

Can antimicrobial peptides scavenge around a cell in less than a second?

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ABSTRACT

Antimicrobial peptides, which play multiple host-defense roles, have garnered increased experimental focus because of their potential applications in the pharmaceutical and food production industries. While their mechanisms of action are richly debated, models that have been advanced share modes of peptide–lipid interactions that require peptide dynamics. Before the highly cooperative and specific events suggested in these models take place, peptides must undergo an important process of migration along the membrane surface and delivery from their site of binding on the membrane to the actual site of functional performance. This phenomenon, which contributes significantly to antimicrobial function, is poorly understood, largely due to a lack of experimental and computational tools needed to assess it. Here, we use ¹⁵N solid-state nuclear magnetic resonance to obtain molecular level data on the motions of piscidin's amphipathic helices on the surface of phospholipid bilayers. The studies presented here may help contribute to a better understanding of the speed at which the events that lead to antimicrobial response take place. Specifically, from the perspective of the kinetics of cellular processes, we discuss the possibility that piscidins and perhaps many other amphipathic antimicrobial peptides active on the membrane surface may represent a class of fast scavengers rather than static polypeptides attached to the water–lipid interface.

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1. Introduction

Antimicrobial peptides (AMPs) play multiple host-defense roles against many pathogens, act quickly, and evidence little if any resistance effects [1–6]. They have garnered increased experimental focus because of their potential applications in the pharmaceutical and food production industries [7–10]. AMPs that are highly antimicrobial (as judged by their minimum inhibitory concentration, MIC) and not lethal to mammalian cells (as shown by low hemolytic effects on red blood cells) are excellent candidates for drug development [4,11–13]. Although more than a thousand natural antimicrobial peptides have been discovered to date and the number is growing rapidly, AMPs appear to adopt a relatively small number of conformational states, with the amphipathic α -helix as a dominating element of secondary structure [14,15]. Despite the overall broad diversity of AMPs in terms of their tertiary structures and molecular

targets, AMPs have key attributes in common: a net positive charge to allow for initial interaction with negatively charged membranes; structural flexibility to facilitate folding into a bio-active conformation upon membrane binding; and hydrophobicity to facilitate partial or total membrane insertion. The subsequent events, such as membrane perturbations and permeation, as well as the mechanisms by which they are achieved, may differ significantly even though interfacial activity is a common theme [2,4,10,13,16–34].

While the mechanisms of action of AMPs are richly debated, the non-receptor-mediated models that have been advanced share modes of peptide–lipid interactions that require peptide dynamics. The Shai–Matsuzaki–Huang model [1–4,6] describes two steps for the initial interactions of AMPs with membranes: (1) adoption of an amphipathic structure upon binding; (2) insertion that leads to thinning of the bilayer. Next, depending on the peptide and targeted membranes, pore formation can ensue when a certain threshold concentration of peptide is reached. In the barrel-stave model, the pores are transmembrane and highly organized. Alternatively, other models describe the formation of supramolecular peptide–lipid systems that can lead to toroidal pores and/or a carpet that disrupts the membrane through detergent or micellar aggregate effects. However, before the highly cooperative and specific events suggested in these models take place, peptides must undergo an important process of migration along the membrane surface and delivery from the site of binding on the membrane to the actual site of functional performance. This process,

Abbreviations: AMPs, antimicrobial peptides; APD, antimicrobial peptide database; CSA, chemical shift anisotropy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerate; E-field, electric-field; HPLC, high performance liquid chromatography; MIC, minimum inhibitory concentration; NMR, nuclear magnetic resonance; p1(or 3)-COO[−], non-amidated piscidin 1 (or 3); p1 (or 3)-NH₂, amidated piscidin 1 (or 3); PC, phosphatidylcholine; PDB, protein data bank; PG, phosphatidylglycerol; TFE, trifluoroethanol

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which contributes significantly to antimicrobial function, is frequently neglected, largely due to a lack of experimental and computational tools needed to assess it. Yet, understanding the rates of these processes would provide valuable information about the overall rate of antimicrobial response under physiological conditions and their deviations.

According to the antimicrobial peptide database (APD), which stores functional, sequence, and structural information about approximately 1400 antimicrobial peptides from all biological sources, the three-dimensional structures of only 13.3% of them have been reported [14]. These structures are largely (>95%) determined by NMR primarily because membrane or membrane-associated proteins and peptides are notoriously difficult to crystallize and therefore X-ray crystallography cannot be readily employed to solve their three-dimensional structures. Solid-state Nuclear Magnetic Resonance (ssNMR), which was solely utilized here to investigate the dynamics of two AMPs, offers the advantage of enabling the study of AMPs in a “native-like” environment that mimics bacterial cell membranes [35,36].

While molecular dynamics simulations make available a wide range of protein dynamics information on the time scale of up to several hundreds of nanoseconds commensurate with increasing computational power, they fall short in identifying dynamic processes of membrane proteins on the time scale of micro- and milliseconds [37,38]. The NMR chemical shift anisotropy (CSA) of ^{15}N sites of the peptide backbone is on the order of 5000–15 000 Hz in modern NMR spectrometers corresponding to the time scale of 60–200 μs . Therefore, the ^{15}N CSA, which can be characterized by ssNMR and supplemented by T_2 and $T_{1\rho}$ relaxation measurements, is an excellent atomic-level probe to target dynamics processes on the time scale of microseconds and distinguish these dynamic events from those on the time scale of milliseconds. Additionally, ssNMR offers a unique advantage for the structural and dynamic characterization of membrane associated peptides and proteins in their “native-like” membrane environment [39,40], as compared to solution NMR methods.

Here, we demonstrate the power of ssNMR on antimicrobial piscidin 1 (FFHHIFRGIVHVGKTIHRLVTG-COO $^-$ /NH $_2$, p1-COO $^-$ /NH $_2$) and piscidin 3 (FIHHIFRGIVHAGRSIGRFLTG-COO $^-$ /NH $_2$, p3-COO $^-$ /NH $_2$) in the presence and the absence of carboxyamidation. Specifically, we used ^{15}N ssNMR to monitor on a molecular level the motions of the amphipathic helices of piscidin 1 and piscidin 3 on the surface of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) hydrated lipid bilayers to mimic the electrostatics on the surface of anionic bacterial membranes where cationic AMPs bind to initiate their antimicrobial activity. Our approach involved the measurement of anisotropic chemical shifts in peptide samples oriented in “native-like” hydrated lipid bilayers and studied in multiple peptide orientations with respect to the external magnetic field B_0 .

Three isoforms of piscidin, piscidins 1, 2, and 3, were discovered in the mast cells of fish as a mixture of amidated and non-amidated peptides. They are highly potent antimicrobial, cationic, and amphipathic peptides that play a vital role in the fight against many aquatic infections [41–44]. Piscidin 1 is the most active, while the least active is piscidin 3. Piscidin 2 differs from piscidin 1 only at position 18 (arginine in piscidin 1 vs. lysine in piscidin 2). Piscidins, which have recently been found in many fish species [45], display common gene structures with pleurocidin and share a number of features with other AMPs, including a broad-spectrum activity against pathogens including multi-drug resistant bacteria, fungi, and viruses [41–44,46,47]; an amphipathic character; the occurrence of amidated and acidic carboxyl-ends [44]; highly conserved structural motifs; and the presence of arginine and lysine residues to increase the cationic character as reflected in a high pI (~12 in the case of piscidin 1). Special features of piscidins [42,44,48] that make them unique targets

for the development of novel therapeutics include: (1) a tolerance to high salt concentrations [44], which may be useful in the treatment of cystic fibrosis [49]; (2) a high content of histidine, a key amino acid in biomolecules due to its pK_a , which is close to physiological pH; (3) an antiviral activity that remains high at low temperature [42–44]; (4) synergistic effects with hepcidin, an important AMP and hormone peptide.

Solid-state NMR was originally applied to piscidins to demonstrate their α -helical structure and residence on the lipid bilayer surface [50–52]. While it was suggested that large amplitude motions existed in the plane of the bilayer [51], no detailed chemical shift tensor analysis was provided. Moreover, since these results were derived from ^{15}N ssNMR data confined to the Val $_{12}$ site of piscidin 1, it was also unclear whether these “in-plane” motions were restricted to a single site or the entire piscidin molecule and whether they were universally present in the whole piscidin family. Interestingly, a recently published solution NMR structure of piscidin's α -helix in zwitterionic micelles has provided context for the ssNMR dynamic results at position 12 since the solution NMR data pointed at significant N-terminal disorder due to fast dynamics [53,54]. Here, position 20 near the well structured and dynamically ordered C-terminus was labeled with ^{15}N in amidated and non-amidated piscidin 1 and piscidin 3. The results are discussed in terms of their relevance to the antimicrobial function of piscidin and other related AMPs.

2. Materials and methods

2.1. Materials

Isotopically labeled carboxyamidated and non-amidated piscidin 1 and piscidin 3 were synthesized by United Biochemical Research (Seattle, WA) using Fmoc chemistry and solid phase peptide synthesis. After in-house protection following previously established protocols [55–57], ^{15}N labeled amino acids (Cambridge Isotope Laboratories, Andover, MA) were incorporated in the peptides at position 20. Following synthesis, the peptides were cleaved from the resin support and purified on a Waters HPLC system using a Terra C18 column and a 25 minute-gradient of acetonitrile/water with 0.1% 2,2,2-trifluoroacetic acid (TFA). Piscidin 1 and piscidin 3 were collected when the acetonitrile concentration reached approximately 30%. Peptide purity was confirmed by mass spectroscopy performed on a Bruker Esquire Ion Trap spectrometer (University of Washington, Seattle, WA). Only piscidin (with charge states of +2, +3, and +4) was detected in the purified fractions. Singly labeled piscidin 1 and piscidin 3 gave rise to signals corresponding to the expected molecular masses of ~2572 and 2492 Da, respectively. Solvents (e.g.; acetonitrile, chloroform, HPLC grade water, methanol, TFA, and 2,2,2-trifluoroethanol) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Preparation of samples for solid-state NMR

Oriented samples were prepared following a procedure reported previously [50,51]. Briefly, lipid films of 3:1 DMPC/DMPG containing piscidin 1 or piscidin 3 (typically 10 to 12 mg) in a 1:20 peptide to lipid molar ratio were dried under nitrogen gas prior to overnight lyophilization. The peptide–lipid films were hydrated at ~40 °C with a 20 mL-phosphate buffer solution (NaH $_2$ PO $_4$ /Na $_2$ HPO $_4$, 3.5 mmol, pH 6). Following mixing to allow for complete suspension, the binding of the peptide to the lipids was allowed to take place overnight at ~40 °C, above the phase transition temperature of the lipids (~24 °C). Next, the samples were centrifuged for 3.0 h at 4 °C and 46 000 $\times$ g, and the pellet was spread on about 40–50 thin glass slides (dimensions 5.7 $\times$ 12 $\times$ 0.03 mm 3 from Matsunami Trading Co., Japan or 5.7 $\times$ 12.0 $\times$ 0.07 mm 3 from Paul Marienfeld GmbH & Co., Germany). After the samples reached equilibrium in a chamber at a relative

humidity greater than 90% in the presence of a saturated solution of K_2SO_4 , some phosphate buffer solution was added to the slides at a ratio of 1 μ L of buffer per 1 mg of the peptide/lipid mixture. The slides were then stacked and inserted into glass cells (internal dimensions $6 \times 20 \times 4$ mm³, Vitrocom Inc., NJ), which were sealed with beeswax to maintain hydration. The samples were incubated at ~ 40 °C until they appeared homogeneously hydrated. Partial hydration of a piscidin 3 sample was achieved by decreasing the amount of buffer present in the sample. Full hydration of this sample was obtained by adding to the glass cell an amount of phosphate buffer equal by weight to the combined amount of dry peptide and lipids and incubating the sample until equilibrium was reached at ~ 40 °C.

2.3. Solid-state NMR experiments

^{15}N cross-polarization experiments were performed at a resonance frequency of 60.83 MHz on a 600 MHz WB Bruker Avance $\times 3$ NMR spectrometer at the National High Magnetic Field Laboratory (NHMFL, Tallahassee, FL). Standard [58] and low electric-field (E-field) [59] flat coil $^{15}N/^1H$ probes built at the NHMFL were used to obtain ^{15}N chemical shifts from oriented samples. The use of the NHMFL low-E field probe and well-sealed samples offered the advantage of preserving sample authenticity during the ssNMR experiments. The NHMFL probes also provided the capability to easily switch between vertical and horizontal coil assemblies. Experimental parameters included a temperature of 40 ± 0.1 °C unless otherwise indicated, a contact time of 0.8 ms, a cross-polarization field of ~ 45 kHz, a decoupling field of ~ 60 kHz, and a recycle delay of 4–6 s. ^{15}N chemical shifts were referenced to a saturated solution of $^{15}NH_4NO_3$, which resonates at 22.3 ppm with respect to liquid NH_3 . The measurements of error bars are based on the linewidth at half height for oriented samples analyzed in the horizontal sample coil, and the signal to noise ratio and spectral resolution for powder patterns obtained in the vertical sample coil [60].

3. Results

In α -helical peptides such as piscidin 1 and piscidin 3, the ^{15}N chemical shift eigenvectors are conserved with δ_{11} being in the peptide plane and deviating from the N-H bond vector by $\sim 20^\circ$, δ_{33} being in the peptide plane as well, and δ_{22} being orthogonal to the peptide plane [60–63]. Since the N-H bond vector is directed approximately along the helical axis, δ_{11} is pointed approximately along the helical axis of piscidin while δ_{22} and δ_{33} are orthogonal to the helical axis, as shown in Fig. 2. While the magnitudes of the principal components of piscidin's chemical shift tensor can be obtained directly from the peaks and shoulders of the powder pattern spectrum from a randomly oriented sample of piscidin (Fig. 1A, red trace), both the magnitudes and orientation of the ^{15}N chemical shift tensor in the molecular frame of piscidin's α -helix determine the spectral appearance of oriented samples (Fig. 1B). In the specific case of $[^{15}N\text{-Leu}_{20}]$ -p3-COO[−] studied under low hydration conditions at $T = 40$ °C (i.e.; above the phase transition of the lipids) (Fig. 1A, red trace), the spectral simulation for the powder pattern (Fig. 1A, dashed line) yielded $\delta_{11} = 186 \pm 5$ ppm, $\delta_{22} = 56 \pm 5$ ppm, and $\delta_{33} = 40 \pm 5$ ppm, which is consistent with values previously obtained on peptides [60,61,64].

When the correlation time of the dynamic events is much larger than $1/CSA$, where the CSA is expressed in Hertz, and the oriented sample of $[^{15}N\text{-Leu}_{20}]$ -p3-COO[−] is macroscopically aligned so that the bilayer plane normal is along B_0 , δ_{11} is approximately orthogonal to B_0 (the so-called “perpendicular” orientation of the sample, which is obtained for a horizontal sample coil orientation) and thus has approximately zero influence on the observed anisotropic chemical shift frequency. The resonance frequency is then determined by the orientation of δ_{22} and δ_{33} (Fig. 2). For example, if the α -helix of

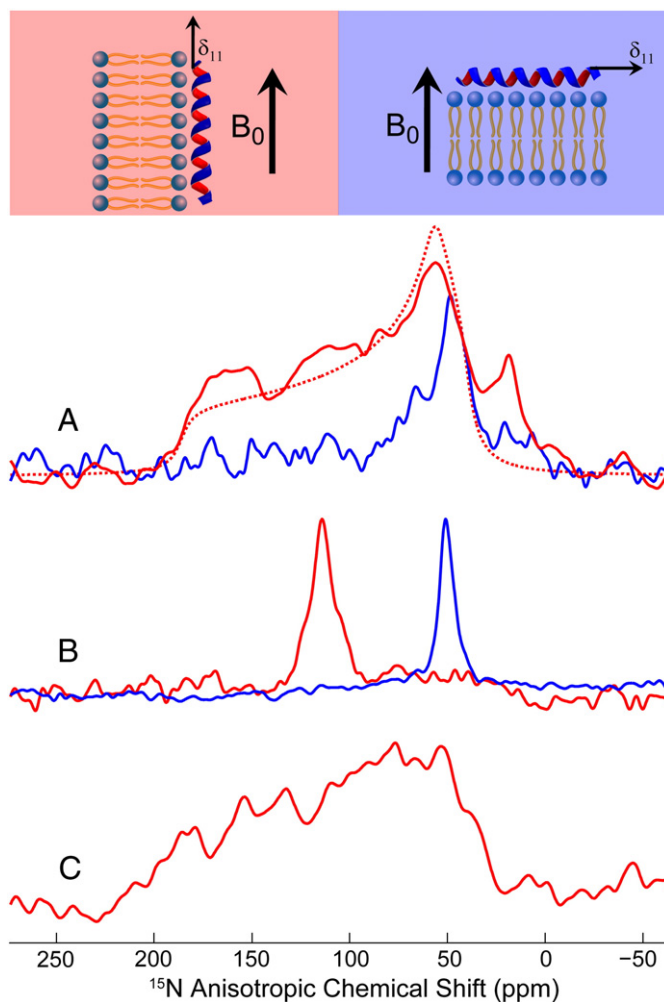


Fig. 1. Proton decoupled ^{15}N spectra of $[^{15}N\text{-Leu}_{20}]$ -p3-COO[−] obtained using the vertical (the so-called parallel orientation of the helix, red trace) and horizontal (the so-called perpendicular orientation of the helix, blue trace) coil orientations with respect to magnetic field B_0 . The spectra were collected under conditions of (A) partial dehydration and 40 °C, (B) full hydration and 40 °C, and (C) high hydration and 20 °C.

piscidin were aligned so that δ_{22} , which is orthogonal to δ_{33} , was pointed along B_0 , the observed resonance frequency would be closer to the value of δ_{22} . This would correspond to the situation when the Leu_{20} peptide plane is parallel to the DMPC bilayer surface. As shown in Fig. 1A, $[^{15}N\text{-Leu}_{20}]$ -p3-COO[−] investigated in the perpendicular orientation at $T = 40$ °C under low hydration conditions gives rise to a relatively broad peak with $\delta_{obs} = 48$ ppm (Fig. 1A, blue trace), which falls between $\delta_{22} = 56 \pm 5$ ppm and $\delta_{33} = 40 \pm 5$ ppm determined above. At first glance, this might be interpreted as indicating that the orientations of δ_{22} and δ_{33} may be unique. However, when this anisotropic sample is studied using high hydration conditions and the same orientation with respect to B_0 (Fig. 1B, blue trace), line narrowing occurs indicating that δ_{22} and δ_{33} are now averaged by a motion about the helical axis. This result also suggests that heterogeneous orientations of δ_{22} and δ_{33} were responsible for the broad line observed under low hydration conditions (Fig. 1A, blue trace). This heterogeneity also explains that a full powder pattern is observed when the low hydration sample of $[^{15}N\text{-Leu}_{20}]$ -p3-COO[−] is tilted by 90° with respect to B_0 (Fig. 1A, red trace, the so-called “parallel” orientation of the sample, which is obtained for a vertical sample coil orientation) and the multiple orientations of δ_{11} , δ_{22} , and δ_{33} with respect to B_0 create the powder pattern expected for a randomly oriented sample.

Under high hydration conditions, the rapid motion of piscidin's α -helix about the lipid bilayer normal is introduced in addition to the

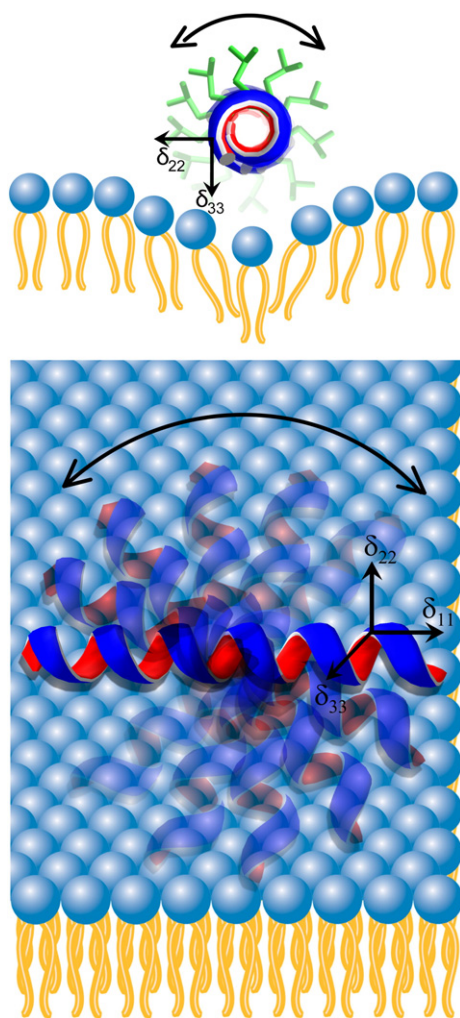


Fig. 2. Diagrams of the α -helical rotations of piscidin about the membrane normal and helical axis, and the effects of these motions on the ^{15}N chemical shift tensor components. As illustrated in the top (side view) and bottom (in-plane view) figures, piscidin experiences motions about two axes, the helical axis of the peptide and the axis along the lipid bilayer normal.

motion about the helical axis. Indeed, when the sample of $[^{15}\text{N}\text{-Leu}_{20}]\text{-p3-COO}^-$ is observed in the parallel orientation of the helix with respect to B_0 , $\delta_{11}=186$ ppm is averaged with $\delta_{\perp}=48$ ppm, the averaged projections of δ_{22} and δ_{33} on the bilayer plane, resulting in $\delta_{\parallel,\text{eff}} = (186 + 48) / 2 = 117 \pm 5$ ppm, which is in excellent agreement with the sharp resonance experimentally observed at 114.5 ± 0.5 ppm (Fig. 1B, red trace; Table 1). While the same motion has very little effect on the linewidth of the spectrum obtained when the sample is oriented with the bilayer normal parallel to B_0 (Fig. 1B, blue trace; Table 1), the motion about the helical axis contributes to the observation of a narrow line. These results indicate that Leu_{20} of

Table 1

^{15}N chemical shift tensors for piscidin 1 and piscidin 3 at $T = 40 \pm 0.2$ °C.

	$\delta_{\perp}$ (ppm)	$\delta_{\parallel,\text{eff}}$ (ppm)	$\delta_{\parallel}$ (ppm)	δ_{iso} (ppm)
$^{15}\text{N-V}_{20}$ p1-COO $^-$	51.4 $\pm$ 0.5	109.4 $\pm$ 0.5	167 $\pm$ 5	90 $\pm$ 5
$^{15}\text{N-V}_{20}$ p1-NH $_2$	51.0 $\pm$ 0.5	107.0 $\pm$ 0.5	163 $\pm$ 5	88 $\pm$ 5
$^{15}\text{N-L}_{20}$ p3-COO $^-$	51.0 $\pm$ 0.5	114.5 $\pm$ 0.5	178 $\pm$ 5	93 $\pm$ 5
$^{15}\text{N-L}_{20}$ p3-NH $_2$	49.5 $\pm$ 0.5	115.5 $\pm$ 0.5	181 $\pm$ 5	93 $\pm$ 5

Referenced to $^{15}\text{NH}_4\text{NO}_3$, which resonates at 22.3 ppm when referenced to $^{15}\text{NH}_3$. p1: piscidin 1; p3: piscidin 3, COO $^-$: free carboxyl end; NH $_2$: carboxyamided end.

Numbers in **bold** are experimental, other numbers are calculated based on experimental values: $\delta_{\parallel}$ is calculated as $(2 \times \delta_{\parallel,\text{eff}} - \delta_{\perp})$; δ_{iso} is calculated as $(\delta_{\parallel} + 2 \times \delta_{\perp}) / 3$.

piscidin 3 undergoes large-scale averaging on a time scale faster than 110 μs , the reciprocal of $\delta_{11} - \delta_{33}$, the tensor span. Interestingly, the bi-axial motion about the bilayer normal and helical axis can be stopped either by using low hydration conditions or decreasing the sample temperature to 20 °C, which is below the phase transition of DMPC (Fig. 1).

Similarly to $[^{15}\text{N}\text{-Leu}_{20}]\text{-p3-COO}^-$, carboxyamided piscidin 3 as well as piscidin 1 with and without carboxyamidation yield high resolution ^{15}N proton decoupled spectra (Fig. 3, Table 1), which are also consistent with the same in-plane large scale motion at position 20, implying that this motion is universal within the piscidin family of AMPs. Combined with the analogous effect observed for Val_{12} in piscidin 1 [51] and the highly ordered α -helical structure of piscidin 1 [51,53,54], our results indicate that this motion is not restricted to a single peptide plane or sub-domain in piscidin 1, but rather is an inherent feature of the entire piscidin α -helix. Neither amidation nor amino acid substitution has any effect on this motion suggesting that this is perhaps a universal feature of antimicrobial amphipathic helices. The universality of the rapid motion in natural AMPs is

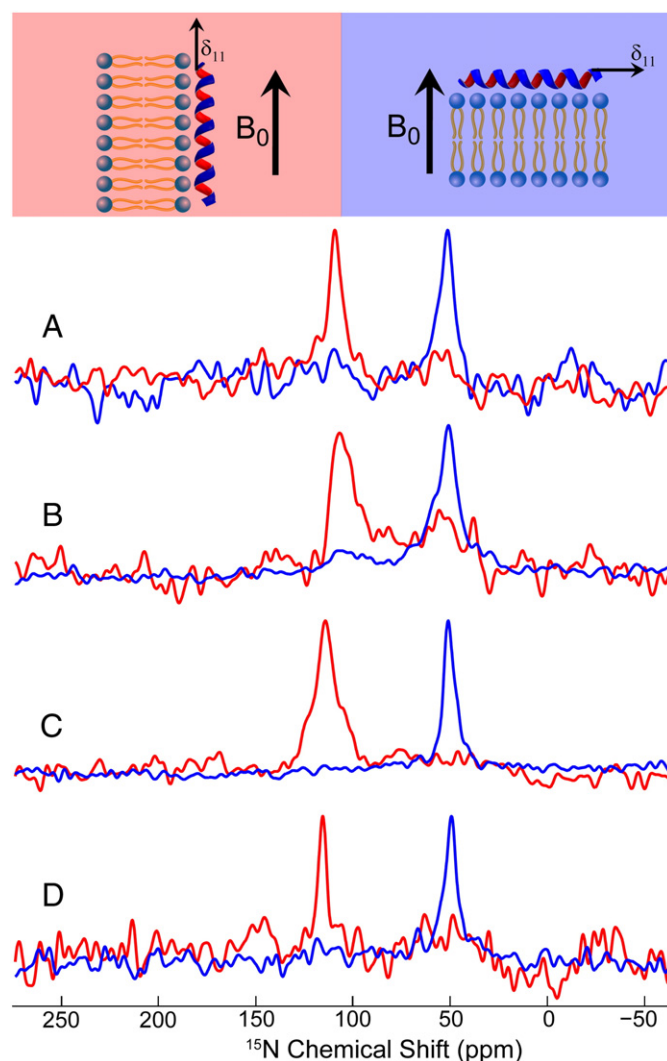


Fig. 3. Proton decoupled ^{15}N spectra of single site ^{15}N labeled piscidin 1 and piscidin 3 acquired with vertical (the so called “parallel” orientation of the helix, red trace) and horizontal (the so called “perpendicular” orientation of the helix, blue trace) sample coil orientations with respect to magnetic field B_0 at full hydration and 40 °C: (A) $[^{15}\text{N}\text{-Val}_{20}]\text{-p1-COO}^-$, $\delta_{\parallel,\text{eff}} = 109.4 \pm 0.5$ ppm, $\delta_{\perp} = 51.4 \pm 0.5$ ppm, (B) $[^{15}\text{N}\text{-Val}_{20}]\text{-p1-NH}_2$, $\delta_{\parallel,\text{eff}} = 107.0 \pm 0.5$ ppm, $\delta_{\perp} = 51.0 \pm 0.5$ ppm, (C) $[^{15}\text{N}\text{-Leu}_{20}]\text{-p3-COO}^-$, $\delta_{\parallel,\text{eff}} = 114.5 \pm 0.5$ ppm, $\delta_{\perp} = 51.0 \pm 0.5$ ppm, and (D) $[^{15}\text{N}\text{-Leu}_{20}]\text{-p3-NH}_2$, $\delta_{\parallel,\text{eff}} = 115.5 \pm 0.5$ ppm, $\delta_{\perp} = 49.5 \pm 0.5$ ppm.

additionally supported by the previous observations of in-plane dynamics in the somewhat shorter antimicrobial peptide ovispirin studied by Yamaguchi and colleagues [65] and the detection of an isotropic type of motion in the peptides from Australian amphibians investigated by Boland and Separovic [66]. Cornell and coworkers used ^{13}C solid-state NMR to characterize the rotation of melittin and gramicidin A in lipid bilayers [67,68] while Aisenbery and Bechinger [69,70] also observed the motional averaging of synthetic peptides in the plane of the bilayer. In addition, Ramamoorthy and coworkers discussed the effect of motion on the mechanism of membrane disruption of several peptides including pardaxin, LL-37, magainin, and derivatives of magainin-2 and melittin [71–74].

4. Discussion

Thanks to new experimental and computational advances, the exploration of protein dynamics has stimulated substantial interest. The significance of this pursuit is multifaceted. It is well accepted that the internal motions of proteins have strong influence on their function. Furthermore, there is increasing awareness that as we characterize the conformational and dynamic substates of peptides and proteins, a better understanding of their functional properties will be obtained [75]. Here ^{15}N chemical shift tensors were used to investigate fast in-plane motions in the piscidin family of AMPs. With the prior knowledge that piscidin is α -helical and oriented parallel to the bilayer surface, the observation of averaged individual tensor components has provided us with the basis to characterize fast motions about both the bilayer normal and helical axis of the peptide. It is interesting to note that Kuttner and colleagues [76] recently discussed the role of translational and rotational diffusion in many aspects of intracellular and multicellular processes that rely on macromolecular interactions. These motions, which share some similarity with piscidin's fast dynamics, facilitate the search for partners within the bilayers as needed for the successful association of complexes that are biologically active.

In addition to the dynamic investigation presented here, the detailed analysis of the averaging process that takes place in piscidin should also be sufficient to delineate the peptide plane orientation with respect to the membrane plane normal. Unfortunately, a resolution of 5 ppm typical for randomly oriented samples and $(\delta_{22}-\delta_{33}) < 15$ ppm or less for ^{15}N amide nitrogens in α -helices [60] would impose large experimental errors and make these measurements impractical. At high magnetic fields, ^{17}O chemical shift tensors that have orientations similar to the one of the ^{15}N amide site shown in Fig. 2 should be of more practical importance since $(\delta_{22}-\delta_{33}) > 400$ ppm [77]. High field studies of the ^{17}O chemical shift tensors of piscidins would also facilitate the assessment of the same molecular motion on a time scale of 0.02 ms rather than the 0.11 ms scale presented here. The outcome of integrating peptide dynamic and structural data is that a higher resolution description of both dynamics and structure can be achieved, which bodes well in terms of enhancing our understanding structure–function relationships in important biomolecules such as piscidins and other AMPs.

Our results support the concept that water activates protein motions [78–80]. Here low hydration as well as low temperature eliminate the fast motions of piscidin. Conspicuously, water also affects the structure, order, and dynamics of the surrounding membrane components. Therefore, while biological membranes are controlled by many complex factors, including membrane composition, hydration, pH, ionic strength, and temperature, water is a versatile agent that couples the components and enables fluctuations, cooperativity, and correlated dynamics such that local order [81,82] and possibly the formation of molecular complexes may be achieved out of disorder. Interestingly, it has been noted that the numerous conformational substates accessed by hydrated proteins provide them with a “reservoir of entropy” that is fundamental to enabling their

function [78]. Here, the entropy generated by the fast dynamics of piscidin may provide some of the energy needed by the peptide to perform its function of disrupting bilayers and killing bacterial cells.

Since the rather unusual bi-axial “in-plane” dynamics presented here for piscidins is largely lateral diffusion of the amphipathic helix along the membrane surface, it is interesting to estimate the role that this motion plays in the biological function of these peptides. A simple calculation using a ~ 3.3 nm α -helical length and the reciprocal of 110 μs (i.e.; the upper limit of the correlation time) suggests that piscidin can travel a trajectory equivalent to more than 30 μm per second; if this is the case, the circumference of a single bacterial cell could be traveled in a fraction of a second. From the perspective of the kinetics of cellular processes, piscidins and perhaps many other amphipathic antimicrobial peptides active at lipid bilayers may represent a class of fast scavengers, rather than static polypeptides attached to the water–membrane interface. Being an active and integral part of the mechanism that removes bacteria from a host organism, these peptides may rely on their high mobility to search for their targets and perform their function of contributing to the immune system's first line of defense against pathogens. This knowledge may benefit the wide range of research currently conducted on amphipathic α -helical peptides. Notably, the approach described here to assess fast peptide dynamics and correlate it to biological function could be extended to other host-defense peptides.

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