Amphipathic Antimicrobial Piscidin in Magnetically Aligned Lipid Bilayers

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ABSTRACT The amphipathic antimicrobial peptide piscidin 1 was studied in magnetically aligned phospholipid bilayers by oriented-sample solid-state NMR spectroscopy. ^{31}P NMR and double-resonance ^{1}H/^{15}N NMR experiments performed between 25°C and 61°C enabled the lipid headgroups as well as the peptide amide sites to be monitored over a range of temperatures. The α-helical peptide dramatically affects the phase behavior and structure of anionic bilayers but not those of zwitterionic bilayers. Piscidin 1 stabilizes anionic bilayers, which remain well aligned up to 61°C when piscidin 1 is on the membrane surface. Two-dimensional separated-local-field experiments show that the tilt angle of the peptide is 80 $\pm$ 5°, in agreement with previous results on mechanically aligned bilayers. The peptide undergoes fast rotational diffusion about the bilayer normal under these conditions, and these studies demonstrate that magnetically aligned bilayers are well suited for structural studies of amphipathic peptides.

INTRODUCTION

Piscidins constitute a family of highly potent amphipathic, cationic, AMPs found in many fish species (1,2). Several studies have documented the efficacy of piscidins against a broad range of pathogens, including multidrug-resistant bacteria, fungi, and viruses such as HIV-1 (2–5). As membrane-active peptides, AMPs can damage or destabilize microbial membranes (6,7), and they are believed to have important immunomodulatory and metabolic effects as well (8,9). AMPs with cancer-selective toxicity have also been described (10). Since the resistance mechanisms found for traditional antibiotics have not been observed with AMPs, these peptides have been subjected to extensive study in the search for new antibiotics with rapid broad-spectrum antimicrobial activity and reduced cytotoxicity (8). More recently, their ability to orchestrate innate immune and inflammatory responses has motivated their use as adjuvants for vaccines and other antimicrobial therapies (9). Important physicochemical properties shared by members of the AMP family of peptides include a net positive charge at physiological pH; a high isoelectric point that facilitates interactions with negatively charged bacterial membranes; significant hydrophobicity that enables partial or complete insertion into lipid bilayers; structural flexibility to convert their predominantly unstructured conformation in aqueous environment to a structured, biologically active conformation (e.g., α-helical, β-sheet) upon membrane-binding (6,11). However, gaps remain in our understanding of the physicochemical characteristics that affect the structure and dynamics of AMPs, the dependence of their efficacy on membrane composition, and their mechanisms of action. Piscidins occur with both amidated and acidic carboxyl termini and display highly conserved structural motifs that include a high density of positively charged residues such as arginine and lysine. The diversity of primary structures in the piscidin family may reflect adaptation to microbes found in various environments and/or defense against evolving microbes (12). It is worth noting that although both piscidin 1 and piscidin 3 are highly homologous, piscidin 1 has significantly stronger antimicrobial and hemolytic activities (1,4). Unique features of piscidins that make them conducive to studies designed to uncover principles important to the design of new therapeutics include their tolerance to high salt concentrations, which is a critical consideration in the treatment of cystic fibrosis patients; a high content of histidine, a residue with a side-chain pKa close to physiological pH; synergistic effects with hepcidin, an important amphipathic cationic antimicrobial and hormone peptide in fish; and antiviral activity (1,2).

Piscidins, like many AMPs, are lytic, i.e., they act by permeabilizing the cell membranes of their targets (6,11,13,14). Molecular recognition and bilayer disruption by lytic AMPs are initiated at the membrane-water interface. Few studies have documented the high-resolution structure, dynamics, and topology of these peptides as a function of the phase behavior and mobility of lipid bilayers (4,15–18). Regarding piscidin, the α-helical character of the membrane-bound peptide and its preferential binding to anionic lipids have been demonstrated by CD (4), whereas its membrane-active properties have been studied using dye-leakage and ion-conductance experiments (3,19). However, the structures of piscidin 1 determined by solution NMR in sodium dodecyl

Submitted December 3, 2010, and accepted for publication July 1, 2011.

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Abbreviations used: AMP, antimicrobial peptide; CD, circular dichroism; OS, oriented sample; 6-O-PC, 1,2-di-O-hexyl-*sn*-glycero-3-phosphocholine; 14-O-PC, 1,2-di-O-tetradecyl-*sn*-glycero-3-phosphocholine; p1, piscidin 1; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt); DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; SLF, separated local field; PISA, polarity index slant angle.

Editor: Paulo F. Almeida.

0006-3495/11/09/1086/9 $2.00

doi: 10.1016/j.bpj.2011.07.015

sulfate (3) and dodecyl phosphocholine (19) micelles differ significantly, which demonstrates the importance of lipid environment in determining peptide structure. To characterize the events pertinent to peptide specificity and efficacy, it is essential to utilize high-resolution methods under native conditions. OS solid-state NMR offers unique advantages to investigate local structure and motions, and to describe the nature of peptide-lipid interactions under varying physiologically relevant conditions (7,20,21). A variety of hydrated preparations of peptides and anionic lipids mechanically aligned on glass plates and studied by OS solid-state NMR have been reported using AMPs such as the α-helical magainin (22,23), aurein (15), pardaxin (18), and piscidin (4,24–26). In previous studies aimed at reproducing native-like conditions, we used OS solid-state NMR to demonstrate that the 22-residue peptide piscidin 1 (FFHHIFRGIVHVGK TIHRLVTG-NH_2) can be refolded in phospholipid bilayers aligned on glass plates (4,24,26). We studied the peptide in bilayers consisting of 3:1 DMPC/DMPG in the liquid crystalline state ($T > 23^\circ C$) with a lipid/peptide molar ratio of 20:1. These studies established that piscidin 1 is α-helical and bound on the membrane surface with a tilt angle of ~86° with respect to the bilayer normal (26). The one-dimensional ^{15}N NMR spectra of piscidin 1 in mechanically aligned bilayers with their normal perpendicular and parallel to the magnetic field demonstrate that the peptide in anionic bilayers undergoes rotational diffusion at a rate of $>10^4$ s^{-1} at 40°C. However, stable sample conditions are not easily maintained in mechanically aligned bilayers for extended periods of time. Although the presence of negatively charged lipids is assumed to be necessary for interaction at the bilayer interface, differences between the alignment of piscidin in zwitterionic and anionic bilayers have not been described in detail, mostly because of the experimental difficulty of obtaining aligned high-resolution ^{15}N spectra in zwitterionic bilayers.

Magnetically aligned bilayers are underrepresented in the study of AMPs, despite the fact that they offer several advantages for OS solid-state NMR studies, the most important feature perhaps being that the experiments can be performed with the peptides in a functional state in fully hydrated lipid bilayers under physiological conditions (21,27–29). Magnetically aligned bicelles are prepared by mixing short- and long-chain phospholipids in water or a buffer solution (28,29); samples for solid-state NMR are typically composed of 20–30% water by weight. The pH, salt concentrations, water content, ionic strength, and temperature can be measured and varied, and hydrogen/deuterium exchange is readily controlled. Peptide-containing magnetically aligned bilayer samples are readily prepared, stable for long periods of time (months), and highly reproducible. The membrane-associated peptides or proteins can be aligned by this method to an extent superior to that obtainable by mechanical alignment between glass plates (29). Furthermore, the range of lipid combinations that can be magnetically aligned is steadily increasing (29–31). Bilayers can be aligned either with the bilayer normal perpendicular to the field, or in the parallel orientation with use of lanthanide ions or biphenyl lipids to flip the bilayers (27,31). Ether-linked phospholipids can be utilized to obtain greater stability over a wide range of sample conditions (29). Detergents such as the relatively mild Triton X-100 (30), CHAPSO (27), or short-chain phospholipids (27) can be used to cap the bilayer edges in the magnetically alignable bilayer samples. Fast rotational diffusion of the peptide about the bilayer normal compared to the timescale of the NMR interactions (10^4 s^{-1}) is a necessary condition for obtaining high-resolution ^{15}N spectra (line widths ~1.5 ppm) of proteins inserted in magnetically aligned lipid bilayers with the bilayer normal perpendicular to the static magnetic field. Therefore, comparisons of experimental spectra of aligned ^{15}N NMR spectra of the peptide in perpendicular bicelles and simulations make it possible to estimate the lower limit of the rotation frequency (32). To our knowledge, there are no structures of amphipathic peptides reported in magnetically aligned bilayers. Thus, our purpose here is not only to complement previous studies of carboxyamidated piscidin 1, but also to demonstrate the feasibility of using magnetically aligned bilayers for structural studies of amphipatic peptides in general. We characterize the oriented lipid phase and the effect of piscidin-lipid interactions as a function of temperature with one-dimensional ^{31}P NMR experiments. The α-helical character, orientation, and dynamics of piscidin 1 are characterized by one-dimensional ^{15}N NMR and two-dimensional $^{1}H/^{15}N$ NMR experiments on selectively ^{15}N-backbone labeled carboxyamidated piscidin 1 in magnetically aligned bilayers. Results obtained on zwitterionic and anionic bilayers are compared over the temperature range between 25°C and 61°C.

MATERIALS AND METHODS

Sample preparation

The preparation of selectively ^{15}N-labeled, carboxyamidated piscidin 1 samples, including F2I5G8-p1-NH_2, V10G13I16-p1-NH_2, F6V12-p1-NH_2, and I5F6G8I9V10V12G13I16L19V20-p1-NH_2, has been previously described (4). Preformed bicelles at pH 7.0 were added directly to the pure, ^{15}N-labeled lyophilized peptide (for details, see Supporting Material). Two types of samples were prepared with lipid compositions (expressed as molar ratios) of 2.6:0.6:1.0 14-O-PC/DMPG/6-O-PC and 3.2:1.0 14-O-PC/6-O-PC. The final lipid/protein molar ratio ranged from ~40:1 to 20:1 for samples containing 4–7 mg of peptide. For each type of sample, NMR spectra within these concentration ranges appeared identical, with no dependence on the amount of peptide. Within 30 min, all the peptide had dissolved and the final pH of the bicelle-piscidin solution was 6.8. In this study, buffers were not added to the sample, but the pH of the samples was monitored closely and found to be stable within a range of 0.2 units.

NMR spectroscopy

The $^{1}H/^{15}N$ solid-state NMR experiments were performed at University of California San Diego (La Jolla, CA) on Bruker (Billerica, MA) Avance

spectrometers with ^{1}H resonance frequencies of 500 MHz, 700 MHz, and 750 MHz. Details of the NMR hardware and experiments are described in the Supporting Material. All samples were first equilibrated in the magnetic field for 24 h, and then equilibrated at each temperature for a period of >30 min before the initiation of NMR measurements. For each sample, the ^{31}P NMR experiments were repeated and the temperature cycled at least three times, to verify reproducibility and ensure that the spectra were reversible with temperature.

Simulations

^{1}H/^{15}N SLF spectra of helical membrane proteins exhibit characteristic circular patterns of resonances called PISA wheels that correspond to helical wheel projections of periodic structures such as helices or β-sheets and serve as indices of secondary structure and topology (20,21). The helical slant (tilt) angle of the peptide in magnetically aligned bilayers was estimated from the experimental spectra by comparison with PISA-wheel simulations calculated using the ^{15}N and ^{1}H chemical shift anisotropy tensors of an ideal α-helix at varying tilt angles (see Fig. 3 *C*). The wheels were calculated using principal values of the ^{15}N chemical shift tensor ($\sigma_{11} = 64$ ppm, $\sigma_{22} = 77$ ppm, and $\sigma_{33} = 222$ ppm (for nonglycine residues)) and uniform dihedral angles ($\Phi = -61°$, $\Psi = -45°$). A bilayer order parameter, S, of ~0.85, was consistent with the breadth and position of the experimental resonances, and in good agreement with previous studies of peptides and proteins (33).

The effect of rotational diffusion rates on oriented peptide spectra in perpendicular bicelles has been described in detail by Nevzorov et al. and implemented as a MATLAB (www.mathworks.com) script (32). Briefly, the numerical simulation program applies the Stochastic Liouville Equation (34) to couple the quantum spin transitions to diffusional reorientations. The numerical simulation was applied here assuming an 18-residue α-helical peptide at a tilt angle of 80° and a bicelle order parameter of S = 0.85. Three different values of rotational diffusion coefficient were chosen ($D_{\|} = 3 \times 10^5\ s^{-1}$, $10 \times 10^5\ s^{-1}$, and $20 \times 10^5\ s^{-1}$) to obtain simulated spectra with line widths similar to the experimental results.

OS solid-state NMR spectra in bilayers aligned at different orientations in the static magnetic field are, in principle, equivalent for identical compositions. Different alignment media can be readily compared by taking into account the overall alignment of the bilayer normal. When the direction of alignment of the bilayer normal is changed by 90°, the observed frequency for each resonance flips around its isotropic frequency, δ_{iso}, and is scaled by a factor of 2. As previously described (35), the relationship between the resonance frequency observed in perpendicular bilayers ($\delta_{\perp}$) and that observed in parallel bilayers ($\delta_{\|}$) is given in Eq. 1 as

$$\delta^{i}_{\|} - \delta^{i}_{iso} = -2\left(\delta^{i}_{\perp} - \delta^{i}_{iso}\right)\left(\frac{S_{\|}}{S_{\perp}}\right), \qquad (1)$$

where S denotes the order parameter of the bilayers in either the perpendicular or parallel alignment. The isotropic chemical shift frequencies can be set approximately equal to their average solution values, i.e., 109 ppm for glycine residues and 121 ppm for all other amino acids. Therefore, from Eq. 1, the ^{15}N chemical shift and ^{1}H/^{15}N dipolar coupling frequencies in SLF spectra obtained in parallel bilayers on glass plates ($S = 1$) can be used to simulate numerically a spectrum in perpendicular bicelles ($S = 0.85$) to facilitate the comparison of results obtained in mechanically oriented bilayers with those in magnetically aligned bilayers.

RESULTS

To investigate the effect of lipid composition on the insertion of piscidin 1 into bilayers that mimic the surfaces of zwitterionic mammalian and negatively charged bacterial membranes, peptide-containing bilayer samples were prepared using either exclusively zwitterionic lipids (3.2:1.0 14-O-PC/6-O-PC mol/mol), or 80:20 14-O-PC/DMPG anionic lipids (2.6:0.6:1.0 14-O-PC/DMPG/6-O-PC mol/mol). The peptide/lipid ratio of 1:20 is consistent with the high concentrations under which the antimicrobial activity and membrane disruption of AMPs occur (36). Control experiments were performed on the same lipid preparation in the absence of peptide. These bilayers align with their normal perpendicular to the magnetic field (29). We monitored the lipid phase, alignment, and dynamics in the presence of piscidin 1 over a range of temperatures by ^{31}P NMR and ^{15}N/^{1}H solid-state NMR. ^{31}P NMR experiments performed on oriented phospholipids were used to monitor the lipid phases present in the sample, the bilayer alignment, and any peptide-induced perturbations of the bilayer (22). ^{15}N chemical shift and ^{15}N/^{1}H dipolar coupling frequencies from SAMPI-4 SLF spectra of ^{15}N-amide-labeled peptide provide information on its topology and structure (7,20,21). These spectra also provide an estimate of the overall rotational diffusion dynamics of the peptide. Membrane proteins uniaxially aligned in lipid bilayers with their normal perpendicular to the static magnetic field yield a single resonance for each amide ^{15}N-^{1}H bond and well-resolved PISA wheel patterns when the proteins are immobilized except for the fast rotation. In perpendicular bilayers spectral line widths are dependent on, among other factors, the overall rotational diffusion rate for a specific peptide orientation, and ^{15}N and ^{15}N-^{1}H spectra can be compared with numerical simulations for an estimate of the rotational diffusion coefficients around the bilayer normal. The presence of fast rotational diffusion around the axis of the α-helix would cause the PISA wheels to collapse into a single frequency, approximately at the center of the ideal wheel in the absence of fast axial motion; therefore, the SLF spectrum would indicate if this type of motion were present.

After adding ^{15}N-labeled peptide to the anionic and zwitterionic preformed bilayers, the sample temperature was changed in a stepwise fashion. Fig. 1 shows ^{31}P NMR spectra of anionic bilayers in the absence of peptide (*left*), and in the presence of ^{15}N-[F2I5G8]-p1-NH_2 (*right*). As expected, anionic bilayers without peptide display optimal alignment at 34°C, which is reversibly lost at temperatures >48°C, where the miscibility of the short-chain phospholipid, 6-O-PC, in the long-chain phospholipid, 14-O-PC, induces the formation of nonorienting lipid structures (37). Peptide-free anionic bicelles at 61°C display a predominantly isotropic phase, as indicated by the presence of a narrow, intense ^{31}P NMR resonance at ~0 ppm, which is ~34% of the total ^{31}P signal. Upon addition of piscidin 1 to anionic bilayers, a strikingly different situation is observed. The addition of the peptide affects the ^{31}P NMR signals from the lipids at all temperatures between 25°C and 61°C. At low temperature, a complex phase behavior, with coexistence of broad signals underneath sharper

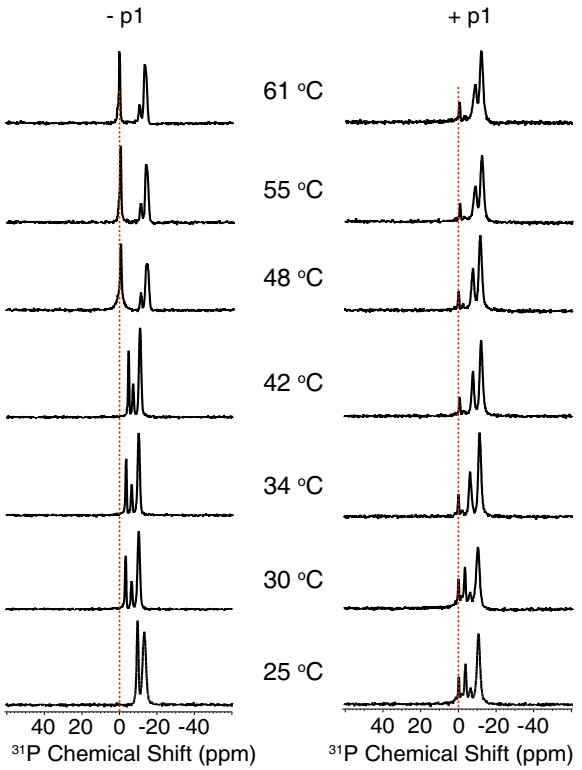

FIGURE 1 One-dimensional ^{31}P NMR spectra as a function of temperature of anionic magnetically aligned bilayers with molar composition 2.6:0.6:1.0 14-O-PC/DMPG/6-O-PC in the absence (−p1) and presence (+p1) of piscidin 1 (*left* and *right*, respectively). As marked, the sample is equilibrated at 25°C, 30°C, 34°C, 42°C, 48°C, 55°C, and 61°C (*bottom to top*). The ^{31}P NMR isotropic chemical-shift frequency (0 ppm) is marked by the vertical dotted lines.

resonances, is observed. A well-aligned lipid phase is present between 34°C and 61°C, the peak at −13 ppm being consistent with the presence of a liquid-crystalline bicelle phase aligned perpendicular to the magnetic field (37). This resonance is noticeably broad, probably reflecting the interactions of the peptide with the headgroups of the phospholipids. In the presence of piscidin, the anionic 14-O-PC/DMPG/6-O-PC bilayers remain well aligned at 61°C. The large isotropic resonance observed at high temperatures is reduced by 80% upon addition of piscidin 1. Therefore, the peptide extends the temperature range for which the bicelles remain aligned. Repetition of the temperature cycling for each sample showed that these results were reversible as a function of temperature. The orientation of anionic bilayers is always thermally stabilized by the presence of piscidin. A small ^{31}P NMR isotropic peak, ~6% of the total ^{31}P intensity, was observed at all temperatures when piscidin 1 was present in the samples. This signal is consistent with disruption, specifically the existence of mobile phospholipids, such as those found in micelles or small unilamellar vesicles (28,37). It is interesting that careful comparisons with control samples confirmed that this signal appears only after the samples containing piscidin 1 have been in the magnetic field >24 h, after which time the intensity is stable and spectra are highly reproducible. This demonstrates that in the presence of piscidin 1, the phospholipid bilayer phase behavior is altered and has a complex dependence on lipid composition.

The interaction of the peptide with the lipid headgroups is also reflected in the ^{15}N NMR signals arising from the ^{15}N-labeled samples. Fig. 2 shows one-dimensional ^{15}N NMR (*left*) and ^{31}P NMR (*right*) chemical-shift spectra of samples containing ^{15}N-(F2I5G8)-p1-NH_2 in aligned bilayers at temperatures between 34°C and 61°C. The ^{31}P NMR spectra provide evidence of the same interactions between the peptide and the negatively charged lipids as observed for the sample in Fig. 1. ^{15}N NMR signals from the labeled peptide are generated by ^{1}H-^{15}N cross-polarization. At low temperatures, the sensitivity is poor, and signals from the three ^{15}N-labeled sites are broad, unresolved, and shifted toward the isotropic frequency (~120 ppm). In contrast, the ^{15}N NMR signals are fully resolved and intense at the relatively high temperature of 61°C. As the temperature is raised, a marked increase in sensitivity and improvement in resolution (~1.5 ppm line widths) is observed along with the significant changes of the chemical shifts that converge toward values associated with an in-plane helix orientation. Two-dimensional SLF spectra were obtained at the higher temperatures, where a single narrow resonance for each labeled ^{15}N-^{1}H bond is observed in ^{15}N chemical-shift spectra.

Fig. 3 displays two-dimensional ^{1}H/^{15}N SLF spectra obtained at 61°C from three selectively ^{15}N-labeled peptide samples: ^{15}N-(F2I5G8)-p1-NH_2 (Fig. 3 *A*), ^{15}N-(V10G13I16)-p1-NH_2 (Fig. 3 *B*), and a piscidin 1 sample labeled at

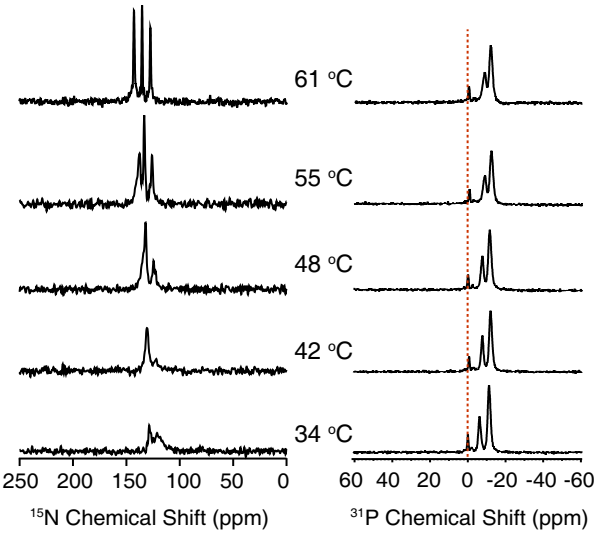

FIGURE 2 One-dimensional ^{15}N NMR (*left*) and ^{31}P NMR spectra (*right*) of ^{15}N-(F2I5G8)-p1-NH_2 in anionic magnetically aligned bilayers with molar composition 2.6:0.6:1.0 14-O-PC/DMPG/6-O-PC, equilibrated at 34°C, 42°C, 48°C, 55°C, and 61°C (*bottom to top*). The ^{31}P NMR isotropic chemical-shift frequency (0 ppm) is marked by the vertical dotted line.

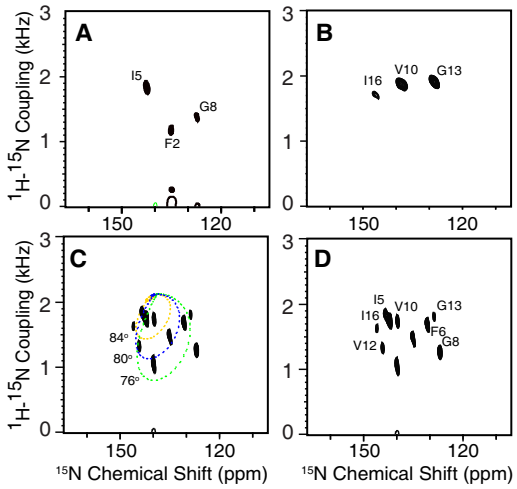

FIGURE 3 Two-dimensional ^{15}N/^{1}H SLF spectra obtained with the SAMPI-4 pulse sequence. (*A*) ^{15}N-(F2I5G8)-p1-NH$_2$. (*B*) ^{15}N-(V10G13I16)-p1-NH$_2$. (*C*) PISA-wheel simulations obtained using the ^{15}N and ^{1}H chemical shift anisotropy tensors of an ideal α-helix that has a tilt angle of 76°, 80°, and 84° with respect to the bilayer normal. The standard order parameter of S = 0.85 is incorporated into the simulations. (*D*) ^{15}N-(I5F6G8I9V10V12G13I16L19V20)-p1-NH$_2$ in anionic 2.6:0.6:1.0 14-O-PC/DMPG/6-O-PC perpendicular bicelles at 61°C. Spectra from ^{15}N-(F2I5G8)-p1-NH$_2$, ^{15}N-(V10G13I16)-p1-NH$_2$, and ^{15}N-(F6V12)-p1-NH$_2$ were used to confirm the bicelle assignments of this sample.

10 amino acids, ^{15}N-(I5F6G8I9V10V12G13I16L19V20)-p1-NH$_2$ (Fig. 3, *C* and *D*). There is a single peak with a ^{1}H-^{15}N heteronuclear dipolar coupling between 1 kHz and 2 kHz for each labeled site, and the resonances fall in the characteristic PISA wheel pattern for tilted helical peptides. Fig. 3 *C* overlays the spectrum for the peptide labeled with ^{15}N in 10 sites and PISA wheels calculated for ideal α-helices (without glycines) at 84°, 80°, and 76° tilt angles, confirming the presence of a bound, structured peptide conformation with a tilt angle of ~80° with respect to the bilayer normal. These spectra confirm that at 61°C, both the phospholipids and the peptide are aligned, and that the peptide undergoes fast rotational diffusion about the bilayer normal (25). Fast rotational motion around the long axis of the α-helix is not observed. Comparison of the spectra in Fig. 3, *A* and *D*, assigns the resonance of F2, which provides a starting point for other resonance assignments using PISA wheels (35). The other assigned resonances are marked in Fig. 3.

Fig. 4 shows one-dimensional chemical-shift NMR spectra obtained from a sample of magnetically aligned 14-O-PC/6-O-PC bilayers. Significantly different lipid behavior is observed in zwitterionic samples upon addition of piscidin 1. The peptide does not appear to alter the bilayer alignment at any temperature. Fig. 4 shows ^{15}N (*left*) and ^{31}P (*right*) NMR chemical-shift spectra of a sample containing ^{15}N-(F2I5G8)-p1-NH$_2$ in aligned zwitterionic bilayers acquired at 42°C and 55°C. The ^{31}P NMR signals are the same as those published throughout the literature for these

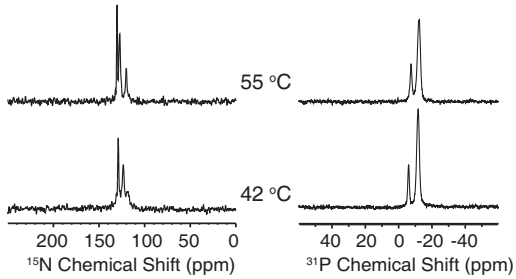

FIGURE 4 One-dimensional ^{15}N NMR (*left*) and ^{31}P NMR (*right*) of ^{15}N-(F2I5G8)-p1 NH$_2$ in zwitterionic magnetically aligned bilayers with molar composition 3.2:1.0 14-O-PC/6-O-PC, acquired at 42°C and 55°C, as designated.

types of bicelles. The ^{31}P NMR signals at −12 ppm (high intensity) and −5 ppm (low intensity) have the expected 3:1 intensity ratio, and no isotropic intensity is observed even after repeated annealing of the samples in the magnet for several days. These spectra can be obtained at temperatures as low as 34°C, and they indicate that the lipids are unperturbed in their structure and alignment by the presence of piscidin 1. The ^{15}N spectra of ^{15}N-(F2I5G8)-p1-NH$_2$ in zwitterionic bilayers show three peaks, as would be expected for an aligned peptide interacting specifically with the lipid bilayer. These spectra exclude the possibilities of isotropic motion of the peptide, such as that seen in micelles or small isotropic bicelles, and of random orientation of the peptide on the bilayer, which would generate a powder pattern. With zwitterionic lipids, in contrast to anionic lipids, the same qualitative behavior as a function of increasing temperature is observed, with sensitivity and resolution increasing at higher temperatures. However, the ^{15}N chemical shifts remain close to the isotropic values (~120 ppm) and are not consistent with an in-plane helix. This is confirmed by results from two-dimensional SLF experiments (Fig. 5), which indicate that the magnitude of the ^{15}N-^{1}H dipolar couplings observed in zwitterionic lipids is very small (<1 kHz). Calculated PISA wheels corresponding to an α-helix with tilt angles of 55° and 60° are drawn below the SLF spectra (Fig. 5 *B*) for comparison. These values of the ^{15}N chemical shifts frequencies and the small ^{15}N-^{1}H dipolar couplings would also be expected if the peptide experienced molecular motions comparable to those seen on the NMR timescale, for example, if piscidin 1 was not anchored to the bilayer but was either partially mobile or in equilibrium between bound and unbound states at the bilayer interface.

Despite the significant differences in the lipid-peptide interactions and peptide orientation for zwitterionic and anionic bilayers, the same qualitative behavior as a function of increasing temperature is observed, i.e., sensitivity and resolution increase at higher temperatures, although this behavior is less marked in zwitterionic bilayers. A possible explanation would be a higher rate of rotational diffusion of the peptide around the bilayer normal at higher

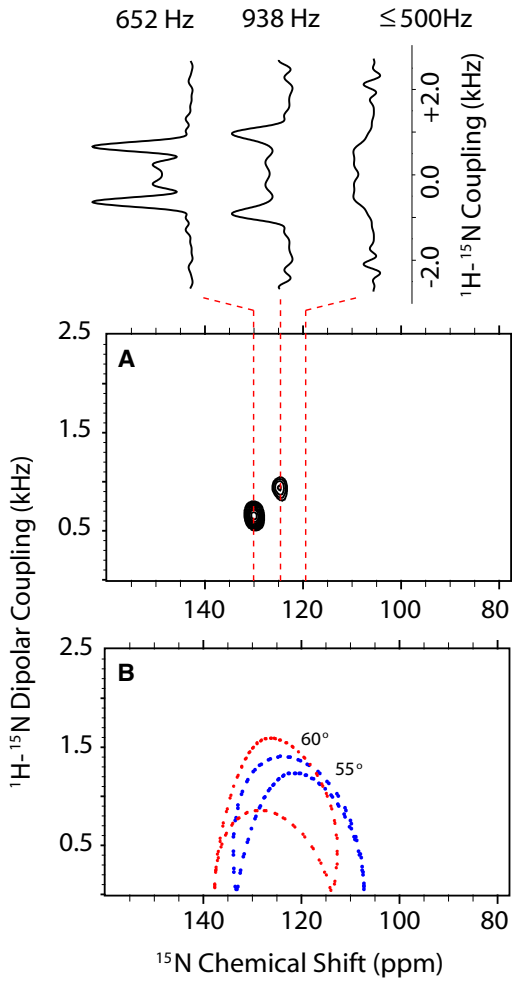

FIGURE 5 (*A*) Two-dimensional ^{15}N/^{1}H SLF SAMPI-4 spectra of ^{15}N-(F2I5G8)-p1-NH_2 in zwitterionic 3.2:1.0 14-O-PC/6-O-PC perpendicular bicelles at 55°C. A third broad, weak resonance with a ^{1}H-^{15}N dipolar coupling of ~500 Hz is visible only at lower contour levels. Slices in the dipolar dimension corresponding to the three observable resonances marked by dotted lines are shown at the top. (*B*) PISA-wheel simulations of an ideal α-helix with tilt angles of 55° (*blue*) and 60° (*red*) with respect to the bilayer normal.

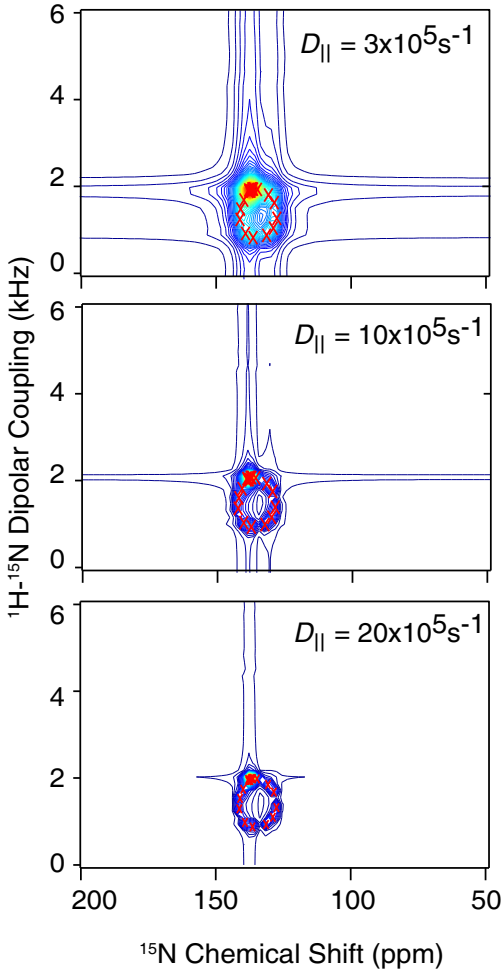

FIGURE 6 Simulations of the effect of uniaxial motional averaging around the bilayer normal on ^{15}N/^{1}H SLF spectra of a uniformly ^{15}N-labeled in-plane 18-residue α-helix with a tilt of 80° with respect to the bilayer normal in perpendicular bicelles, at rotational diffusion rates of: 3×10^5 s^{-1}, 10×10^5 s^{-1}, and 20×10^5 s^{-1} (*top* to *bottom*). Red crosses show the predicted positions of the resonances.

temperatures. We conducted numerical simulations of SLF spectra in magnetically aligned bilayers with an order parameter of S = 0.85 for a peptide with an in-plane orientation of 80°. Fig. 6 shows calculated SLF spectra obtained for increasing values of the rotational diffusion coefficient. These simulations indicate that to obtain resolved PISA wheel patterns in this peptide orientation, the rotational diffusion needs to be higher than that predicted for a transmembrane α-helix. This would at least in part explain why in aligned bilayers the sharpest line widths are always obtained at the highest temperature sampled. Molecular dynamics simulations that take into account other slow motions (38) may give a more comprehensive description of the system at low temperatures and in zwitterionic bilayers.

DISCUSSION

Piscidin 1 was studied in magnetically aligned lipid bilayers and characterized by one- and two-dimensional OS solid-state NMR experiments. Piscidin 1 was aligned in the presence of magnetically oriented zwitterionic and anionic phospholipid bilayers, and significant differences were observed between the two bilayer types with respect to the peptide's effects on their phase behavior. The peptide orientation and rotational dynamics were also characterized by NMR experiments.

A conclusion from these studies is that piscidin 1 interacts very differently with zwitterionic (PC) and anionic (PC/PG) phospholipid bilayers. ^{31}P NMR spectra show that the orientation and phase behavior of zwitterionic bilayers are not altered significantly in the presence of the peptide. Using a fully hydrated, magnetically aligned bilayer medium

allowed us to observe the ^{15}N NMR signals arising from the amphipathic ^{15}N-labeled peptide, even when it is not anchored in an in-plane conformation in zwitterionic bilayers. The small dipolar couplings and chemical shifts near ^{15}N isotropic values observed in zwitterionic samples at all temperatures are consistent with an ~57° tilt angle, or indicative of a weak interaction with the peptide in equilibrium between bound and unbound states at the bilayer interface, or both. A small insertion angle in zwitterionic bilayers compared to anionic bilayers would suggest that the peptide may insert more deeply in zwitterionic lipids, which mimic mammalian cells. The low sensitivity observed in the zwitterionic samples is consistent with a weakly bound state or fast exchange dynamics that interfere with efficient ^{1}H-^{15}N cross-polarization.

In contrast, in negatively charged bilayers, which are better mimics of bacterial cell membranes, the ^{31}P NMR spectra show marked differences in the presence and absence of piscidin 1. Without peptide, the temperature window for the existence of stable anionic bilayers that are aligned is narrower. When piscidin 1 is present, the bilayer is predominantly aligned, with its normal perpendicular to the magnetic field, at all temperatures >34°C. The peptide effectively stabilizes a significant portion of the sample, reducing the isotropic resonance intensities by ~80%, and the bilayers remain aligned at temperatures as high as 61°C. Preliminary studies of samples containing piscidin 3 indicate that it exhibits similar behavior (A. A. De Angelis, C. V. Grant, M. K. Baxter, J. A. McGavin, S. J. Opella, and M. L. Cotten, unpublished data). Recently, Ouellet et al. also observed similar bicelle stabilization by a synthetic 21-mer cytotoxic peptide that forms transmembrane channels (39). Although zwitterionic bilayers are not affected by piscidin 1, in anionic bilayers, the 6-O-PC, 14-O-PC, and DMPG ^{31}P NMR resonances are resolved in the absence of the piscidin 1 peptide but appear to merge into broader signals when piscidin 1 is added to the sample. This observation is not unprecedented, since Crowell et al. noticed that the presence of charged amphiphiles leads to more negative chemical shift values, as well as shorter T_2 relaxation times and broader signals for DMPC in the presence of DMPG (37). Strong interactions of the peptide with the headgroups of DMPG would affect its ^{31}P chemical shift, since the peptide's positive charges have a neutralization effect on the anionic charges of DMPG. It is of interest that piscidin 1 exhibits the characteristics required for the mechanism of action by charge clustering, recently described by Epand and colleagues (40): it has a high concentration of positive charges, structural flexibility, and sufficient hydrophobicity to insert in the hydrophobic core of lipid membranes. Furthermore, it contains arginine residues that can form hydrogen bonds with the lipid headgroups. These results suggest that not only does the cationic peptide preferentially interact with the negatively charged lipid molecules, but it may also cluster them such that peptide-lipid interactions are optimized at the bilayer interface, possibly as a prerequisite for bilayer disruption. A similar effect was discussed when piscidin was studied in the presence of 3:1 DMPC/DMPG oriented on glass plates (4). The small isotropic resonance observed in anionic bilayers in the presence of piscidin 1 at all temperatures is completely absent in the case of zwitterionic bilayers. The persistence in PC/PG planar bilayers of a small percentage of mobile lipid assemblies such as those observed for melittin, a highly lytic peptide (41), is consistent with a recently proposed mechanism of action of piscidin 1 involving a carpet mechanism leading to toroidal pore formation (19). Although the disruptive effect of piscidin 1 may seem small in these spectra, it is important to note that small aggregates form as part of the equilibrium that is established within the boundaries of sealed samples. In bacterial cells exposed to piscidin 1, equilibrium may not be achieved, since the law of mass action predicts that the continuous removal of the aggregates from bacterial cell membranes would favor further formation of these aggregates to compensate for their disappearance in the surroundings. Furthermore, the isotropic signals induced by piscidin 1 in anionic bilayers make up ~6% of the total intensity of the ^{31}P spectrum, which is still twice as large as that observed in melittin.

The results not only of comparing the signals arising from lipid bilayers, but also of direct observation of piscidin 1 in bilayers with different compositions, demonstrate directly that the composition of the lipid bilayers modulates membrane recognition and binding, which are prerequisites to the deployment of antimicrobial activity. In the unbound state in an aqueous environment, the peptide is unstructured (4). In the presence of anionic lipids, the membrane binding of piscidin 1 is enhanced, which results in a stabilization of its membrane-bound secondary structure and amphipathic character, both of which are biophysical parameters known to affect antimicrobial efficacy. Since not only the peptide but also the anionic lipids are stabilized (Figs. 1 and 2), it is possible that the formation of a peptide-lipid complex allows piscidin to initiate its disruptive behavior in the form of small aggregates.

The well-resolved single-line ^{15}N NMR spectra of piscidin 1 at high temperatures in both types of lipid bilayers studied here is consistent with the peptide undergoing fast rotational diffusion about the bilayer normal and rules out the possibility of fast rotational diffusion around the helical axis. Numerical simulations of SLF spectra (Fig. 6) for an in-plane peptide show well-resolved resonances, comparable to the experimental results of Fig. 3, for rotational diffusion rates of the order of 10^6 s^{-1}. Diffusion rates of this magnitude provide a possible explanation for why higher temperatures are conducive to narrow resonances of the aligned bilayer samples of piscidin 1. We previously proposed that piscidins may rely on their fast diffusion to locate their bacterial membrane targets and perform their immune function against pathogens (25). Therefore, the

high rates of diffusion detected here may have some relevance to the biological effects of piscidin.

The spectra obtained in anionic bilayers reveal a tilt angle of the peptide that is very close to that previously measured in mechanically aligned bilayers of similar composition (26). An SLF spectrum of the peptide obtained in perpendicular bicelles is equivalent, in principle, to that in mechanically aligned bilayers of identical composition, once corrections are made to account for the different bilayer orientation and order parameter. Within the limits of experimental differences between these two types of oriented samples, the correlation between NMR spectra in magnetically and mechanically aligned bilayers is approximated by Eq. 1. Fig. 7 *A* shows the SLF spectrum of ^{15}N-labeled peptide in a perpendicular bicelle spectrum back-calculated with Eq. 1 from data obtained in mechanically aligned glass plates (26). Fig. 7 *B* shows the experimental results of Fig. 3, *A* and *C*, combined. The overall topologies (helical tilt and rotation) of the peptide in the two aligned media are extremely similar; in fact, the spectral patterns are in very good agreement. In addition, our individual assignments correlate well with those on glass plates, despite some differences in the composition and the conditions of the alignment media. These results demonstrate the validity of magnetically aligned bilayers as a medium to study amphipathic peptides by NMR.

Magnetically aligned bilayers with the normal parallel to the static magnetic field are also available (27,31); therefore, both perpendicular and parallel orientations can be obtained in magnetically aligned media and used simultaneously for resonance assignments (35). Despite the fact that well-established mechanically aligned bilayers on glass plates still offer a wider variety of compositions, especially in terms of bilayer thickness, the number of magnetically alignable bilayers is steadily increasing. Although the preliminary observations reported here did not focus on other physicochemical parameters, the versatility of the bilayers offers opportunities for future studies as a function of important physiological parameters such as bilayer composition and electrolytes. The pH of magnetically aligned samples can be easily measured and controlled by using suitable buffers, thus enabling future pH-dependent studies of piscidin structure and activity by NMR. This may prove particularly important for comparisons between piscidin 1 and piscidin 3, which differ most notably in one histidine residue. Preliminary ^{31}P NMR and ^{15}N NMR spectra of piscidin 3 in magnetically aligned bilayers show that this method can be used to make comparisons between these two piscidins.

In summary, these studies demonstrate that the alignment and effects on the host bilayer from an antimicrobial peptide vary over a wide range of temperatures in magnetically aligned phospholipid bilayers of either zwitterionic or anionic character. The significant differences between the effects of piscidin 1 on zwitterionic and anionic membranes are paralleled by changes in the alignment of the peptide in these two lipid environments. The results presented here demonstrate the complexity of relatively short peptides interacting with phospholipid bilayers. This suggests that primary structure may play a role in these peptides that is normally played by the tertiary fold of globular proteins in terms of specificity of interactions and effects on other constituents of the biological system. It is remarkable that the relatively few biophysical parameters derived from the primary structure of host-defense peptides are required for their notable immunomodulatory effects and antimicrobial efficacy against a broad range of microbes.

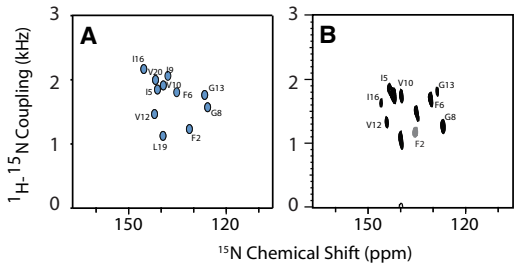

FIGURE 7 Comparison between solid-state NMR data of piscidin 1 in 3:1 DMPC/DMPG lipid bilayers mechanically aligned on glass plates at 40°C and piscidin 1 in 2.6:0.6:1.0 14-O-PC/DMPG/6-O-PC magnetically aligned bilayers at 61°C. (*A*) Simulated bicelle spectra calculated from the ^{15}N/^{1}H SLF PISEMA data for piscidin 1 in mechanically aligned bilayers (A. A. De Angelis, C. V. Grant, M. K. Baxter, J. A. McGavin, S. J. Opella, and M. L. Cotten, unpublished results) using Eq. 1 and bicelle order parameter S = 0.85. (*B*) Experimental results in magnetically aligned bilayers combining data from two samples: ^{15}N-(I5F6G8I9V10V12G13I16L19V20)-p1-NH_2 and ^{15}N-(F2I5G8)-p1-NH_2. Phe^{2}, which provides a starting point for resonance assignment using PISA wheels, is shown in gray.

SUPPORTING MATERIAL

Additional text and references are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(11)00845-9.

We thank Alexander A. Nevzorov for assistance with the MATLAB programs and for insightful discussions.

This research was supported by a grant from the National Science Foundation (CHE-0832571). It utilized the NMR Facilities at the University of California at San Diego, supported by grants from the National Institutes of Health (EB002031 and S10RR23773).

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