Intermolecular Packing and Alignment in an Ordered β-Hairpin Antimicrobial Peptide Aggregate from 2D Solid-State NMR

Ming Tang,† Alan J. Waring,‡ and Mei Hong*†

Contribution from the Department of Chemistry, Iowa State University, Ames, Iowa 50011, and Department of Medicine, University of California at Los Angeles School of Medicine, Los Angeles, California 90095

Received April 25, 2005; E-mail: mhong@iastate.edu

Abstract: The aggregation and packing of a membrane-disruptive β-hairpin antimicrobial peptide, protegrin-1 (PG-1), in the solid state are investigated to understand its oligomerization and hydrogen-bonding propensity. Incubation of PG-1 in phosphate buffer saline produced well-ordered nanometer-scale aggregates, as indicated by 13C and 15N NMR line widths, chemical shifts, and electron microscopy. Two-dimensional 13C and 1H spin diffusion experiments using C-terminus strand and N-terminus strand labeled peptides indicate that the β-hairpins in these ordered aggregates are oriented parallel to each other with like strands lining the intermolecular interface. In comparison, disordered and lyophilized peptide samples are randomly packed with both parallel and antiparallel alignments. The PG-1 aggregates show significant immobilization of the Phe ring near the β-turn, further supporting the structural ordering. The intermolecular packing of PG-1 found in the solid state is consistent with its oligomerization in lipid bilayers. This solid-state aggregation approach may be useful for determining the quaternary structure of peptides in general and for gaining insights into the oligomerization of antimicrobial peptides in lipid bilayers in particular.

Introduction

Protegrin-1 (PG-1) is a small β-hairpin peptide from porcine leukocytes that has potent and broad-spectrum antimicrobial activities.1 Its minimum inhibitory concentrations lie in the range of a few micrograms per milliliter, more than 2 orders of magnitude stronger than existing antibiotics such as vancomycin.2 PG-1 carries out this remarkably efficient microbicidal function by destroying the cell membranes of the target organisms. Yet, how the peptide interacts with lipid bilayers functionally and structurally, remains a mystery. Understanding PG-1 structure can shed light on the structure–function relationships of a large class of similar β-sheet antimicrobial peptides.3

Using solid-state NMR chemical shift anisotropy and dipolar coupling measurements, we recently found that PG-1 is immobilized in POPC bilayers, where the lipid acyl chains contain 16–18 carbons, but undergoes rigid-body uniaxial rotation in DLPC bilayers, where the lipid chains have only 12 carbons.4 The immobilization in the biologically relevant membrane thickness of POPC bilayers suggests that the peptide is aggregated. Using 19F spin diffusion NMR, we found that PG-1 is dimerized in POPC bilayers.5 This prompted questions pertaining to the dimer structure: are the β-hairpins aligned parallel or antiparallel to each other? Which strand of the hairpin forms the dimer interface? Understanding the detailed membrane-bound dimer structure, which is essentially determined by intermolecular hydrogen bonding, is important for deciphering the mechanism of action of the peptide. Because PG-1 is highly cationic, similar to most antimicrobial peptides,6 the dimer structure can also provide useful insights into the energetic driving force for the insertion of PG-1 into the hydrophobic membrane.

The crystal structures of two β-sheet antimicrobial peptides outside the lipid or detergent environments have been determined to understand the oligomerization and mechanisms of action of these peptides in the membrane.7,8 It was found that these β-sheet peptides form dimers in the crystal, stabilized by a combination of hydrophobic interactions and hydrogen bonds. Because the crystal structure of PG-1 is not available, an alternative approach for gaining insights into the oligomerization of this peptide in the membrane is to create well-ordered and lipid-free peptide aggregates whose intermolecular packing can be determined by solid-state NMR. Studying the structure of lipid-free ordered aggregates has the practical advantages that it has high sensitivities due to the avoidance of lipid dilution and that it does not suffer from the dynamic disorder common to membrane systems. Further, the solid-state aggregate structure

---

1 Iowa State University.
2 University of California at Los Angeles School of Medicine.
can be compared with independently determined membrane-bound oligomeric structure to shed light on the importance of various noncovalent interactions and the environment to peptide oligomerization.

In addition to antimicrobial peptides, other examples of β-strand peptide oligomerization include the amyloid peptide fibrils found in neurodegenerative diseases such as the Alzheimer’s disease.9 The packing and high-resolution structure of the Alzheimer’s β-peptide Aβ1-40 have recently been determined using solid-state NMR.10 Whether PG-1 can form similar extended fibrils is not obvious, because the 18-residue disulfide-linked peptide has a much smaller shape anisotropy than typical amyloid-forming peptides, making the free energy reduction of oligomerization less significant than the longer β-strand peptides. The β-hairpin fold of PG-1 also presents an additional degree of complexity and novelty to the oligomerization: because the two strands of the hairpin share intramolecular hydrogen bonds in the plane of the β-sheet, oligomerization can occur either with like strands or with unlike strands lining the intermolecular interface.

Two general NMR strategies are available for determining the oligomeric structure of peptides. The first involves distance measurements on site-specifically labeled samples.11–13 A number of solid-state NMR techniques already exist for measuring site-specific distances with high accuracy.14,15 However, the success of this approach depends crucially on the labeling positions, otherwise one may not be able to extract measurable distances even for closely packed molecules. The second approach bypasses this difficulty by increasing the number of labeled sites in the peptide and uses more qualitative methods such as spin diffusion16 to determine the proximity of spins between different molecules.17,18

In this work, we show that β-hairpin PG-1 can indeed form ordered aggregates on the tens-of-nanometer scale by suitable solution incubation, and we have determined the molecular packing and alignment in these aggregates using 2D 13C and 1H spin diffusion NMR. Several residues in PG-1 are uniformly labeled in 13C and 15N. Distance-dependent 13C and 1H spin diffusion produces cross-peaks in the 2D spectra whose intensities provide semiquantitative constraints on the intermolecular distances. In this way, we have determined both the identity of the β-strand lining the intermolecular interface and the mutual alignment of the strands.

Materials and Methods

Uniformly 13C,15N-labeled Gly, Leu, Phe, and Val were purchased from Isotec (Miamisburg, OH) and Cambridge Isotope Laboratory (Andover, MA) and converted to Fmoc derivatives by Synpep Corp. (Dublin, CA). PG-1 (NH2-RGGRLYCRRRFCCVGCGR–CONH2) was synthesized using Fmoc solid-phase peptide synthesis protocols and purified by HPLC as described previously.19 The labled amino acids were incorporated at residues F12, V14, and G17 on one sample, and G3 and L5 on another sample (Figure 1).

Preparation of PG-1 Samples. Ordered PG-1 aggregates were prepared by dissolving the purified and lyophilized peptide in pH 7 phosphate buffer saline (PBS) containing 10 mM phosphates and 100 mM sodium chloride. The concentration of the peptide was typically 2–3 mM. The solution was incubated at room temperature for 2–3 weeks with gentle shaking. The solution was then centrifuged, and the precipitate was collected and dried for ~8 h before being packed into NMR rotors for magic-angle spinning (MAS) experiments. Mixed aggregates and 20% diluted aggregate samples were prepared by co-incubating appropriate amounts of the starting compounds in the PBS.
solution. The untreated PG-1 samples were taken directly from the purified and lyophilized peptide without solution incubation.

Solid-State NMR Experiments. NMR experiments were carried out on a Bruker (Karlsruhe, Germany) DSX-400 spectrometer operating at a resonance frequency of 400.49 MHz for 1H, 100.70 MHz for 13C, and 40.58 MHz for 15N. A triple-resonance MAS probe equipped with a 4 mm spinning module was used for the experiments. Low-temperature experiments were conducted by cooling the bearing air through a Kinetics Thermal Systems XR air-jet sample cooler (Stone Ridge, NY). The temperature was maintained within ±1 K of the desired value, and the spinning speed was regulated to within ±3 Hz. Typical 90° pulse lengths were 5 μs for 1H and 10μs for 13C and 15N chemical shifts were referenced externally to the α-Gly 13C signal at 176.4 ppm on the TMS scale and the N-acetylvaline 15N signal at 122.0 ppm on the NH3 scale, respectively. Secondary shifts were calculated after converting the random coil chemical shift values20 onto the same scales.

2D 1H-driven 13C spin diffusion (PSSD) and 1H spin diffusion (CHHC) experiments were carried out at a spinning speed of 5.4 kHz to minimize sideband overlap and to avoid rotational resonance effects25 between directly bonded 13C labels. A 0.5°, spectral window of 20 kHz and a maximum 1τ evolution time of 11.2 ms were used. The mixing time 1τSD was 400 ms for 13C spin diffusion and 200 μs for 1H spin diffusion. For the CHHC experiment, a short 13C–1H CP contact time 1τCP of 120 μs was used before and after the 1H mixing period to ensure site-specific detection of the 1H–1H distances. The short 1τSD for the CHHC experiment minimizes the relay mechanism for strong cross-peaks.21

The 2D wide-line separation (WISE) experiment22 was used to measure 1H–1H dipolar couplings in various PG-1 samples. After 1H evolution under 13C–1H and 13C–15N dipolar couplings for a maximum of 0.13 ms, the 1H magnetization is transferred site-specifically to 13C by a 200 μs Lee-Goldburg (LG) CP period.2,3,4

13C–1H dipolar couplings between directly bonded C–H spins were measured using the 2D LG-CP experiment.25 The evolution time (1τ) is the LG-CP contact time, during which 1H spin diffusion is suppressed by the magic-angle spin lock. At short contact times (<1 ms), only directly bonded 13C–1H dipolar couplings are observed. 13C detection during 1τ resolves these 13C–1H couplings according to the 13C isotropic chemical shifts. The spinning speed was 10 kHz, and the maximum 1τ was 2.56 ms. To achieve polarization transfer, the first sideband matching condition, a1C ≈ a13C − a1H, was used, where a1C is the 13C spin-lock field strength and a13C is the 1H effective spin-lock field strength. Due to the short 1H T1s values, which make it difficult to measure small C–H couplings, we used a constant-time version of the experiment where a variable 1H LG spin-lock period was added before CP to make the total 1H spin-lock time constant.24,26

1H rotating-frame spin–lattice relaxation times (1T1s) were measured using a 13C-detected 1H LG spin-lock experiment. Again, the use of magic-angle spin lock suppresses 1H spin diffusion so that only the 1T1s of protons directly attached to the 13C is detected. The 1H spin-lock field strength was 70 kHz.

Electron Microscopy. Aliquots of incubated PG-1 solutions were applied to Formvar coated nickel grids. After adsortion for ~2 min, the excess fluid was wicked off and the samples were negatively stained by applying a drop of 1% phosphotungstic acid (PTA; pH 6.2) for ~1 min. Excess fluid was wicked off, and grids were air-dried. TEM images were collected using a JEOL 1200EX II scanning and transmission electron microscope (Japan Electron Optics Laboratory, Peabody, MA) at 80 kV and were digitally collected with a Megaview III camera and SIS Pro software (Soft Imaging Systems, Inc., Lakewood, CO).

Results

Figure 1 shows a schematic diagram of the possible modes of intermolecular packing of the β-hairpin PG-1. For simplicity, only two molecules are shown in each model, but the pattern is expected to repeat in a well-ordered aggregate on the tens of nanometer scale. In addition to the possibilities of parallel and antiparallel alignment, the β-hairpins can arrange themselves either with like strands facing each other, NCCN, or with unlike strands facing each other, NCNC. This results in four distinct packing motifs. These different modes of packing can be distinguished with suitably labeled peptides. If the peptide is labeled solely on one strand, then the presence of intermolecular cross-peaks will prove the existence of the like-strand NCCN packing. If such cross-peaks are absent, and NCNC packing is suspected, then a mixture of N-strand-labeled and C-strand-labeled peptide should give rise to intermolecular cross-peaks. To determine whether the strands align in a parallel or antiparallel fashion, the labeling positions on each strand should include both ends. Figure 1 highlights the labeled residues in two PG-1 samples: one incorporates uniformly 13C,15N-labeled F12, V14, and G17 (red), while the other contains uniformly labeled G3 and L5 (green). The figure also shows the short intermolecular distances expected for each packing motif (dashed lines): F12−V14 for NCCN parallel packing (a), F12−G17 for NCCN antiparallel packing (b), G17−L5 and possibly V14−L5 for NCNC parallel packing (c), and F12−G3, V14−G3, and V14−L5 for NCNC antiparallel packing (d).

Preparation and Characterization of Ordered PG-1 Aggregates. To obtain well-ordered PG-1 aggregates, we incubated the peptide in PBS solution for an extended period of time with gentle agitation. Representative TEM images of the resulting aggregates (Figure 2) show a network of strands that are ~10 nm wide and ~100 nm long. These are shorter and thicker than the amyloid fibrils of Aβ peptides27 and distinct in morphology. To assess the local order and secondary structure of the aggregates on the subnanometer length scale, we compared the 13C and 15N line widths and chemical shifts of the incubated and untreated peptide. Figure 3a,b shows the 13C CP-MAS spectra of [U-F12, V14, G17] PG-1 in the two different states. Several changes are observed. First, the spectral resolution is much enhanced by incubation: for example, V14 Cα and F12 Cα became much better resolved, and the C′ peak narrowed. Second, the Cα and C′ peaks in the PG-1 aggregate shifted upfield as compared to the untreated peptide, while the resolved Val Cβ shifted downfield. Based on the known 13C secondary shifts of proteins,27,28 these indicate that the incubation procedure makes the β-strand conformation of PG-1 more ideal. In comparison, the N-strand labeled peptide, [U-G3, L5] PG-1, showed less pronounced chemical shift and line width differ-

ences between the incubated and the untreated peptide, suggesting that incubation has less influence on the N-strand structure than the C-strand.

Similar to the 13C spectra, the 15N CP-MAS spectra of [U-F12, V14, G17] PG-1 show pronounced line narrowing and chemical shift changes for the aggregate sample. The most significant line narrowing occurs at G17 15N, while F12 undergoes the largest chemical shift change, ~8 ppm upfield as compared to the untreated peptide. Because this change is larger than the typical 15N secondary shift range of Phe,27 we suspect that it results from the location of Phe 15N at the β-turn, whose chemical shift trend is not as well represented in the protein database as the canonical R-helix and β-sheet structures.

Figure 4 summarizes the line widths (a) and 13C isotropic shift (b) differences between the aggregated and untreated PG-1. The aggregate sample exhibits narrower line widths and stronger β-sheet secondary shifts for most resolved sites, especially for the C-strand residues. For the untreated PG-1, residues in the middle of the strands such as V14 and L3 have narrower lines than terminal residues such as G17. The residue experiencing the most significant ordering is F12, whose Cα and Cβ line widths both decreased, while the terminal G17 Cα showed slightly increased disorder in the aggregate. Thus, the β-turn region of the peptide is most strongly structured by incubation. Taken together, the NMR chemical shifts and the microscopy data indicate that the PG-1 aggregates prepared by solution incubation are well ordered on the tens of nanometer scale but do not have the micrometer-length order typical of amyloid fibrils. Because the purpose of this study is to determine the molecular-level packing and hydrogen bonding of PG-1, the nanometer-scale order evident from the NMR line widths and chemical shifts is sufficient for further analysis using 2D 13C correlation experiments.

**Packing Motif of PG-1 Aggregates.** To determine the packing of PG-1 β-hairpins in the ordered aggregate, we carried out 2D 1H-driven 13C spin diffusion (PDSD) experiments. Figure 5 shows the spectra of [U-F12, V14, G17] PG-1 as 100% labeled aggregates (a), 20% diluted aggregates (b), and untreated 100% labeled peptide (c). A mixing time of 400 ms was used in all experiments to achieve complete exchange. The spectrum of the 100% PG-1 aggregate (Figure 5a) shows significant cross-peaks between F12 and V14 such as α→α, α→β, and α→γ. These immediately suggest that the C-strand of one β-hairpin packs closely with another C-strand, causing intermolecular spin diffusion. There are no visible V14→G17 cross-peaks and only a weak F13→G11 α→α peak, suggesting that the two C-strands are mainly aligned in a parallel fashion. Because the G11→α signal is broad and partially overlaps with the F13→β peak at room
confirming that the three labeled residues are sufficiently close. The only remaining strong cross-peaks are intra-residue ones, as the 2D spectrum of a diluted sample. Indeed, the F12–G17 peak decreased even further, to about half the intensity of the room-temperature peak (Table 1), thus confirming that the F12–G17 α–α peak is long.

To rule out the possibility that the observed F12–V14 cross-peaks are intramolecular, we measured the 2D spectrum of a 20% diluted PG-1 aggregate sample, prepared by co-dissolving 20% labeled peptide with 80% unlabeled peptide in the incubation buffer. Dilution removes intermolecular 1H–13C spin diffusion, so that any inter-residue cross-peaks in the spectra must result from intramolecular spin diffusion. Figure 5b shows the 2D spectrum of this diluted sample. Indeed, the F12–V14 cross-peaks are either significantly attenuated or disappeared. The only remaining strong cross-peaks are intra-residue ones, confirming that the three labeled residues are sufficiently separated along the β-strand not to cause intramolecular 13C spin diffusion within 400 ms.

To determine whether the NCCN parallel packing of the PG-1 aggregate is specifically caused by incubation, we measured the 2D spectrum of the untreated peptide. The spectrum (Figure 5c) shows much weaker F12–V14 cross-peaks but a stronger F12–G17 α–α peak. These indicate that in the absence of incubation, PG-1 does not adopt any preferential alignment in the solid state, but has a combination of parallel and antiparallel alignments. The fact that the F12–G17 α–α peak is visible while V14–G17 cross-peaks are not suggests that it is easier for the two ends of the β-hairpin to contact each other than for the peptide to align in an out-of-registry fashion, which is necessary for forming V14–G17 contacts.

Because cross-peaks in the long-mixing-time PDSD experiment can arise from both direct and relay transfer, we carried out a 1H spin diffusion experiment (CHHC) with a short τSD of 200 μs18,21 to verify the direct nature of the intermolecular F12–G17 contact. It has been shown that within a τSD of ∼200 μs, strong cross-peaks in the CHHC spectrum reflect direct 1H–1H distances of within ∼3 Å. Figure 6 shows the CHHC spectra of aggregated (a) and untreated (b) PG-1. The F12–V14 Hα–Hα cross-peak is strong in the aggregate but is absent in the untreated peptide. In fact, the inter-residue α–α cross-peak in the aggregate is higher than some of the intra-residue cross-peaks such as Vα–Vγ (see 1D cross sections in Figure 6c). As compared to the highest intra-residue cross-peak, Fα–Fβ, which has a distance of ∼2.5 Å, the F12–V14 cross-peak is visible while V14–G17 cross-peaks are not visible. The only visible intermolecular cross-peaks due to NCCN parallel packing are the F12–V14 cross-peak intensity is ∼70%, strongly suggesting a direct F12–V14 distance of ∼3 Å.

If the NCCN packing motif is correct, then there should be N-strand to N-strand interfaces in the PG-1 aggregate in addition to the C-strand to C-strand interfaces. To test this, we measured the 2D 13C spin diffusion spectrum of the N-strand labeled aggregate, [U-G3, L5] PG-1 (Figure 7a). The spectrum shows well-resolved and clearly visible G3–L3 C′–α and C′–γ peaks, indicating the existence of short intermolecular distances. Again, the contribution of intramolecular spin diffusion is negligible based on the 2D spectrum of a 20% diluted sample (Supporting Information). Thus, the N-strand does form hydrogen bonds with another N-strand and in a parallel fashion. However, the cross-peaks of the peptide aggregate are not significantly stronger than those of the untreated peptide (Table 1), suggesting that the N-strand is not as tightly packed as the C-strand or that the N-terminus is more disordered than the C-terminus in the aggregate.

The presence of the NCCN packing does not in itself rule out the alternative NCNC packing. To determine if the ordered PG-1 aggregate contains a mixture of NCCN and NCNC packing motifs, we prepared an equimolar mixture of N- and C-strand labeled PG-1 aggregates. If NCNC packing is present, then cross-peaks between the N-strand residues on one molecule and the C-strand residues on another molecule are expected. The 2D spectrum of this mixture is shown in Figure 7b. No N-strand to C-strand cross-peaks such as V14–L3 (dashed circles) and V14–G3 are detected. The only visible intermolecular cross-peaks are the F12–V14 peaks due to NCCN parallel packing. These F12–V14 peaks are about a factor of 2 weaker than the C-strand sample (Table 1), consistent with the 1:1 molar ratio of the two labeled peptides. Thus, NCCN parallel packing is the sole repeat motif in the ordered PG-1 sample.

Table 1 lists the normalized cross-peak intensities of the 100% N- and C-strand labeled PG-1 aggregates and their untreated equivalents, and of the 1:1 mixture. The cross-peak intensities for forming V14–G17 contacts.

J. AM. CHEM. SOC. • VOL. 127, NO. 40, 2005 13923
The 2D CHHC spectra of (a) aggregated and (b) untreated [U-F12, V14, G17] PG-1 aggregates, and (b) 1:1 mixture of [U-G3, L5] PG-1 and [U-F12, V14, G17] PG-1 aggregates. Inter-strand cross-peaks such as V14−L5 (dashed circles) are absent in (b).

Segmental Mobility of PG-1 Aggregates. The 13C line widths of the untreated and aggregated PG-1 samples (Figure 4) indicate that the F12 at the β-turn experiences the most significant line narrowing upon aggregation, while the G17 Cα signal broadened rather than narrowed, suggesting chain-end disorder in the aggregate. To determine the origin of the order and disorder in the aggregate, we measured the 1H−1H dipolar coupling, 13C−1H dipolar coupling, and 1H T1ρ of untreated and aggregated PG-1. These dynamic parameters are resolved by the 13C isotropic shifts and the use of spin-diffusion free LG-CP. Figure 8 shows the 2D 1H WISE spectrum of the aggregate (a) and its Phe cross sections (solid lines, b), which are superimposed with the cross sections of the untreated peptide.
ordered and rigid in the aggregate. The untreated PG-1 exhibits narrower 1H widths than the aggregate, indicating larger-amplitude motion. Moreover, the mobility difference increases down the Phe side chain. Both PG-1 samples are more mobile than amino acid Phe: the latter has an aromatic 1H-1H coupling of 53 kHz, as compared to 25 kHz for the aggregate sample and 9 kHz for the untreated peptide. Cooling the aggregated PG-1 to 253 K increased the 1H-1H dipolar coupling of 53 kHz, as compared to 25 kHz for the aggregate sample and 9 kHz for the untreated peptide. The untreated PG-1 exhibits narrower 1H widths than the aggregate, indicating larger-amplitude motion. Moreover, the mobility difference increases down the Phe side chain.

The 1H T1p values of F12 Hα and Hβ increased in the aggregate (Table 2), while the T1p of G17 Hα decreased. These suggest that the broadening of G17 Cα signal in the aggregate (Figure 3) results from increased microsecond-time scale motions of the C-terminus, which interfere with CP, while the opposite occurs at F12 Cα and Cβ, making the β-turn more ordered and rigid in the aggregate.

Discussion

The NMR line widths and chemical shifts and microscopy images indicate unambiguously that well-ordered PG-1 aggregates on the scale of at least tens of nanometers can be created by appropriate solution incubation. The fact that these aggregates do not show micrometer-length order may result from a combination of the highly charged nature of the peptide and the low aspect ratio of the molecule.

The cross-peak patterns in the 2D 13C correlation spectra indicate that the β-hairpins in the ordered aggregate pack and hydrogen bond in a parallel fashion with like strands facing each other. Both 1H-driven 13C spin diffusion and direct 1H spin diffusion support this conclusion. The 13C spin diffusion experiment detects C−C distances up to ~7.5 Å within a mixing time of 500 ms, as shown by a recent study of α-spectrin SH3 domain,29 while the 1H spin diffusion experiment can detect H−H distances within ~3 Å in a short t1p of ~200 ms. Thus, the absence or weakness of 13C spin-diffusion cross-peaks such as $F_{12}^\alpha$−$G_{17}$ (Figure 5a) and $V_{14}$−$L_{5}$ (Figure 7b) in the peptide aggregates indicates $13C$−$13C$ distances longer than 7.5 Å. These rule out the antiparallel packing and the alternate strand packing (NCNC) models. The strongest constraint in favor of NCCN parallel packing is the significant $F_{12}^\alpha$−$V_{14}$ cross-peak in the peptide aggregate. Although this cross-peak in the 13C spin diffusion spectrum could arise from both direct and relay transfer, the fact that in the 1H spin diffusion spectrum this $\alpha$−$\alpha$ peak is stronger than most inter- and intra-residue side chain cross-peaks (Table 1) rules out the possibility of side chain-mediated relay transfer. In addition, the untreated peptide shows clear PDSD intra-residue side chain cross-peaks (Figure 5c) but negligible $F_{12}^\alpha$−$V_{14}$ intensity, indicating that relay transfer alone is insufficient to produce backbone $\alpha$−$\alpha$ cross-peaks if the distance is large.

The fact that the N-strand $G_{3}$C−$L_{5}$α peak is lower than the $F_{12}^\alpha$−$V_{14}$ peak in the PDSD spectra is partly due to the larger isotropic shift difference between C′ and Cα, which attenuates 13C spin diffusion. It may also reflect true looser packing of the N-strand interface, which is also manifested in the less dramatic line narrowing of the N-strand residues as compared to the untreated peptide. This looseness likely results from the more hydrophilic nature of the N-strand due to the presence of an additional Arg residue (R3) in the middle of the strand (Figure 1). In comparison, the C-terminal strand is almost entirely hydrophobic, thus stabilizing the C-strand interface.
packing motif yields $F_{12}-V_{14}$ $C\alpha-C\alpha$ distances of $\sim 13\, \text{Å}$, well beyond the detection limit of $^{13}$C spin diffusion.

This NCCN packing model shows the direction of the intermolecular contacts to be sideways in the $\beta$-hairpin plane rather than perpendicular to the plane. This reflects the fact that the side chains occupy space above and below the $\beta$-hairpin plane, which makes it difficult to establish close inter-plane backbone contacts. In amyloid fibrils, the typical distances between adjacent $\beta$-sheet planes are $9-10\, \text{Å}$ according to fiber diffraction studies.\(^ {32,33}\) Such a large distance is beyond the detection limit of $^{13}$C spin diffusion. Moreover, since inter-plane packing is not driven by hydrogen bonding, any accidental close contact between $\beta$-sheet planes would be nonspecific in nature; thus the untreated peptide should show similarly strong backbone cross-peaks as the peptide aggregate if inter-sheet contact were the cause of these backbone cross-peaks. This is inconsistent with the experimental data.

The NCCN parallel alignment of PG-1 in the ordered solid-state aggregate determined from these 2D experiments is consistent with the results obtained in the membrane.\(^ {34}\) There, intermolecular $\text{C}--\text{H}$, $\text{C}--\text{N}$, and $\text{C}--\text{F}$ dipolar couplings between site-specifically labeled residues constrained the PG-1 dimer structure to be parallel with two C-strands lining the dimer interface. Thus, the PG-1 aggregate formed from solution incubation outside the membrane has similar packing and hydrogen bonding to the membrane-bound PG-1 oligomer. This suggests that the common driving force for the oligomerization of this $\beta$-hairpin peptide inside and outside the membrane is hydrogen bonding. This approach of preparing ordered aggregates may thus be useful for studying the oligomerization of other membrane-active $\beta$-sheet antimicrobial peptides whose crystal structures are not available.

It is interesting to note that a solution NMR study of PG-1 in DPC micelles\(^ {35}\) showed that the peptide forms antiparallel dimers with the C-strand lining the dimer interface. The reason for the different alignment between the micelle environment, on one hand, and the aggregate and lipid bilayer environments, on the other, is presently unclear. However, because detergent micelles are well known to impose curvature strains onto peptides, one possible reason for the difference may be the different shape anisotropies of the parallel and antiparallel PG-1 dimers. The parallel NCCN packing observed in the aggregate and in the lipid bilayer puts six Arg residues at two adjacent $\beta$-turns in close proximity, forming a strongly amphipathic structure. The electrostatic repulsion between these $\beta$-turns may make the parallel dimer a bulkier structure than the antiparallel dimer, where the Arg-rich $\beta$-turns are spaced apart. The compact antiparallel dimer structure may thus be favored in the constrained micelle environment, while the parallel packing may be stabilized in the bilayer because the stronger amphipathic structure facilitates peptide insertion into the membrane.

**Conclusion**

We demonstrated the preparation and quaternary-structure determination of well-ordered aggregates of the $\beta$-hairpin antimicrobial peptide PG-1. 2D $^{13}$C correlation experiments mediated by both $^{13}$C and $^1$H spin diffusion showed intermolecular backbone cross-peaks that are consistent with parallel packing of the $\beta$-hairpins, with like strands lining the intermolecular interface. The C-strand interfaces in the aggregate are more tightly packed and ordered than the N-strand interfaces, which may result from the stronger hydrophobic nature of the C-strand. The ordered packing of the aggregate is supported


by the reduced mobility of the Phe ring at the $\beta$-turn as compared to the untreated peptide. This is the first time a $\beta$-hairpin peptide is shown to be able to form ordered aggregates on the length scale of tens of nanometers. The hydrogen-bonding propensity of PG-1 in the solid state determined from this study sheds light on the oligomerization of this peptide in lipid bilayers, which will be presented elsewhere.\textsuperscript{34}

Acknowledgment. We thank Dr. Tracey M. Pepper for help in the electron microscopy measurements. This work is supported by the National Institutes of Health grant GM-066976 to M.H. and grants AI-22839 and AI-37945 to A.J.W.

Supporting Information Available: 2D $^{13}$C correlation spectra of [U-F\textsubscript{12}, V\textsubscript{14}, G\textsubscript{17}] PG-1 aggregates at 253 K and of 20\% diluted [U-G\textsubscript{13}, L\textsubscript{6}] PG-1. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0526665
Supporting Information

Intermolecular Packing and Alignment in an Ordered β-Hairpin Antimicrobial Peptide Aggregate from 2D Solid-State NMR

Ming Tang, Alan J. Waring, and Mei Hong

Figure S1. Lack of F₁₂-G₁₇ α–α cross peak in the 100% [U-F₁₂, V₁₄, G₁₇] PG-1 aggregate at 253 K. (a) 1D $^{13}$C spectrum of the peptide at 293 K. (b) 1D $^{13}$C spectrum of the peptide at 253 K. The G₁₇α peak has higher intensity and is better resolved from the F₁₂β signal at 253 K. (c) 2D $^{13}$C spin diffusion spectrum of the peptide aggregate at 253 K with a mixing time of 400 ms. Despite the prominent diagonal G₁₇α peak, the F₁₂-G₁₇ α–α cross peak is negligible (dashed circles), confirming that the C-strands are aligned in a parallel fashion in the aggregate.
Figure S2. (a) 2D $^{13}$C PDSD spectrum of 20% diluted and untreated [U-G$_3$, L$_5$] PG-1 at 293 K. Negligible G$_3$-L$_5$ C’-Cα cross peak is observed. (b) PDSD spectrum of the 100% [U-G$_3$, L$_5$] PG-1 aggregate is reproduced from Figure 7(a), where a clear G$_3$-L$_5$ C’-Cα cross peak is observed.