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Published on Web 03/30/2006

High-Field NMR Studies of Molecular Recognition and Structure−Function Relationships in Antimicrobial Piscidins at the Water−Lipid Bilayer Interface

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Piscidins are the first amphipathic, cationic, antimicrobial peptides (ACAPs) to be found in the mast cells of fish, and they are believed to play a crucial role in the fight against many aquatic infections.[1] Many ACAPs have been characterized functionally, and some models for their mechanism of action exist, including the barrel-stave model, the wormhole model, the carpet mechanism, and the intracellular activation of fatal pathways.[2−9] Much information remains to be learned about the details of their structure, initial interactions with membranes, and the ultimate mechanism for disrupting cellular function. To this end, we employed solid-state NMR (ssNMR) to probe the structure and topology of isotopically labeled piscidins in the membrane-bound state.

Detailed characterization of piscidin structure is a necessary step in the search for relationships between structural motifs, interactions with biological membranes, potency, and mechanisms of action. Many ACAPs presumably bind to phospholipid bilayers because their amphipathic structures are well suited for partitioning at water−bilayer interfaces.[2−7,9,10] Electrostatic interactions may contribute to the high specificity of ACAPs for anionic bacterial membranes, and both hydrophobic interactions and aggregation may also play a role.[2−7,9,10] More detailed structural knowledge of ACAPs, such as piscidins, could help future selection and design of peptides with increased antimicrobial activity and reduced toxicity.[9,11] However, performing traditional structural/dynamic methods on physiologically relevant peptide−lipid samples is challenging and may not lead to site-specific results. ssNMR using isotopically labeled peptides offers major advantages for probing local structure and dynamics and for determining the precise nature of peptide−lipid interactions under changing conditions.[3,12]

The special features of piscidins 1, 2, and 3 (p1, p2, and p3), which have been isolated from hybrid striped bass,[1] include (1) a carboxyamidated form; (2) tolerance to high salt concentrations; (3) highly conserved amino ends; (4) high cationic character; and (5) high histidine content.[1] The antimicrobial activities of p1 and p3 are comparable, but p1 is more active against some bacteria[1] and p3 is considerably less hemolytic than p1.[1] Here, complementary ssNMR distance and orientational restraints were obtained from hydrated piscidin−lipid samples to investigate molecular recognition and structure−function relationships in nonamidated and amidated p1 (p1-COOH/NH_2) and p3 (p3-COOH/NH_2).

Intramolecular distance restraints derived from nuclear dipolar couplings (DC) can be measured by ssNMR and used to diagnose the secondary structure of peptides.[12] REDOR (Rotational Echo DOuble Resonance)[13] can be performed to measure carbonyl $^{13}C_i$ to amide $^{15}N_{i+4}$ distances. A $^{13}C_i/^{15}N_{i+4}$ distance of ∼4.1−4.2 Å is characteristic of backbone hydrogen-bonded α-helical structures,

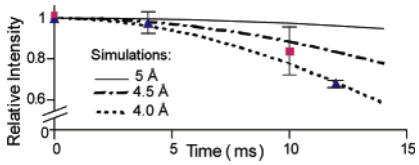

Figure 1. ^{13}C detect REDOR dephasing (S/S_0 vs dephasing time) for $^{13}C-G_8/^{15}N-V_{12}$ p1-COOH (squares) and $^{13}C-I_{16}/^{15}N-V_{20}$ p1-COOH (triangles) in hydrated 3:1 DTPC/DTPG at 1:20 peptide-to-lipid ratio, and $T = -50$ °C.

while $^{13}C_i/^{15}N_{i+4}$ distances are too long to be measured in the case of β-sheet and unstructured peptides. To supplement distances, orientational restraints can be used to investigate the topology and the structure of peptides in the presence of oriented phospholipid bilayers.[3,12] Since ^{15}N-amide-oriented chemical shift (CS) frequencies of α-helical peptides conveniently reflect the orientation of the peptide planes with respect to the bilayer normal, these CSs directly characterize peptide topology when the type of secondary structure is already known.[3,12] The PISEMA (Polarization Inversion Spin Exchange at the Magic Angle) experiment[14] applied to oriented samples containing ^{15}N-amide site(s) advantageously provides both ^{15}N CSs and $^{15}N-^1H$ DCs. Analysis of PISA wheel patterns observed in PISEMA spectra from helical segments can yield their tilt, polarity, and high-resolution structure.[12,15]

We performed ^{13}C-detect REDOR on p1 in the presence of C14 ether-linked DTPC (1,2-*O*-ditetradecyl-*sn*-glycero-3-phosphocholine) and DTPG (1,2-*O*-ditetradecyl-*sn*-glycero-3-phosphoglycerate) bilayers, which allow the selective detection of peptide carbonyl signals. On the basis of ^{15}N NMR line shape analysis at various temperatures, fast, large amplitude backbone motions in piscidins stop below the phase transition of the lipids (∼25 °C). Hence, we carried out REDOR at ∼−50 °C to quench dynamics that are problematic at 25 °C in hydrated samples.[12b] Interdigitation can occur in ether-linked lipid bilayers in the gel state, but these lipids have been reliable for the structural and topological studies of other lipid-bound helical ACAPs[16] and the stabilization of bicelles.[17] Interdigitation reduces bilayer thickness which may affect transmembrane (TM) species, but as shown next, piscidin has an in-plane orientation in C14 ester-linked lipids. The REDOR data for $[^{13}C_1-I_{16}/^{15}N-V_{20}]$ and $[^{13}C_1-G_8/^{15}N-V_{12}]$ p1-COOH shown in Figure 1 indicate that, within experimental error, p1 is α-helical where probed. While a full investigation of the peptide's structural preferences as a function of membrane composition and temperature has not been performed yet, α-helicity characterized by REDOR is consistent with circular dichroism carried out using ester-linked lipids and similar sample conditions (not shown).

Once we assigned the secondary structural motif, we used oriented samples and PISEMA to initiate high-resolution structural

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10.1021/ja058385e CCC: $33.50

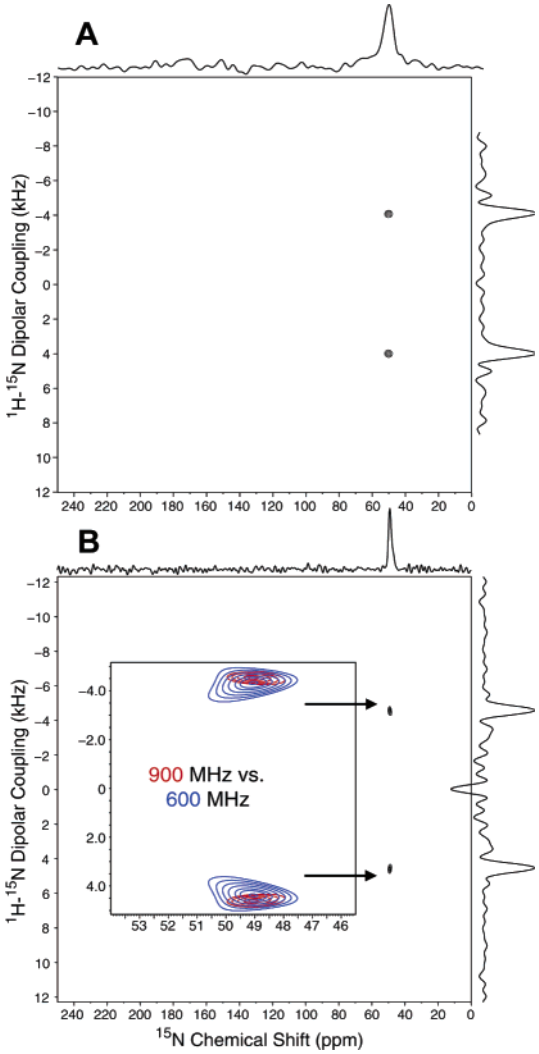

Figure 2. ^{15}N−^{1}H PISEMA spectra of (A) ^{15}N−V_{20} p1-NH_2 and (B) ^{15}N−L_{20} p3-NH_2 in hydrated 3:1 DMPC/DMPG at 1:20 peptide-to-lipid ratio, pH 6, and T = 40 °C. (A) 600 MHz; (B) 900 (red) and 600 MHz (blue). The ^{15}N chemical shift scale is referenced to a saturated solution of $^{15}NH_4NO_3$.

studies. Specifically, we determined that at position 20 the helix is oriented parallel to the bilayer surface; p1 and p3 were studied in the presence of oriented, hydrated bilayers of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerate) in the fluid state (T > 23 °C). In Figure 2, sharp ^{15}N-oriented peaks at 50.7 and 49.2 ppm for ^{15}N−V_{20} p1-NH_2 and ^{15}N−L_{20} p3-NH_2, respectively, indicate that each piscidin is very well oriented and its helical axis is perpendicular to the magnetic field B_0 and bilayer normal.[3,12,15] In contrast, CSs for TM peptides are ∼180 ppm. At position 20, where p1 and p3 are α-helical, the DCs vary from 8.12 (p1) to 9.20 (p3) kHz, suggesting that the tilt differs slightly for these amidated peptides, which according to both CS and DC data nevertheless adopt an in-plane bilayer orientation. The line width at half-height for p1-NH_2 is only 600 Hz in the ^{15}N−^{1}H DC dimension (Figure 2A). Notably too, the anisotropic ^{15}N CS of p3-NH_2 is narrower at 900 than at 600 MHz (Figure 2B). Overall, the PISEMA data shown here, including the 2.2 ppm CS line width for p3-NH_2 at 900 MHz, represent outstanding results for biological samples studied by ssNMR under physiologically relevant conditions. Variations in line widths may be related to enhanced resolution and sample alignment at higher field and slight differences in sample hydration and/or salt level.[18]

To our knowledge, this is the first high-resolution structural characterization of bilayer-bound piscidins. Distance and orientational ssNMR restraints provided site-specific information establishing that the piscidins we studied are α-helical and oriented parallel to the bilayer surface. Few natural membrane-bound peptides with this orientation have been structurally studied in detail in the presence of bilayers.[19] The topology we observed was anticipated since the partitioning of ACAPs at the water−bilayer interface allows for favorable peptide−lipid interactions, and it may be related to the mechanism of action of piscidins, as it seems to be the case for other ACAPs.[2−7,9,10] Peptide concentration and lipid composition could affect this peptide orientation. While challenges exist for structural study of helices with an in-plane orientation, the line widths and sensitivity obtained in our PISEMA experiments support the feasibility of determining full high-resolution structures for piscidins, as well as characterizing structural motifs likely to be involved in biological activity and functional diversity among variants. Enhanced resolution from an ultra-high field gives a critical advantage needed for structural determination using multiple-labeled peptides. High-resolution data from our study of piscidins will serve to advance research on ACAPs and other species active at membranes.

Acknowledgment. We acknowledge support from Research Corporation and the Dreyfus Foundation, and NIH (GM-64676 for E.Y.C.'s salary). We are grateful for NMR time allocated at the Environmental Molecular Sciences Laboratory (EMSL), sponsored by the Department of Energy, and the NHMFL supported by Cooperative Agreement (DMR-0084173) and the State of Florida. We particularly thank Dr. Cross (NHMFL).

Supporting Information Available: Materials and Methods. Complete ref 18. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA058385E