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Membrane-Bound Structure and Topology of a Human Alpha Defensin Indicates A Dimer Pore Mechanism for Membrane Disruption

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Abstract

Defensins are cationic and disulfide-bonded host defense proteins of many animals that target microbial cell membranes. Elucidating the three-dimensional structure, dynamics and topology of these proteins in phospholipid bilayers is important for understanding their mechanisms of action. Using solid-state NMR spectroscopy, we have now determined the conformation, dynamics, oligomeric state and topology of a human α -defensin, HNP-1, in DMPC/DMPG bilayers. 2D correlation spectra show that membrane-bound HNP-1 exhibits a similar conformation to the water-soluble state, except for the turn connecting the β 2 and β 3 strands, whose sidechains exhibit immobilization and conformational perturbation upon membrane binding. At high protein/lipid ratios, rapid ^1H spin diffusion from the lipid chains to the protein was observed, indicating that HNP-1 was well inserted into the hydrocarbon core of the bilayer. Arg C ζ -lipid ^{31}P distances indicate that only one of the four Arg residues forms tight hydrogen-bonded guanidinium-phosphate complexes. The protein is predominantly dimerized at high protein/lipid molar ratios, as shown by ^{19}F spin diffusion experiments. The presence of a small fraction of monomers and the shallower insertion at lower protein concentrations suggest that HNP-1 adopts concentration-dependent oligomerization and membrane-bound structure. These data strongly support a “dimer pore” topology of HNP-1 in which the polar top of the dimer lines an aqueous pore while the hydrophobic bottom faces the lipid chains. In this structure R25 lies closest to the membrane surface among the four Arg residues. The pore does not have large lipid disorder, in contrast to the toroidal pores formed by protegrin-1, a two-stranded β -hairpin antimicrobial peptide. These results provide the first glimpse into the membrane-bound structure and mechanism of action of human α -defensins.

Keywords

Human alpha defensins; solid-state NMR; depth of insertion; guanidinium-phosphate interaction; antimicrobial peptides

Defensins are host-defense antimicrobial proteins present in many animals and plants. These cationic polypeptides contain three intramolecular disulfide bonds, whose linkage patterns

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Supplementary Information

Additional 2D spectra and ^{13}C - ^{31}P REDOR analyses are included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

distinguish the α -, β -, and θ -defensins (1). Humans have six α -defensins of about 30 residues long (2). The human neutrophil peptides (HNP) 1–4 were found in the azurophilic granules of neutrophils (3–5), while HD-5 and HD-6 are present in intestinal epithelial cells (6,7). Except for HD-6, the human α -defensins show wide-spectrum antimicrobial activities in the microgram per milliliter range (8,9). Similar to many other antimicrobial peptides (AMPs), the general mechanism of antimicrobial action is believed to be permeabilization of the microbial cell membrane (10,11).

High-resolution crystal structures of all six human α -defensins in the absence of membrane-mimetic solvents have been determined (12,13). The structures are very similar despite significant sequence differences between HNP 1–3 and the other three defensins. The proteins consist of three antiparallel β -strands (β 1, β 2, β 3) connected by turns and a longer loop (Figure 1a). The proteins are dimerized in the crystal through intermolecular H-bonds between the two β 2 strands, extending the triple-stranded β -sheet to a six-stranded β -sheet. The dimer has a basket shape and is amphipathic, with a polar top and an apolar base.

Based on the crystal structure of HNP-3 (12), several mechanistic models had been proposed to account for the antimicrobial activities of HNPs. The wedge model (12) suggests that the dimer inserts into the bilayer with the hydrophobic bottom contacting the hydrocarbon region of the membrane, thus disrupting lipid packing (Figure 1b, c). The depth of the wedge was unspecified, so the dimer may be partly or completely immersed in the lipid membrane. Alternatively, HNPs might form membrane-spanning pores with the hydrophobic base facing the lipids. In the so-called “dimer pore” model (12) (Figure 1d), the polar tops of two dimers are oriented to favor transmembrane (TM) orientation of a small solvent channel observed at the dimer interface in the crystal structure (12). In the “general pore” model (12), the dimers are rotated by 90° around the horizontal axis (Figure 1e), so that the side view, parallel to the membrane normal, shows the basket shape of the dimer. A multimeric pore model was also proposed based on vesicle leakage and dextran permeability experiments (14). Here 6–8 HNP dimers may form a large pore with an inner diameter of 20–25 Å. The dimers are oriented with the long axis of the basket top at about 45° from the bilayer normal, so that the Arg residues are located in two rings separated by about 16 Å along the bilayer normal, promoting favorable electrostatic interactions with the lipid phosphates (Figure 1f).

Despite many crystal structures of HNPs, no high-resolution structure information for membrane-bound HNPs has been reported. To elucidate the mechanism of action of human defensins, we have initiated a solid-state NMR study of uniformly ^{13}C and ^{15}N labeled recombinant HNP-1. Since membrane-bound proteins typically have broad linewidths than proteins outside the membrane, due to the conformational disorder of the lipids, we found it necessary to first conduct resonance assignment of the protein in the more ordered water-soluble and lipid-free state. Unlike the bactericidal channel-forming colicins (15–17), which exhibit abundant membrane-induced flexibility, HNPs cannot undergo large conformational rearrangements due to their multiple disulfide bonds. We thus first determined the NMR structure of lipid-free microcrystalline HNP-1 using 2D and 3D magic-angle-spinning (MAS) ^{13}C - ^{13}C and ^{13}C - ^{15}N correlation experiments (18,19). Together with distance constraints, these data led to the first solid-state NMR structure of a human α -defensin, which confirmed the three-dimensional fold seen in the crystal structures (PDB code: 2KHT). The NMR structure determination yielded the complete ^{13}C and ^{15}N chemical shifts of the protein (19), with which the membrane-bound HNP-1 chemical shifts can now be compared. In this work, we investigate the conformation and dynamics of HNP-1 in the membrane, its oligomeric state (20,21), and most importantly, its interactions with lipids and water. These results yielded the global topology of HNP-1 in the lipid bilayer, allowing us to rule out most structural models and propose the membrane-disruptive mechanism of HNP-1.

Materials and Methods

HNP-1 expression and synthesis

Recombinant ^{13}C , ^{15}N -labeled HNP-1 (ACYCRIPACIAGERRYGTCTIYGQRLWAF-C (Figure 3a) was obtained as a cleavage product from its precursor protein, proHNP1. The residues are numbered from A2 to C31 due to sequence alignment with other mammalian defensins, many of which contain an additional N-terminal residue before A2 (12). Using this numbering system also facilitates comparison with HNP-3, which was the first structure determined in the HNP family. Briefly, the expression protocol starts with expressing proHNP1 as a GST-fusion protein in *E. coli* using ^{13}C , ^{15}N -labeled Spectra 9 media (Cambridge Isotope Laboratories) (19, 22). GST-proHNP1 was folded and then cleaved using thrombin, producing proHNP1. ProHNP1 was purified by reversed-phase HPLC, then cleaved using cyanogen bromide to yield correctly folded HNP-1. Antimicrobial assays confirmed the activity of the protein. For example, 100% killing of *S. aureus* is reached at 64 $\mu\text{g/ml}$ HNP-1 (19, 22). Crude HNP-1 was purified by reversed-phase HPLC. About 1.5 mg HNP-1 (molecular weight: 3634 Da) was purified from 1 L culture. ^{19}F -labeled HNP-1 for CODEX experiments was synthesized using t-Boc chemistry (23), where Tyr4 was replaced by 4- ^{19}F -Phg. The use of phenylglycine removes the possibility of sidechain χ_1 motion that could complicate the interpretation of the CODEX data (24, 25). The choice of Tyr4 as the ^{19}F labeled site was based on the crystal structures of various HNPs, all showing close inter-subunit contact of this residue (12, 13, 26). Previous studies of 4- ^{19}F -Phg mutants of the related β -hairpin antimicrobial peptide, PG-1 (24), suggest that the mutation generally does not perturb the antimicrobial activities of the peptides.

Membrane sample preparation

DMPC and DMPG lipids (Avanti Polar Lipids, Alabaster, AL) were codissolved in chloroform at a molar ratio of 3:1, dried under a stream of nitrogen gas, redissolved in cyclohexane and lyophilized overnight. The dry and homogeneous lipid powder was suspended in a pH 7 phosphate buffer, vortexed and then freeze-thawed five times. The vesicle solution was then extruded through polycarbonate filters with pore sizes of 400 nm and 100 nm to obtain large unilamellar vesicles. HNP-1 was dissolved in 1 mL phosphate buffer, added to the lipid vesicle solution and dialyzed overnight. The mixture was centrifuged at 200,000 g for 3 hours at 4°C to obtain a membrane pellet. The pellet was pipette-transferred into 4 mm MAS rotors for NMR experiments.

Three samples were prepared: two U- ^{13}C , ^{15}N -labeled HNP-1 samples at protein : lipid (P/L) molar ratios of 1: 18 and 1: 40, and one ^{19}F -labeled HNP-1 sample at P/L = 1: 18. The 1:18 molar ratio corresponds to a mass ratio of 1 : 3.3, thus there was sufficient lipid to ensure appropriate protein-lipid interactions.

Solid-state NMR spectroscopy

Most 2D resonance assignment experiments were carried out on a Bruker (Karlsruhe, Germany) AVANCE-600 (14.1 Tesla) spectrometer operating at a ^{13}C Larmor frequency of 150.92 MHz. A Bruker DSX-400 (9.4 Tesla) spectrometer was used for ^{13}C - ^{31}P REDOR experiments, ^1H spin diffusion and ^{13}C - ^1H dipolar coupling measurements. Triple-resonance MAS probes with 4-mm rotors were used for all experiments. ^{13}C and ^{31}P chemical shifts were referenced externally to the α -glycine ^{13}CO signal at 176.49 ppm on the TMS scale and the hydroxyapatite ^{31}P signal at 2.73 ppm on the phosphoric acid scale, respectively. ^{15}N chemical shifts were referenced to the ^{15}N signal of N-acetyl-valine at 122.0 ppm on the liquid ammonia scale.

2D ^{13}C - ^{13}C DARR correlation spectra (27) were measured at 273 K under 5 kHz MAS with a ^{13}C spin diffusion time of 40 ms. 2D N(CO)CX and N(CA)CX correlation spectra (28,29) were measured at 273 K under 7.5 kHz MAS with a ^{13}C mixing time of 40 ms for NCACX and 60 ms for NCOCX. The ^{15}N - ^{13}C SPECIFIC (30) cross-polarization (CP) contact times were 3 ms. In the intra-residue NCACX experiment, ^{15}N - $^{13}\text{C}\alpha$ magnetization transfer was achieved using a ^{15}N spin-lock field of 17 kHz and an on-resonance $^{13}\text{C}\alpha$ spin-lock field of 25 kHz. In the inter-residue NCOCX experiment, ^{15}N - ^{13}CO magnetization transfer was accomplished with a ^{15}N on-resonance spin-lock field of 27 kHz and a tilted ^{13}CO spin-lock field strength of 35 kHz, which was the result of a 30 kHz applied field on resonance with $\text{C}\alpha$ and an 18 kHz offset for CO.

^{13}C - ^1H dipolar couplings were measured at 303 K using the 2D dipolar chemical-shift (DIPSHIFT) correlation experiment (31). The sample was spun at 4.5 kHz, and the MREV-8 sequence (32) was used for ^1H homonuclear decoupling. The t_1 -curves were fit using a Fortran program, and the fit values were divided by the MREV-8 scaling factor to obtain the true couplings. To account for uncertainties in the MREV-8 scaling factor, we measured the rigid-limit C-H dipolar couplings using the crystalline model peptide formyl-Met-Leu-Phe (f-MLF) (33,34), where Leu $\text{C}\alpha$, Met $\text{C}\beta$, and Leu $\text{C}\delta$ signals represented CH, CH_2 , and CH_3 groups, respectively. The CH and CH_2 peaks gave C-H rigid-limit dipolar couplings of 11.2 kHz (fit values), which corresponded to true couplings of 23.8 kHz when the theoretical scaling factor of 0.47 for MREV-8 was used (35). For the Leu CH_3 signal, the fit value was 3.5 kHz. The ratio of the measured HNP-1 couplings with the rigid-limit f-MLF couplings yielded the order parameter S_{CH} .

To determine the depth of insertion of HNP-1, we carried out 2D ^{13}C -detected ^1H spin diffusion experiments (36,37) at 303 K or 308 K under 5 kHz MAS. For the P/L = 1:18 sample, ^1H spin diffusion mixing times (t_m) of 2.25 – 225 ms were applied to transfer the ^1H magnetization of mobile lipids and water to HNP-1. For the P/L=1:40 sample, two mixing times of 100 ms and 225 ms were measured since the sensitivity was too low to measure a complete buildup curve. The ^1H magnetization transfer was detected through the protein ^{13}C signals. To ensure that only the mobile lipid and water polarization served as the ^1H magnetization source, we suppressed the ^1H magnetization of the rigid protein by a ^1H T_2 filter of 0.4×2 ms before the t_1 evolution period. For the 2.25 ms spectrum of the 1:18 sample, the water ^1H cross section was extracted to analyze the water-proximal residues in the protein.

A frequency-selective rotational-echo double-resonance (REDOR) experiment (38) was used to measure protein ^{13}C distances to lipid ^{31}P . The experiments were performed at 233 K under 4.5 kHz MAS to suppress lipid motions as well as protein sidechain motions. A rotor-synchronized ^{13}C Gaussian 180° pulse of 444 μs was applied in the middle of the REDOR period to suppress ^{13}C - ^{13}C J-couplings between the on-resonance ^{13}C and its directly bonded spins. ^{31}P 180° pulses of 9 μs were applied every half a rotor period to recouple the ^{13}C - ^{31}P dipolar coupling. The time-dependent REDOR intensities were fit using two-spin simulations, which had been shown (39) to correspond to the vertical distance between the ^{13}C spin and the ^{31}P plane for long distances of greater than ~ 6 Å.

^{19}F CODEX experiments (20,40,41) for determining the oligomeric state of HNP-1 were carried out at 233 K under 7 kHz MAS. The ^{19}F chemical shift anisotropy (CSA) was recoupled by two rotor periods of 180° pulses. During the mixing time, ^{19}F spin diffusion changes the ^{19}F CSA and prevents complete refocusing of a stimulated echo. A z-filter after the second 180° -pulse train allows the correction of ^{19}F T_1 relaxation effects by conducting two experiments for each t_m , a control experiment (S_0) in which the short z-filter period (10 μs) was applied between the two 180° -pulse trains while the long t_m occurred after, and a

dephasing experiment (S) where the long t_m occurs between the two 180° -pulse trains. The intensity ratio, S/S_0 , at equilibrium yields the number of ^{19}F spins in close proximity. Two mixing times, 500 ms and 1 s, were measured.

Results

Conformation and dynamics of HNP-1 in the lipid membrane

Figure 2a,b shows the ^{31}P spectrum of the DMPC/DMPG (3:1) membrane with bound HNP-1 above (303 K) and below (253 K) the membrane phase transition temperature. At 303 K the ^{31}P spectrum has the classical uniaxial lineshape indicative of lamellar bilayers and is identical to the protein-free lipid spectrum, indicating that HNP-1 does not cause observable orientation disorder to the lipid bilayer. Below the phase transition temperature (23°C) the ^{31}P spectrum exhibited the expected broadening, with a rigid-limit span of 210 ppm at 253 K.

We examined the membrane-bound conformation of HNP-1 by first comparing the 1D ^{13}C spectra of the membrane-bound and microcrystalline states. Figure 2c,d show the ^{13}C spectra of DMPC/DMPG bound HNP-1 at 298 K and 273 K. The intensity and linewidths were largely unaffected by temperature, suggesting that HNP-1 was immobilized at ambient temperature. Compared to the microcrystalline protein spectrum (Figure 2e), the membrane-bound sample exhibited similar intensity distributions except for the additional lipid peaks. Thus, HNP-1 adopts similar overall conformation in the membrane as in the microcrystalline state, as expected for this disulfide-bonded protein.

To better resolve the resonances, we measured 2D ^{13}C - ^{13}C and ^{15}N - ^{13}C correlation spectra. The 2D ^{13}C - ^{13}C DARR spectrum at 40 ms mixing (Figure 3b) showed mostly intra-residue cross peaks, which were readily assigned by comparison with the microcrystalline spectra (19). 2D N(CO)CX and N(CA)CX spectra (Figure S1) further corroborated the assignments. Most residues exhibited similar chemical shifts in the membrane as in the microcrystalline state, except for the sidechains of I21 and R25. The I21 C_γ and C_ϵ peaks moved downfield from the microcrystalline state: the C_γ chemical shift increased from 24.6 ppm to 25.5 ppm while the C_ϵ peak moved from 11.5 ppm to 12.4 ppm (Figure 3c). In the microcrystalline state, the R25 sidechain resonances were not visible in the 2D ^{13}C - ^{13}C spectrum and much weaker than the R6 and R16 signals in the 3D NCC spectra (19). In comparison, in the membrane-bound state, the R25 sidechain resonances became much stronger, with clear C_β - C_γ and C_δ - C_β cross peaks (Figure 3d, e). I21 and R25 are both located in the $\beta 2$ - $\beta 3$ turn at the hydrophobic base of the dimer (Figure 1a). This turn was seen in the HNP-3 crystal structure to deviate significantly from crystallographic symmetry and was thought to exhibit flexing motions (12). Thus, the membrane-induced conformational and dynamical changes of I21 and R25 sidechains are likely significant, and may reflect site-specific interactions of this $\beta 2$ - $\beta 3$ turn with lipids (see below). Figure 3f shows the C_α and C_β secondary chemical shifts (42) of HNP-1 for both the membrane-bound and microcrystalline states, confirming the triple-stranded motif of the protein and the similarity of the protein conformation without and with the lipids. The secondary shifts suggest that the Y17-G18 junction in the middle of the $\beta 2$ strand deviates from the ideal β -sheet structure, as noted before (19), which may reflect the role of G18 as a hinge for the $\beta 2$ - $\beta 3$ hairpin (13).

To evaluate the mobility of membrane-bound HNP-1 quantitatively, we measured the ^{13}C - ^1H dipolar couplings using the 2D DIPSHIFT experiment. Figure 4 shows the ^{13}C chemical shift dimension of the 2D spectrum, with partial resolution of the sites. Several dipolar cross sections representing the backbone C_α - H_α , sidechain CH_2 and CH_3 groups are shown. We obtained rigid-limit S_{CH} 's of 0.95–1.05 for C_α and methylene groups, while the methyl groups of Ile and Ala exhibited S_{CH} values of 0.26–0.28. Since methyl three-site

jumps alone give an order parameter of 0.33, the small degree of additional scaling, by a factor of 0.78–0.85, indicates that other torsional degrees of freedom in the aliphatic sidechains of these residues are insignificant. Thus the DIPSHIFT data is consistent with the temperature insensitivity of the ^{13}C spectra and indicate that HNP-1 backbone is immobilized in the liquid-crystalline phase of the bilayer, and only the sidechains exhibit moderate motion. This finding is not only consistent with the conformational rigidity of this disulfide-bonded protein, but more importantly indicates that HNP-1 is not monomeric in the membrane. If the protein were monomeric, the small molecular weight would cause fast whole-body uniaxial diffusion of the protein in the membrane, as observed in many small and medium-sized membrane proteins with molecule weights up to several tens of kDa (43–47). Since all HNPs form dimers in the crystal (12,13,48), the immobilization suggests that when bound to the membrane, HNP-1 is at least dimeric and may form higher-order oligomers.

Depth of insertion of HNP-1 from lipid-protein ^1H spin diffusion

To determine the immersion depth of HNP-1, we carried out a 2D ^{13}C -detected ^1H spin diffusion experiment (36). The experiment measures inter-proton distances from the mobile water and lipid acyl chains to the rigid protein through distance-dependent ^1H magnetization transfer. TM proteins in contact with both the membrane surface and the hydrocarbon core exhibit fast ^1H spin diffusion from both lipid acyl chains and water (37), while surface-bound proteins have much slower ^1H spin diffusion from the lipid chains.

Figure 5 shows the ^1H spin diffusion data of membrane-bound HNP-1 (P/L = 1:18) at 305 K. A representative 2D spectrum (mixing time of 100 ms) shows the characteristic cross peaks between the protein ^{13}C signals (63 – 37 ppm) and the lipid CH_2 protons at 1.3 ppm, indicating that HNP-1 is within spin diffusion reach of the acyl chains. Strong water-protein cross peaks were also observed. To quantify the distances, we measured the 2D spectra as a function of the mixing time. Figure 5b shows the integrated ^1H cross sections for ^{13}C chemical shifts of 63–37 ppm for mixing times of 9 to 225 ms. After correcting for ^1H T_1 relaxation of the water and CH_2 protons, we plotted the cross-peak intensities as a function of the square root of the mixing time. Figure 5c shows that both the water and lipid cross peaks reached a plateau by 100 ms, qualitatively indicating that HNP-1 is in close contact with both the hydrophobic core of the membrane and water. The equilibration allows both the water and lipid cross peaks to be normalized with respect to their own maximum intensities at 100 ms.

We simulated the CH_2 buildup curve using a 1D lattice model to obtain the minimum lipid-protein separation. In this model, ^1H magnetization (M_i) transfer was modeled as a discrete process along the bilayer normal (49). The transfer rate, $\Omega = D/a^2$, depends on the lattice spacing a , fixed at 2 Å, and the diffusion coefficients of the various membrane components. Based on previous calibrations we used diffusion coefficients of 0.012 nm^2/ms for lipids (D_L), 0.03 nm^2/ms for water (D_W) and 0.3 nm^2/ms for the protein (D_P) (24,36,50). For the interfacial D_{LP} between the lipid and the protein, we used a value of 0.0025 nm^2/ms , which was within the range reported before (24,36,50). The simulation yields the minimum lipid-protein or water-protein distances because once ^1H magnetization crosses the interface from the soft lipid matrix (or water) to the nearest protein residues, it is rapidly equilibrated within the immobilized protein. The lack of site specificity allowed us to use the integrated intensities for the protein ^{13}C signals to obtain global information about the depth of insertion of HNP-1. Figure 5c shows that the CH_2 buildup curve for the P/L=1:18 sample was best fit to a distance of 2 Å, indicating that HNP-1 was well inserted into the hydrophobic region of the DMPC/DMPG bilayer, in immediate contact with the acyl chains. This close proximity rules out the surface-bound wedge model (Figure 1b): if the basket-shaped dimer is exposed to the aqueous phase to any significant extent, for example one

third of its 18 Å height, then the membrane-immersed part of the protein would be ~12 Å, which would make the protein mostly outside the hydrophobic region of the bilayer.

Some antimicrobial peptides have been found to exhibit concentration-dependent interactions with the lipid bilayer: they bind to the surface of the lipid bilayer at low concentrations but insert into the membrane at high concentrations (51,52). To determine whether the depth of insertion of HNP-1 changes with concentration, we measured the ^1H spin diffusion spectra at P/L = 1:40. We measured two mixing times, 100 ms and 225 ms, which were sufficiently long to give equilibrium intensities for the 1:18 sample. The lipid-protein cross peaks at 225 ms were clearly higher than at 100 ms after correcting for ^1H T_1 relaxation, indicating that the CH_2 cross peaks had not equilibrated by 225 ms. To obtain the correct normalization, we scaled the CH_2 cross-peak intensities with the water cross-peak intensities in the 100 ms spectrum, since the water intensities were already equilibrated at 100 ms (Figure 5d), consistent with all membrane proteins studied so far (53–55). This ratio was further scaled by the equilibrium intensity ratio (0.61) between the CH_2 peak and the water peak in the 1D ^1H spectrum:

$$I_{\text{CH}_2}^{\text{norm}}(t_m) = \frac{I_{\text{CH}_2}^{\text{obs}}(t_m) e^{+t_m/T_{1,\text{CH}_2}}}{\left(I_{\text{H}_2\text{O}}^{\text{obs},100\text{ms}} \cdot e^{+t_m/T_{1,\text{H}_2\text{O}}}\right) \left(I_{\text{CH}_2}^{\text{eq}}/I_{\text{H}_2\text{O}}^{\text{eq}}\right)} \quad (1)$$

The normalized CH_2 intensities at P/L = 1:40 are significantly lower than those of the 1:18 sample, indicating that HNP-1 inserts more shallowly at lower protein concentrations. Thus, HNP-1 has concentration-dependent insertion, similar to several other antimicrobial peptides (51,52).

Membrane topology of HNP-1 from ^{13}C - ^{31}P distances

To further define the membrane topology of HNP-1, we measured ^{13}C - ^{31}P distances between the Arg residues and the lipid headgroups. The well resolved Arg C ζ signal at 157 ppm provides a site-specific probe of the interaction of the four Arg's with the lipid phosphates. Figure 6a shows the normalized C ζ intensities, S/S_0 , as a function of REDOR mixing time. The intensities decayed quickly to 0.67 in the first 10 ms, then more slowly to ~0.5 by 18 ms. The bimodal decay cannot be fit to a single distance, but to a short distance of 4 Å for the initial fast regime and three longer distances of ~7 Å for the long-time regime. The weighting factor of 1 : 3 reflects the intensity plateau at 0.67 between 8 ms and 14 ms. Thus, the data suggests that one out of four guanidinium ions forms tight H-bonded complexes with the lipid phosphates, while the other three Arg's are more distant.

We now consider the compatibility of the various membrane topological models of HNP-1 with the Arg C ζ -P distances. In the fully immersed wedge model (Figure 6b), R6 and R15 are closest to the membrane surface, with their C ζ depths differing by only ~2 Å when the axis of the basket-shaped dimer is oriented parallel to the bilayer normal (Table 1) (12). Thus, if R6 C ζ is 4 Å away from ^{31}P , then R15 C ζ should be about 6 Å away. Using this approach, we estimated all four C ζ -P distances for the immersed wedge model (Table 1) and simulated the resulting REDOR curve. Figure 6b shows that the simulated REDOR curve decays faster than the experimental intensities, especially in the 8 – 14 ms regime.

Since Arg residues have multiple rotameric states with similar energetic stabilities (56) and the lipid bilayer is expected to exert a strong influence to Arg sidechain conformation through salt bridge formation (39,57), the Arg sidechains may adopt different conformations in the membrane than in the crystal. However, any rotamer differences should shorten the C ζ -P distances and speed up the dipolar dephasing, rather than slowing down the dephasing

as required to fit the experimental data. When the four C ζ -P distances were estimated based on the relative positions of the four Arg backbone C α atoms to eliminate sidechain conformational effects, the simulated REDOR curve (dashed line) (Figure 6b) still decayed too fast compared to the observed data.

In the general pore model (Figure 6c), more than one Arg sidechain was close to the ^{31}P atoms. Specifically, R6, R15, and R16, all have similar C ζ depths based on the crystal structure (Table 1) (12). Keeping the least inserted R15 C ζ -P distance at 4 Å, as constrained by the REDOR data, we obtained R16 and R6 C ζ -P distances of ~6 and 7 Å. The resulting REDOR curve again decays faster than the measured data, and cannot be remedied by changing the sidechain conformations.

Finally, we considered the dimer pore model, where the dimer orientation was rotated by 90° from that of the general pore model (Figure 6d). Here the situation is qualitatively different: R25 is now closest to the membrane surface, with the next nearest Arg, R15 in the other monomer, ~4 Å deeper in the membrane. Using the rotamers in the HNP-3 structure, we found the simulated curve to now decay more slowly than the measured data (Figure 6d), thus allowing us to modify the sidechain conformations to minimize the C ζ -P distances. In addition, ^{13}CO - ^{31}P and $^{13}\text{C}\alpha$ - ^{31}P REDOR data suggested a slight membrane thinning from 35 Å to 30 Å (Figure 7). Combining these two changes, we obtained Arg rotamers that gave C ζ -P distances of 4, 6.5, 9, and 10 Å (Table 1), which gave excellent fit to the experimental data (Figure 6e). Moreover, R25, the residue with the shortest C ζ -P distance, is also the residue showing significant immobilization upon membrane binding (Figure 3). Thus, the conformational changes also support the dimer pore topology of HNP-1.

Backbone CO and C α distances to ^{31}P are consistent with the Arg C ζ REDOR data. Both signals decay slowly, with average S/S₀ values of ~0.8 at 14 ms. Simulations yielded average distances of 7.6 Å for CO and 7.2 Å for C α . Since no site resolution was attempted, we added the REDOR dephasing of every CO and C α atoms in each structural model based on their distances to the membrane surface. Figure S2 shows the distance distribution for the dimer pore model, where the shortest distance to ^{31}P was fixed at 4 Å. The simulated REDOR curves fit the measured dephasing well. In reaching this agreement, it was necessary to thin the membrane to 30 Å, suggesting that HNP-1 insertion slightly reduces the bilayer thickness. Although the backbone ^{13}C - ^{31}P REDOR did not exclude the other structural models, it was consistent with the dimer pore structure. In addition, the ^{13}CO - ^{31}P REDOR data verified that HNP-1 was inserted into the gel-phase membrane: simulations using a surface-bound wedge model were inconsistent with the experimental result (Figure S3).

Residue-specific water-protein distances

To complement the topology information from the lipid-protein ^1H spin diffusion experiment, we examined the water-protein ^1H spin diffusion profile at a short mixing time of 2.2 ms, when the ^1H magnetization has not equilibrated in the protein, thus giving residue-specific information about water proximities. Figure 8a shows the water-edited ^{13}C spectrum (red) extracted from the water ^1H cross section of the 2D spectrum. This water-edited spectrum was superimposed with the ^{13}C CP spectrum that reflects the equilibrium ^{13}C magnetization. The two spectra were scaled such that the water-edited intensities at best equaled but never exceeded the CP intensities. It can be seen that the intensity distribution of the water-edited ^{13}C spectrum clearly differs from that of the CP spectrum, indicating selectivity of the water-protein spin diffusion at this mixing time. For example, the lipid chain signals from 33 ppm to 22 ppm were significantly attenuated, as expected because of the immiscibility of water with the hydrocarbon core. The protein

signals in the 53–40 ppm region, which mainly result from $\text{C}\alpha$ and $\text{C}\beta$, were also reduced compared to the $\text{C}\alpha$ intensities in the 60–55 ppm range.

To simulate the water-edited ^{13}C spectrum, we first reproduced the equilibrium CP spectrum by using appropriate weighting factors for each carbon. These weighting factors accounted for different CP dynamics of CH_n ($n=1, 2, 3$) groups and the different mobilities between the backbone and sidechains (Figure 8b). Next, we assigned each carbon a spin-diffusion weighting factor based on the proximity of each site to water on the membrane surface and water in the pore. Both the crystal structure of HNP-3 (12) and the solid-state NMR structure of HNP-1 (19) were considered in estimating the carbon-water distances, and the simulation was insensitive to the exact starting structure (Figure S4). Three categories of spin diffusion weighting factors were used. Carbons close to water were given a weighting factor of 1, while those far from water a weighting factor of 0.1. Residues in the middle were assigned a weighting factor of 0.55. Combining the water-proximity weighting factors with the CP weighting factors, we simulated the water-edited spectrum expected for the dimer pore topology. Figure 8a shows that the simulated stick spectrum agrees well with the measured spectrum. The RMSD difference between the two was 0.22, normalized to the 54-ppm peak intensity (Figure 8c). This RMSD value is comparable to the experimental RMS noise of 0.18, indicating that the measured spectrum is consistent with the dimer pore topology. We did not consider the intensities in the 33 – 22 ppm region due to the significant overlap between the lipid and protein signals.

Oligomeric structure of HNP-1 in the lipid membrane

So far we have assumed that HNP-1 assembles into a basic unit of dimers in the membrane, upon which loose higher-order oligomers may form. Although the immobilization result supports this assumption, we verified this hypothesis using the ^{19}F CODEX experiment, which detects ^{19}F magnetization exchange between orientationally different fluorinated molecules. The oligomeric number n is obtained from the equilibrium CODEX echo intensity of $1/n$ at long mixing times. Tyr4 in the $\beta 1$ strand was replaced by 4- ^{19}F -Phg, since crystal structures suggest this residue to lie in the dimer interface, with inter-monomer distances of less than 10 Å (PDB codes: 1DFN, 3HJ2). Figure 9 shows the ^{19}F CODEX control (S_0) and exchange (S) spectra of HNP-1 in DMPC/DMPG membranes (P/L = 1:18) at a mixing time of 1 s. The S/S_0 value was 0.66 ± 0.06 and was the same between 1 s and 500 ms within experimental uncertainty, indicating that intermolecular spin diffusion has equilibrated. The slightly larger than 0.5 final value indicates that the majority (66%) of Tyr4 exists in a dimer state ($n = 2$) while the rest (33%) has ^{19}F - ^{19}F distances greater than ~15 Å. Thus, when the protein concentration is about 5 mol% in the membrane, the majority of HNP-1 is dimerized, while the rest either exists in a monomer state or forms loose dimers with Tyr4 separations greater than the detection limit of this ^{19}F spin diffusion technique.

Discussion

Evidence of pore formation by HNPs in lipid membranes from biochemical data

Since the discovery of human α -defensins (3,4), many antimicrobial assays, lipid vesicle experiments, and high-resolution structures have been reported to understand the mechanisms of action of these host-defense proteins (1,58). Similar to smaller β -hairpin antimicrobial peptides, bactericidal activity by HNPs followed inner membrane permeabilization (59). Dye release and fluorescence spectroscopy experiments found that HNP-1 caused both fusion and lysis of negatively charged lipid vesicles through electrostatic interactions (60). For rabbit neutrophil defensins, vesicle leakage depends on the membrane composition: it is all-or-none for whole *E. coli* lipids but graded for POPG vesicles (61). Electron micrographs of human parasite cells *Trypanosoma cruzi* in the

presence of micromolar concentrations of HNP-1 showed distinct 25-nm sized pores in the cellular and flagellar membranes (62), through which HNP-1 appears to enter the trypanosome cells, causing subsequent DNA fragmentation and cell destruction. Vesicle leakage experiments also showed that HNP-induced pores increase with the concentration of the anionic lipid (63). These biochemical studies all indicate pore formation by human α -defensins in anionic lipid membranes. However, the exact structure and topology of HNP-1 at the pore and the type of lipid disorder have remained elusive.

Structural constraints on HNP-1-induced pores in anionic lipid membranes

The ^1H spin diffusion, ^{13}C - ^{31}P distances, and ^{19}F spin diffusion results gave the following constraints to HNP-1 structure in the anionic lipid membrane. At high protein concentrations ($\text{P/L} = 1:18$), HNP-1 fully spans the membrane and contacts the hydrophobic chains. Among the four Arg's, one Arg forms hydrogen-bonded complexes with the lipid phosphates. This Arg is most likely R25 located in the $\beta 2$ - $\beta 3$ turn, since its signal was enhanced in the 2D spectra by lipid-induced immobilization. The DMPC/DMPG bilayer is thinned slightly upon HNP-1 binding. Seen at Tyr₄, the majority of the protein is at least dimerized at $\text{P/L} = 1:18$. These observations support the dimer pore model for HNP-1. The fully immersed wedge model (Figure 1c) and the general pore model (Figure 1e) would place R25 to be the furthest Arg from the membrane surface, which is inconsistent with the 2D ^{13}C spectra, because the hydrocarbon core is the most fluid region of the lipid bilayer and should not immobilize R25. The depth distribution of the four Arg sidechains in the fully immersed wedge and general pore models also do not agree with the stoichiometry that only one Arg C ζ is H-bonded to the lipid phosphates. On the other hand, the surface-bound wedge model (Figure 1b) does not agree with the fast ^1H spin diffusion from the lipid chains to the protein. In the multimeric pore model (Figure 1f), the Arg backbones lie at roughly the same depths in each lipid leaflet, with the two C α rings separated by ~ 16 Å. Thus, the C ζ atoms would be similarly close to the membrane surface ^{31}P , which does not agree with the distance distribution of the C ζ -P REDOR data. At lower protein concentrations ($\text{P/L}=1:40$), HNP-1 is less inserted into the membrane, as manifested by the slower lipid-to-protein ^1H spin diffusion. Although sensitivity limitations preclude the determination of the exact topology, we expect the surface-bound wedge model to be the most likely scenario at lower protein concentrations.

In model-specific fitting of the various experimental data, we primarily used the crystal structure of HNP-3 (PDB: 1DFN) because the solid-state NMR structure (19) (PDB: 2KHT) is that of the monomer, with no direct intermolecular constraints for the dimer. Nevertheless, Figure S4 shows that the hypothetical dimer NMR structure would give similar conclusions as the HNP-3 crystal structure for the water-edited ^{13}C spectrum, suggesting that the exact input structure does not affect the conclusion of the global topology of HNP-1.

Taken together, the solid-state NMR data shown here indicate a dimer pore topology of HNP-1, where the β -sheet dimers span the membrane with the R25-containing $\beta 2$ - $\beta 3$ turn pointing towards the membrane surface (Figure 10). When water on the membrane surface and in the pore is both considered, the dimer pore topology reproduces the observed water-edited protein ^{13}C spectrum. The ^{19}F spin diffusion data verifies the dimerization of the majority of the protein. The small fraction of monomers is likely related to the observed shallower insertion of the protein at lower concentrations, and suggests that concentration-dependent oligomerization may be important for the membrane-disruptive activity of HNP-1.

Does the dimer pore mechanism of HNP-1 apply to other human α -defensins? Comparisons of the activities and specificities of the six human α -defensins indicate that their interactions with lipid membranes are diverse. Against Gram-positive *S. aureus*, the relative potencies

were HNP-2 > HNP-1 > HNP-3 > HNP-4, while the relative potencies against the Gram-negative *E. coli* were HNP-4 > HNP-2 > HNP-1 = HNP-3 (8). Among HNP 1, 2, and 3, whose amino acid sequences differ only in their N-terminal residue, HNP-3, which possesses an N-terminal polar residue Asp, has weaker activities than HNP-1 and HNP-2, which have a hydrophobic N-terminal residue (5,8). Consistently, acetylation and amidation of HNP-2 to remove the terminal charges modulated the protein's antimicrobial activity and vesicle leakage (64). Thus, the density and distribution of the positive charge have a significant effect on the membrane interaction of HNPs. In comparison, HD5 and HD6, which are found in intestinal epithelial cells, have much lower sequence homologies to HNPs (6,7). HD6 has nearly two orders of magnitude weaker activities than the HNPs (8), while HD5, while localized on the cell membrane, was suggested to interact with the cells in a receptor-mediated fashion (65). Based on the high sequence homology and functional similarity between HNP-1 and HNP-2, we speculate that the dimer pore mechanism may apply to HNP-2 and should be relevant to HNP-3 as well, but the other defensins in this family may adopt different orientations and insertions in the lipid membrane.

Comparison of HNP-1 with small β -hairpin antimicrobial peptides

Our previous studies of the two-stranded disulfide-bonded β -hairpin antimicrobial peptide, PG-1, indicated that the strong interactions between Arg guanidinium ions and the lipid phosphate groups drove the formation of toroidal pore defects in the membrane, where PG-1 lines the pore as a TM β -barrel (24,39,55). When the guanidinium ions were dimethylated, the mutant showed 3-fold weaker antimicrobial activities and no longer formed large β -barrels in the membrane (66). A PG-1 mutant with only half the number of Arg residues inserted only partly into the anionic membrane and exhibited much weaker interactions with the lipid headgroups (50). Guanidinium-phosphate interactions were also observed in two Arg-rich cell-penetrating peptides, penetratin (67) and the HIV TAT peptide (68). In HNP-1, although only one guanidinium ion is within H-bonding distance to the lipid phosphates, the average distance for the other three Arg residues is 7 Å, which is short compared to the bilayer thickness (Figure 6a). Lu and coworkers have shown by mutagenesis that when three of the four Arg's were converted to Lys, HNP-1 activity was significantly weakened, and the effect was more pronounced against the Gram-positive *S. aureus* than the Gram-negative *E. coli* (69).

It is interesting that no isotropic peak was observed in the ^{31}P NMR spectra, indicating that high-curvature defects such as micelles or toroidal pores were absent in the HNP-bound DMPC/DMPG membrane (Figure 2). Thus, in the DMPC/DMPG membrane, the HNP-1 dimer pores exist in regular lamellar bilayers, consistent with the classical barrel-stave model. The retention of the bilayer integrity contrasts with the behavior of PG-1, which caused substantial membrane disorder to phosphocholine (PC)/phosphatidylglycerol (PG) membranes as well as phosphatidylethanolamine (PE)/PG membranes. The HNP-1 interaction with the DMPC/DMPG membrane is more akin to that of tachyplesins (70) and a synthetic antimicrobial arylamide (71), which did not disrupt PC/PG membranes. However, tachyplesin-1 caused clear disruption to the PE/PG membrane (70). The extent of membrane disorder depends on both the distribution of Arg residues in the protein sequence and the composition of the lipid membrane. It is possible that HNP-lipid interactions are sensitive to the membrane composition, similar to tachyplesins, so that while HNP-1 does not disrupt PC/PG membranes, it may disrupt PE/PG membranes. Similarly, an increased percentage of the negatively charged PG lipids may increase the amount of membrane disorder. A study of rabbit neutrophil defensins suggested that the presence of PE and cardiolipin lipids increased the membrane disruption (61). Given the increased complexity of HNPs over two-stranded β -hairpin antimicrobial peptides, structural investigations as a function of membrane

composition will be useful to further elucidate the membrane interaction and mechanism of action of this class of defensins.

Finally, the precise antimicrobial mechanism of HNP-1 in vivo may depend on factors other than HNP-phospholipid interaction. It was recently reported that HNP-1 activity correlates with the amount of lipid II, a bacterial cell wall precursor (63): inhibition of lipid II synthesis weakened the HNP-1 antibacterial activity. This result suggests that interaction of cell wall components may be involved in the antimicrobial action of HNPs, and may explain why D-amino-acid analogs of HNPs appeared to have weaker bactericidal activities but similar membrane-disruptive abilities compared to their L-amino acid counterparts. Thus, the mechanism of action of HNPs, similar to a number of other defensins (72–75), may involve multiple targets in the bacteria. The dual mechanisms may also contribute to the lack of significant disorder seen in the ^{31}P NMR spectra.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HNP-1	human neutrophil peptide 1
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphatidylcholine
DMPG	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphatidylglycerol
MAS	magic-angle spinning
CP	cross-polarization
DARR	dipolar-assisted rotational resonance
DIPSHIFT	dipolar-chemical-shift correlation
REDOR	rotational-echo double-resonance

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Figure 1.

Different structural models of HNPs in lipid bilayers. The basic oligomeric state of the protein is a basket-shaped dimer. (a) Structure of the HNP monomer (12) with the four Arg sidechains indicated. (b) Surface-bound wedge model with the hydrophobic basket bottom inserted into the bilayer and the polar basket top in contact with water. (c) Membrane-inserted wedge model. (d) Dimer pore model, where the hydrophobic basket bottom contacts the lipids while the polar top faces the water pore. (e) General pore model, where the dimers are rotated by 90° from the dimer pore orientation. (f) Multimeric pore model, where six dimers form a pore with a ~25 Å inner diameter. The height of the multimer, measured between two Pro8 residues, is 27 Å. The approximate dimensions of the various oligomeric assemblies are indicated in (b-f).

Figure 2.

1D ^{31}P and ^{13}C spectra of membrane-bound HNP-1. (a) ^{31}P static spectra of DMPC/DMPG membranes with HNP-1 (P/L = 1:18) (thick line) and without HNP-1 (thin line), measured at 303 K. The protein-bound spectrum exhibits no isotropic peak and is identical to the non-protein-containing spectrum. (b) ^{31}P spectrum of HNP-1-bound DMPC/DMPG membrane at 253 K. (c, d) ^{13}C MAS spectrum of membrane-bound HNP-1 at 298 K (c) and 273 K (d). The intensities and linewidths are little affected by temperature, indicating that HNP-1 is immobilized in the liquid-crystalline membrane. (e) ^{13}C spectrum of microcrystalline HNP-1 without the lipids at 268 K. The intensity distribution of the membrane-bound HNP-1 is similar to that of the microcrystalline protein, indicating similar conformations.

Figure 3.

Resonance assignment of membrane-bound HNP-1 and comparison with the microcrystalline protein. (a) Amino acid sequence of HNP-1. (b) 2D ^{13}C - ^{13}C DARR spectrum of membrane-bound HNP-1 at 273 K with a mixing time of 40 ms. (c-e) Regions that show chemical shift changes between the membrane-bound (black) and microcrystalline (red) states. (c) I21 sidechain. (d) R25 sidechain. (e) R25 and R16 sidechains. Note the significant increase of the R25 sidechain intensities in the membrane-bound state in (d) and (e). (f) $\text{C}\alpha$ and $\text{C}\beta$ secondary chemical shifts of HNP-1 in the membrane-bound and microcrystalline states, confirming the β -strand-rich nature of the protein and the overall similarity of the protein structure in the two states.

Figure 4.

^{13}C - ^1H dipolar couplings of DMPC/DMPG-bound HNP-1 at 303 K under 4.5 kHz MAS. (a) ^{13}C chemical shift dimension of the 2D spectrum. (b) C-H dipolar coupling curves of two $\text{C}\alpha$ cross sections and their best-fit, compared with the Leu $\text{C}\alpha$ data of f-MLF. (c) C-H dipolar coupling curves of two CH_2 peaks and their best-fit, compared with the Met $\text{C}\beta$ data of f-MLF. (d) C-H dipolar coupling curves of two CH_3 cross sections and their best-fit simulations at 3.0 and 3.2 kHz, compared to the Leu $\text{C}\delta$ signal of f-MLF. All couplings here are fit values, and convert to true couplings after they are divided by the homonuclear decoupling scaling factor.

Figure 5.

^1H spin diffusion from lipid chains and water to HNP-1 in DMPC/DMPG bilayers. (a) Representative 2D spectrum for $P/L = 1:18$ at 303 K, with a ^1H mixing time of 100 ms. (b) Integrated ^1H cross sections from ^{13}C chemical shifts of 63 – 37 ppm as a function of ^1H mixing times, compared with the 1D ^1H spectrum at the top. (c) ^1H spin diffusion buildup curves for lipid CH_2 and water at $P/L=1:18$. (d) ^1H spin diffusion intensities for lipid CH_2 and water at $P/L=1:40$. The CH_2 buildup is much slower at the lower protein concentration.

Figure 6.

Arg C ζ - ^{31}P distances of HNP-1 in DMPC/DMPG bilayers. (a) C ζ ^{13}C - ^{31}P REDOR S/S_0 values as a function of mixing time and representative S_0 and S spectra. Best-fit simulation used a 1:3 combination of a 4 Å distance and a 7 Å distance. (b)-(e) Solid lines represent REDOR simulations using C ζ -P distances based on the vertical distance differences among the four Arg C ζ atoms. Dashed lines represent simulations based on the Arg C α vertical distance differences. (b) Membrane-immersed wedge model and the best-fit Arg C ζ -P REDOR curve. Simulated curves decay faster than the experimental data. (c) General pore model and the best-fit C ζ -P curves, which decay faster than the measured data. (d-e) Simulation of the Arg C ζ -P REDOR data using the dimer pore model. (d) Dimer pore model based on the crystal structure of HNP-3 and a 35 Å bilayer thickness. The simulated C ζ -P REDOR curves decay slower than the experimental data. (e) Dimer pore model after adjusting the Arg sidechain rotameric states (Table 1) and using a membrane thickness of 30 Å. The calculated C ζ -P curve fits the data very well.

Figure 7. ^{13}C - ^{31}P REDOR dephasing of backbone CO and C α of membrane-bound HNP-1. (a) CO peak. (b) C α peak. Best-fit curves represent average two-spin distances.

Figure 8.

Water-protein ^1H spin diffusion to determine the membrane topology of HNP-1. (a) ^{13}C spectrum after 2.25 ms ^1H spin diffusion from water (red), superimposed with the ^{13}C CP spectrum (black). Simulated stick spectrum based on the dimer pore model is shown in blue. (b) ^{13}C CP spectrum and simulation to fix the CP weighting factors. (c) Difference between the experimental and simulated water-edited spectrum from (a). (d) Dimer pore model, where residues close to water are shown in blue, interfacial residues in gray, and residues far from water in red. The respective intensity weighting factors are 1, 0.55 and 0.1.

Figure 9. ^{19}F CODEX control (S_0) and dephased (S) spectra of 4- ^{19}F -Phg₄ HNP-1 in DMPC/DMPG (3:1) membranes at P/L=1:18. The mixing time was 1 s. The S/S_0 value was 0.66 ± 0.06 .

Figure 10.

Membrane topology of HNP-1 in anionic lipid bilayers. At high protein concentrations HNP-1 is mostly dimerized and spans the membrane, lining a central water pore. The R25 sidechain lies closest to the membrane surface interacting with the phosphate groups. The lipid chains near the pore do not exhibit significant disorder. (a) Side view. (b) 90° rotated side view from (a), showing the pore behind one dimer. (c) Top view. The distances between the two dimers and the total oligomeric number of the assembly are not probed by the experiments here.

Table 1
Arg C ζ distances to lipid ^3P (R_{CP} , Å) in various topology models of HNPs in lipid bilayers

Residue ¹	Original HNP-3 structure ² 35 Å bilayer thickness				Arg-modified structure, 30 Å bilayer thickness	
	Rotamer	R_{CP} , wedge	R_{CP} , general pore	R_{CP} , dimer pore	Rotamer	R_{CP} , dimer pore
R6	mmt180 ³ mtt-85	4	7	15	tpt180	9
R15	mtt180	6	4	8	mtt85	6.5
R16	ttt180	16	6	16	ptt85	10
R25	mtt-85	18	15	4	ttt180	4

¹ Each residue in principle has four distances to the membrane surfaces due to the dimer state and two bilayer surfaces. Tabulated here is the shortest distance, which is the one relevant for the ^{13}C - ^3P distance experiment.

² The crystal structure of HNP-3 was used to model the dimer pore (12), since the NMR structure is for the HNP-1 monomer and has lower resolution for the sidechains.

³ The symbols m, t, p indicate -60° , 180° , and $+60^\circ$, respectively, for the consecutive χ torsion angles from the backbone (56).