-crystalline phase, and finally sealed in a glass cell. POPC/DOPE samples of Ala-9 and Phe-9 were prepared with a final lipid/protein (L/P) ratio of 200:1, whereas distinctin labeled at chain 1 (Ala-11-Leu-12-Ile-13-Leu16) and distinctin labeled at chain 2 (Gly-5-Leu-6-Tyr-12-Leu13) were reconstituted at a L/P ratio of 50:1. For the POPC/DOPA sample of Ala-9, the L/P ratio was 50:1.

2.3. Solid-state NMR spectroscopy

All spectra were acquired on a Varian spectrometer operating at field strength of 14.1 T (¹H frequency of 600.1 MHz). ³¹P single-pulse experiments were performed for all the samples to evaluate the macroscopic orientation of the lipid bilayers [21]. A doubly tuned flat coil probe (Varian Inc., Palo Alto, CA, BioStatic™) was used with the temperature for all experiments regulated at 5 °C. The following parameters were used: spectral width of 100 kHz, acquisition time of 5 ms, recycle delay time of 4 s, SPINAL64 decoupling [33] field strength of 50 kHz, and a 90° pulse length of 6 μ s. All spectra were processed with a 50-Hz line-broadening window function. For each ³¹P spectrum, a total of 64 scans were collected and zero filled to 2048 points.

¹⁵N cross-polarization (CP) spectra of static aligned samples were acquired with the same low-*E* double-resonance flat-coil probe (Varian Inc., Palo Alto, CA) using the following parameters: spectral width of 100 kHz, acquisition time of 5 ms, CP contact time of 1 ms, SPINAL64 decoupling [33] field strength of 50 kHz, and 5 μ s ¹H 90° pulse length. For each spectrum, a total of 24,000–56,000 scans were recorded and zero filled to 2048 points with a 100-Hz line-broadening window function applied. The two-dimensional ¹⁵N chemical shift/¹H–¹⁵N dipolar correlations were acquired using the SAMPI4 experiment [34] with 6400–7200 scans in the direct dimension and 8–10 increments used to evolve the dipolar coupling on the coupling. All of the spectra were referenced to the ¹⁵NH₄Cl (solid) signal (41.5 ppm) as an external standard.

3. Results and discussion

The first step in the present study was to assess the effect of different concentrations of distinctin peptide on the planarity of the lipid bilayers. Fig. 2 shows ³¹P spectra of mechanically aligned POPC/DOPE lipid bilayers in the presence of distinctin at 200:1 L/P molar ratio. The presence of a single peak at ~30 ppm is indicative of well aligned lipid bilayers with the normal parallel to the external magnetic field. This is consistent with a recent report by Bechinger et al. [32] that shows no effect of distinctin peptide on the alignment of zwitterionic (POPC) lipid bilayers. Since distinctin interacts more strongly with membranes containing charged lipids (PA, PS, and PG) [29,31], we investigated the effects of charged lipids by adding DOPA to the lipid preparations up to a molar ratio of 1:1, POPC/DOPA. Both the presence of the charged lipid and the low lipid–peptide ratio used (up to 50:1) did not cause a disruption of the lipid bilayers. Fig. 3A shows the presence of a single resonance at ~30 ppm, confirming the

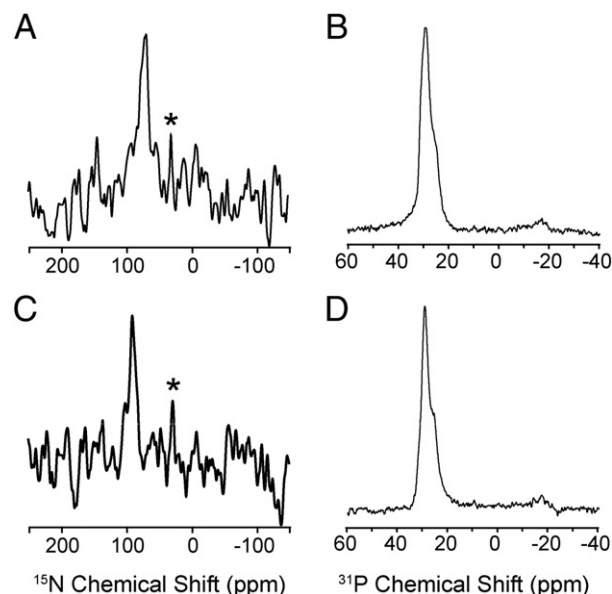


Fig. 2. (A) and (C) One-dimensional cross-polarization spectra of ¹⁵N Ala-9 (chain 2) and ¹⁵N Phe-9 (chain 1) distinctin, respectively in POPC/DOPE (4:1 mol/mol) at 200:1 lipid–protein. (B) and (D) One-dimensional ³¹P spectra of aligned lipid bilayers for ¹⁵N Ala-9 (chain 2) and ¹⁵N Phe-9 (chain 1) distinctin, respectively. Asterisks indicate natural abundance ¹⁵N from lipid head groups.

macroscopic orientation and integrity of the lipid bilayer. These data are consistent with the static ³¹P experiments carried out in lipid vesicles under the same lipid composition (POPC/DOPA) and lipid–peptide molar ratio (50:1) [29]. In those experiments, no changes in the ³¹P powder pattern were detected, ruling out detergent-like mechanism of action of distinctin or other major perturbations of the lipid membranes.

To ascertain the ability of each distinctin chain to penetrate the membrane, several selectively ¹⁵N labeled samples were prepared (Fig. 1A). The ¹⁵N chemical shift of amide groups

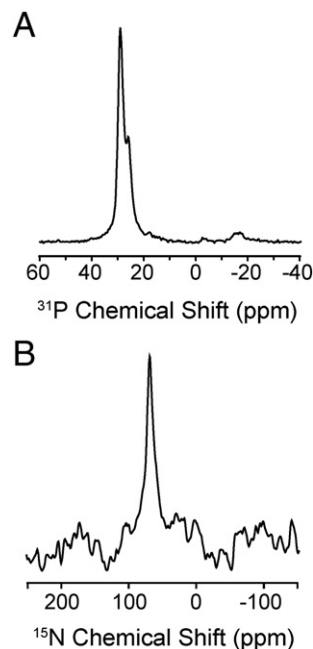


Fig. 3. (A) ³¹P spectrum of POPC/DOPA (1:1 mol/mol) containing ¹⁵N Ala-9 (chain 2) distinctin at 50:1 lipid–peptide. (B) ¹⁵N proton-decoupled cross-polarization spectrum of ¹⁵N Ala-9 (chain 2) distinctin at 50:1 lipid–peptide.

in helical segments is very sensitive to the orientation of the amide group with respect to the external magnetic field, which in our experimental set-up is collinear with the membrane normal. Qualitatively, chemical shifts greater than 200 ppm indicate that the helix is nearly perpendicular to the lipid bilayer plane (transmembrane). Conversely, chemical shifts smaller than 100 ppm entail that the helix is almost parallel to the membrane plane. Shifts around ~110–120 ppm are usually indicative of isotropic or disordered regions of the protein.

Figs. 2A and C show the proton-decoupled cross-polarization spectra for the two singly labeled distinctin samples (^{15}N Phe-9 of chain 1 or ^{15}N Ala-9 of chain 2) in POPC/DOPE bilayers at a lipid-peptide molar ratio of 200:1. Assuming both distinctin chains are in a helical configuration, the Ala-9 amide nitrogen shift of ~72 ppm corresponds to an almost parallel orientation of chain 2 with respect to the membrane plane, while Phe-9 resonates at 92 ppm, also indicating a parallel configuration for chain 1. The slight change in chemical shift can be attributed to a somewhat altered tilt angle of chain 1 with respect to chain 2, a different relative position of Phe-9 on the helical wheel, or alternatively, a difference in dynamics between chain 1 and chain 2, which would cause a scaling of the chemical shift toward the isotropic value (~110–120 ppm). The extra peak at ~30 ppm present in the Phe-9 spectrum was assigned to the natural abundance ^{15}N of the phospholipid head-groups [35]. The Phe-9 spectrum has a signal/noise ratio ~4 times lower than Ala-9.

The experiments were repeated in POPC/DOPA-oriented lipid bilayers. Fig. 3B shows the proton-decoupled cross-polarization spectrum of ^{15}N Ala-9 (chain 2). In this case, we were able to reach a L/P molar ratio of up to 50:1, without disturbing the macroscopic orientation of the lipid bilayer (see Fig. 3A). Predictably, the signal-to-noise ratio of this ^{15}N spectrum is much higher than the previous spectra obtained at 200:1 molar ratio. However, the chemical shifts did not change substantially (~72 ppm for distinctin in POPC/DOPE at L/P of 200:1 and ~72 in POPC/DOPA at L/P of 50:1). This demonstrates that the addition of the DOPA in the lipid preparations does not substantially modify the topological orientation of distinctin. The higher concentration of peptide reached under these conditions made it possible to carry out a two-dimensional separated local field experiment that resolves ^{15}N chemical shifts with ^1H - ^{15}N dipolar couplings. Given the predicted orientation based on ^{15}N chemical shifts, we opted for the SAMPI4 experiment, which has a smaller ^1H offset dependence on the measured dipolar coupling values [36]. Fig. 4B shows the SAMPI4 spectrum of Ala-9 in mechanically oriented POPC/DOPA lipid bilayers. The measured dipolar coupling for Ala-9 is 2.0 kHz, a value typical of well-structured alpha helix absorbed on the lipid bilayer surface [37]. In order to ensure homogeneity of our sample preparations, we show the solution NMR [^1H - ^{15}N]-HSQC spectrum of Ala-9 ^{15}N -labeled distinctin in phosphate buffer at 37 °C in Fig. 4A, indicating homogenous linewidths and the isotropic chemical shift values for both ^1H and ^{15}N of Ala-9.

To determine the topology of the distinctin heterodimer more accurately, two other samples were prepared: 1) ^{15}N labels at Ala-11, Leu-12, Ile-13, Leu-16 of chain 1 and chain 2 unlabeled (Distinctin1) and 2) ^{15}N labels at Gly-5, Leu-6, Tyr-12, Leu-13 of chain 2 and chain 1 unlabeled (Distinctin2).

Both samples were reconstituted in POPC/DOPE lipid bilayers at a L/P ratio of 50:1. Figs. 5A and B show the SAMPI4 spectra of Distinctin1 and Distinctin2, respectively. Distinctin1 has four resolved resonances ranging from ~75 to ~110 ppm. The four resonances are well dispersed in the nitrogen dimension, indicative of a tilted orientation of the helical axis.

Distinctin2, corresponding to the longer chain, presents only two clearly resolved resonances at ~74 and ~86 ppm, which were assigned to Tyr-12 and Leu-13 respectively. Although the sample had four labeled sites, two of them (Gly-5 and Leu-6) are located at the beginning of the α -helix [29], therefore highly dynamic resulting in

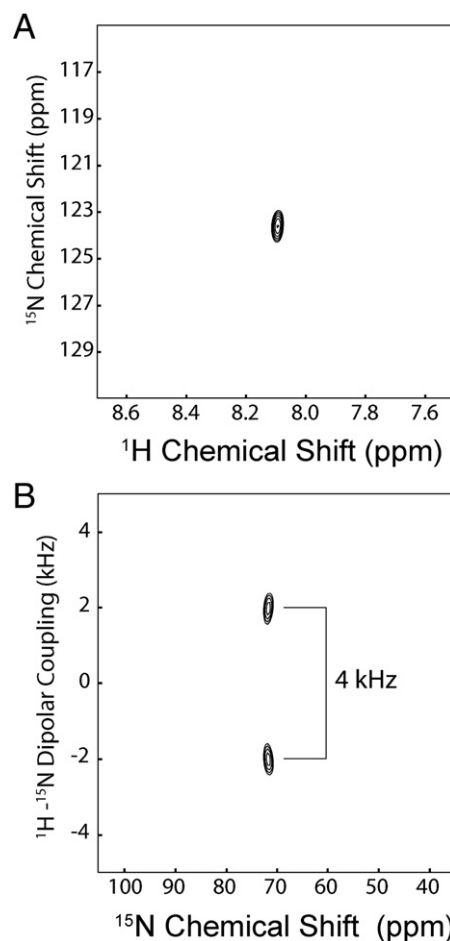


Fig. 4. (A) Solution NMR [^1H - ^{15}N]-HSQC spectrum acquired in phosphate buffer pH = 6.5, showing the isotropic chemical shift of the ^{15}N Ala-9 (chain 2) distinctin. (B) Solid-state NMR [^1H - ^{15}N]-SAMPI4 spectrum of ^{15}N Ala-9 (chain 2) distinctin reconstituted in oriented POPC/DOPA lipid bilayers.

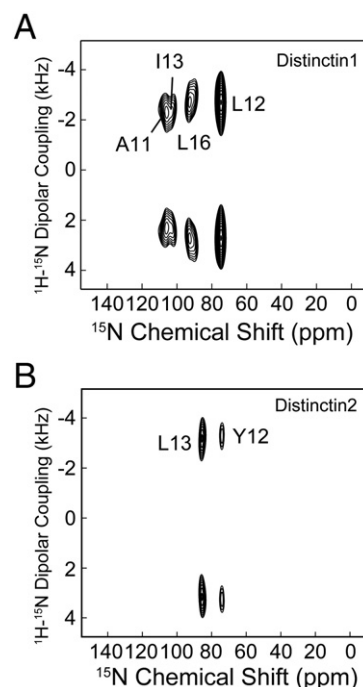


Fig. 5. SAMPI4 spectra of distinctin ^{15}N selectively labeled at (A) Ala-11, Leu-12, Ile-13, Leu-16 of chain 1 and (B) Gly-5, Leu-6, Tyr-12, Leu-13 of chain 2.

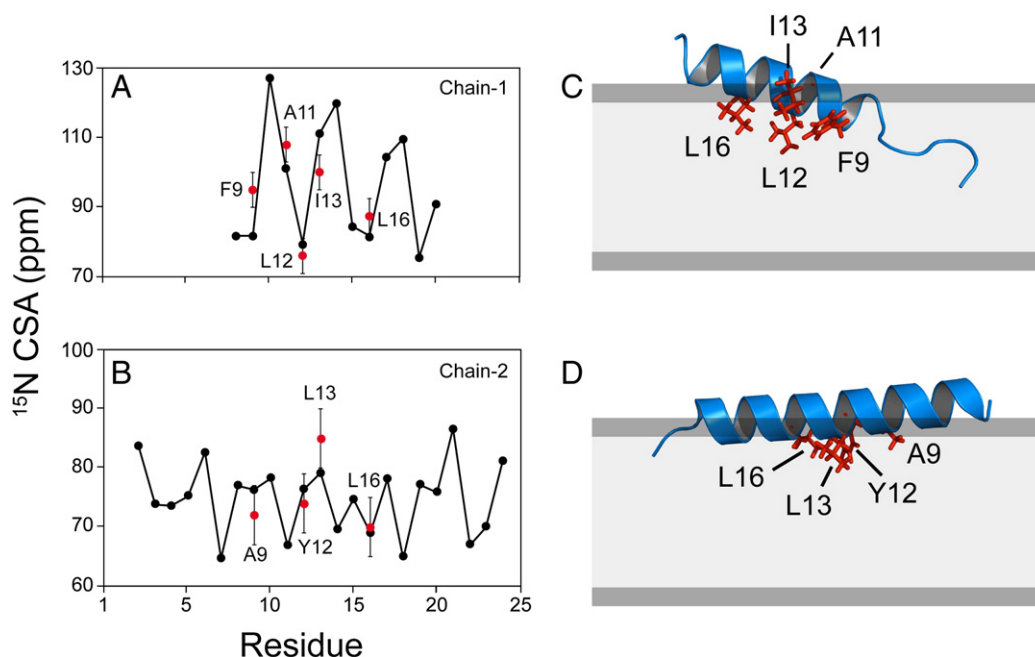


Fig. 6. Calculated (black circles) and experimental (red circles) chemical shift anisotropy of distinctin chain 1 (A) and distinctin chain 2 (B). Error values in the experimental values are ± 5 ppm. The chemical shift of Leu-16 in chain 2 is taken from Bechinger et al. [32]. Refined molecular structures of distinctin chain 1 (C) and chain 2 (D) obtained using the energy minimization protocol of Shi et al. [41].

inefficient cross-polarization. In contrast, the more structured residues Tyr-12 and Leu-13 are efficiently cross-polarized in the SAMPI4 spectrum.

To determine the tilt and rotation angles of the two distinctin chains in lipid bilayers more quantitatively, we implemented the chemical shift anisotropy values in the simulated annealing protocol according to Shi et al. [38] and refined the published solution structure of the distinctin heterodimer [29]. After refinement, the anisotropic chemical shifts for each residue were back-calculated from the energy minimized structure. Figs. 6A and B show the results calculated from the minimized structures (black traces) and the experimental values determined by oriented solid-state NMR (red points). Almost all of the experimental points are within the error of ± 5 ppm, following the periodic pattern typical of α -helices [39–43]. By fitting the chemical shift values we obtained tilt angles of $\sim 24^\circ$ and 5° with respect to the bilayer plane, for chains 1 and 2, respectively.

To elicit antimicrobial activity (cell lysis), the two distinctin amphipathic helices need to interact strongly with bacterial membranes. This interaction is modulated by negatively charged lipids (DOPA1,2-dioleoylphosphatidylglycerol, phosphatidylinositol and phosphatidylserine) [29,31]. However, our measurements show that lipid interactions occur even with the neutral lipids POPC and DOPE [31], without disrupting the lipid membranes. At a molar ratio of 50:1, the peptide is still absorbed on the surface of the membrane and there is no evidence of a transmembrane orientation of the longer distinctin chain (chain 2). This would support a “carpet-like” model of interaction with lipid bilayers [4].

A possible reconciliation of these observations is that distinctin might adopt a mixed mechanism of action between two extremes represented by the *all-or-none* [44,45] and *graded* [9] antimicrobial mechanisms, an intermediate situation that Almeida et al. [9] defined as the “grey zone.” In other words, while distinctin may form transient pores even at lower concentrations, there is a substantial population of the peptide present on the surface of the lipid membrane, which is absorbed in a “carpet-like” fashion (see Fig. 7). Increases in peptide concentration may drive the system to the formation of more organized structures similar to pores [31]. This ambivalence of

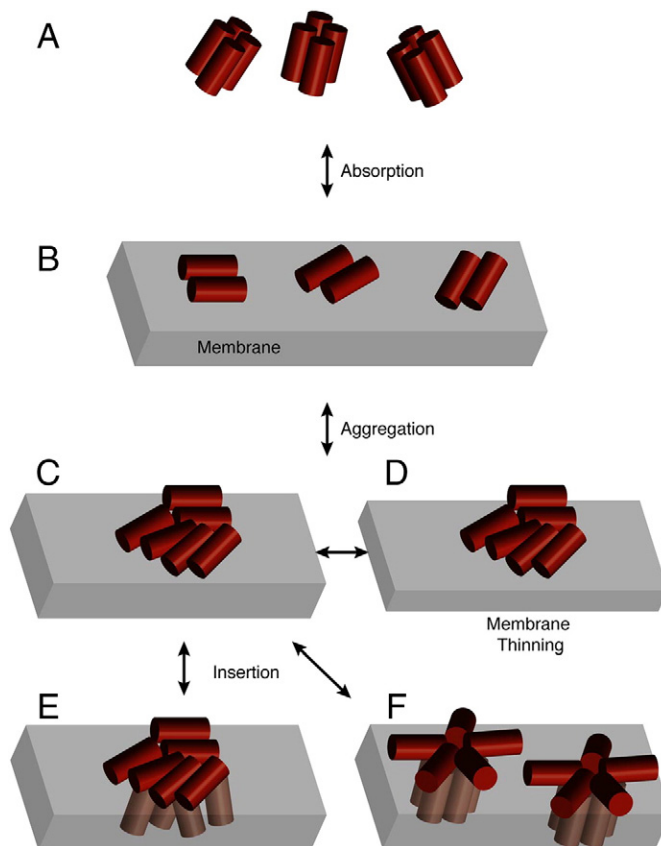


Fig. 7. Proposed model of distinctin interacting with cell membranes: (A) In the soluble form, distinctin forms well-structured helical bundle (dimer of dimers) that are resistant to proteases. (B) The helical bundle deaggregates in the presence of the membrane to interact with the surface of the lipid bilayer. (C–F) Aggregation of the peptide on the surface of the bilayer and possible models to explain the channel-like behavior.

distinctin may find a justification in its affinity for both zwitterionic and charged lipids. The latter does not fully justify the selectivity of distinctin for bacterial membranes versus erythrocytes and suggests that more experiments for the characterization of the thermodynamics and kinetics of membrane binding of distinctin need to go along with the structural studies to elucidate the mechanism of action of this important peptide.

4. Conclusions

In conclusion, we report the ssNMR investigation of the distinctin peptide in three model membranes. We found that this peptide is absorbed on the surface of either zwitterionic or negatively charged lipid bilayers at a peptide to lipid ratio as high as 50:1. In agreement with previous studies [31], ^{31}P spectra show that these high peptide concentrations do not disrupt model lipid bilayers (POPC/DOPE or POPC/DOPA). Two-dimensional ^{15}N chemical shift/ ^1H - ^{15}N dipolar correlation spectra of several residues in chain 1 and chain 2 show a dipolar coupling of ~ 2.0 kHz, confirming that distinctin, behaves as a typical amphipathic peptide, with the helical domains absorbed on the surface of the membrane and the helical axes approximately perpendicular to the normal of the membrane. In particular, the short chain 1 has a tilt angle of $\sim 24^\circ$ with respect the plane of the bilayer, whereas chain 2 is almost parallel ($\sim 5^\circ$). Taken with the recent electrochemical data on single-channel conductivity [29], this study suggests a mixed mechanism of action of distinctin, which is intermediate between the *all-or-none* and *graded* antimicrobial mechanism identified by Almeida et al. [9]. Even at low concentrations, distinctin can form stochastic pores that justify the ion conductivity; however, the peptide has high propensity to interact with the lipid membranes in a carpet-like topology.

Finally, our ssNMR investigation was carried out in mechanically aligned lipid bilayers spread onto glass plates. Although this system is widely employed in the structural biophysics of membrane proteins, further studies on distinctin with model membranes having a higher degree of hydration are preferable. The latter will be part of our future endeavors.

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