Resonance Assignment and Three-Dimensional Structure Determination of a Human α-Defensin, HNP-1, by Solid-State NMR

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Human α-defensins [human neutrophil peptides (HNPs)] are immune defense mini-proteins that act by disrupting microbial cell membranes. Elucidating the three-dimensional (3D) structures of HNPs in lipid membranes is important for understanding their mechanisms of action. Using solid-state NMR (SSNMR), we have determined the 3D structure of HNP-1 in a microcrystalline state outside the lipid membrane, which provides benchmarks for structure determination and comparison with the membrane-bound state. From a suite of two-dimensional and 3D magic-angle spinning experiments, 13C and 15N chemical shifts that yielded torsion angle constraints were obtained, while inter-residue distances were obtained to restrain the 3D fold. Together, these constraints led to the first high-resolution SSNMR structure of a human defensin. The SSNMR structure has close similarity to the crystal structures of the HNP family, with the exception of the loop region between the first and second β-strands. The difference, which is partially validated by direct torsion angle measurements of selected loop residues, suggests possible conformational variation and flexibility of this segment of the protein, which may regulate HNP interaction with the phospholipid membrane of microbial cells.

Introduction

Antimicrobial peptides (AMPs) are small cationic peptides that constitute part of the innate immune system of many plants and animals to rapidly kill invading microbial pathogens. In mammals, the two main classes of AMPs are cathelicidins and defensins.¹ While most cathelicidins are α-helical in structure, defensins contain β-strands stabilized by three disulfide bonds.²–⁴ Based on the disulfide-linkage patterns, three classes of defensins, α-, β-, and θ-defensins, are identified in vertebrates. Humans have six α-defensins that contain 29–32 residues. Four of these proteins are present in the azurophilic granules of neutrophils and are called human neutrophil peptides 1–4 (HNP-1–HNP-4).²,⁵ and two are expressed in intestinal Paneth cells and are called HD-5 and HD-6.⁶,⁷ Except for HD-6, all five human α-defensins have wide-spectrum antimicrobial activities with LD₅₀ in the microgram-per-milliliter range.⁸,⁹ Similar to most other AMPs, the main mechanism of action of HNPs is

Abbreviations used: HNP, human neutrophil peptide; 2D, two-dimensional; 3D, three-dimensional; SSNMR, solid-state NMR; AMP, antimicrobial peptide; MAS, magic-angle spinning; PEG, polyethylene glycol; DARR, dipolar-assisted rotational resonance; DQ, double-quantum; PDB, Protein Data Bank; PDSD, proton-driven spin diffusion.
believed to be permeabilization of the microbial cell membrane.\textsuperscript{10,11}

The presence of three antiparallel disulfide-stabilized $\beta$-strands makes defensins larger and more complex proteins than most other AMPs. In particular, they are natural extensions of the two-stranded $\beta$-hairpin AMPs such as protegrins from porcine leukocytes and tachyplesins from horseshoe crabs, whose membrane-bound structure and lipid interaction have been characterized in detail by solid-state NMR (SSNMR) recently.\textsuperscript{12–21} Compared to these smaller $\beta$-sheet AMPs, HNPs have weaker antimicrobial activities. Moreover, although HNP-1–HNP-3 have nearly identical sequences, differing only in the N-terminus residue, their activities differ by as much as 4-fold.\textsuperscript{8}

Understanding the activity differences among human defensins requires structural investigations in the lipid membrane. However, so far, most high-resolution structures of human defensins come from X-ray crystallography in the absence of any membrane-mimetic solvents.\textsuperscript{3,4} A few biophysical studies that investigated the interactions of defensins with lipid membranes have also been reported but did not contain high-resolution structural information.\textsuperscript{22–24}

SSNMR magic-angle spinning (MAS) NMR has been recently shown to be able to elucidate the atomic-resolution structure of $^{13}$C- and $^{15}$N-labeled proteins in microcrystalline states,\textsuperscript{25–28} in fibrillar forms,\textsuperscript{29–31} and in lipid membranes.\textsuperscript{32,33} A wide variety of two-dimensional (2D) and three-dimensional (3D) correlation techniques have been developed to obtain resonance assignment, torsion angles,\textsuperscript{34–38} and inter-atomic distances.\textsuperscript{39} Therefore, the technology is now in place to determine the full structures of human $\alpha$-defensins in lipid membranes to elucidate the mechanism of action of this important class of immune defense molecules.

In this work, we report the 3D structure determination of recombinant HNP-1, whose sequence is shown in Fig. 1, by SSNMR. We have carried out resonance assignment and internuclear distance measurements of HNP-1 in an ordered microcrystalline state, in preparation for structure determination of the protein bound to the lipid membrane, since membrane-bound proteins usually give lower-resolution spectra due to the disorder created by the lipid bilayer. The conformation of microcrystalline HNP-1 also represents the protein structure before binding to the lipid membrane and is thus necessary for understanding potential conformational changes of HNPs induced by the lipid bilayer. We compare the SSNMR structure with various published crystal structures of HNPs.\textsuperscript{3,4,40}

Results

Preparation of microcrystalline HNP-1 samples for SSNMR experiments

The SSNMR data presented here were obtained from microcrystalline samples of HNP-1 precipitated from urea solution as described in Materials and Methods. A typical sample was prepared at a protein concentration of 200–300 mg/ml. The average size of the crystals was $\sim 20 \mu$m.

Fig. 2. An image of microcrystalline HNP-1. The average size of the crystals is $\sim 20 \mu$m.

Fig. 3. 1D CP-MAS spectra of microcrystalline U-$^{13}$C, $^{15}$N-labeled HNP-1. (a) $^{13}$C spectrum, measured at 258 K at a $^{13}$C Larmor frequency of 225 MHz. (b) $^{15}$N spectrum, measured at 268 K at a $^{15}$N Larmor frequency of 60 MHz.
ed from a polyethylene glycol (PEG) solution. Two samples were prepared using different concentrations of the protein stock solution. The high-concentration stock solution (36 mg/ml) yielded microcrystals 1 h after mixing with PEG, while the low-concentration stock solution (30 mg/ml) produced microcrystals gradually in a 4-day period, with ~80% of the protein eventually precipitating from the supernatant. The slower precipitation produced larger crystals of ~20 μm diameter compared to 2 μm for the rapidly precipitated sample. Figure 2 shows an image of HNP-1 microcrystals obtained from the slower precipitation procedure.

Both microcrystalline HNP-1 samples yielded well-resolved $^{13}$C and $^{15}$N MAS spectra, shown in Fig. 3. For the rapidly precipitated sample in the 4-mm rotor, the $^{13}$C full widths at half maxima were 0.6–1.0 ppm and the $^{15}$N linewidths were 1.3–1.7 ppm. For the slowly precipitated sample in the 2.5-mm rotor, the linewidths improved to 0.4–0.8 ppm for $^{13}$C and 1.0–1.5 ppm for $^{15}$N. Faster spinning frequencies and stronger $^1$H decoupling fields further contributed to the narrower linewidths of the 2.5-mm rotor sample.

**Resonance assignment of HNP-1 by 2D and 3D SSNMR**

We first assigned the amino acid spin systems of HNP-1 using 2D $^{13}$C–$^{13}$C correlation experiments, including a dipolar-assisted rotational resonance (DARR) experiment with 40 ms mixing, and double-quantum (DQ)-filtered experiments using SPC541 and CM5RR sequences. The 40-ms DARR spectrum allowed the assignment of most amino acid types (Fig. 4a, b, and d), including Cys, Ala, Ile, Arg, Leu, Pro, and Thr, which have distinct cross-peak patterns. The aromatic residues were assigned based on the $^{13}$C$\alpha$/C$\beta$ cross peaks to the aromatic carbons. For example, the C$\gamma$ of the unique tryptophan W27 resonates at 107.8 ppm (Supplementary Fig. S1),

**Fig. 4.** Representative regions of 2D $^{13}$C–$^{13}$C correlation spectra of HNP-1. (a) Ala C$\alpha$/C$\beta$ region from a 40-ms DARR spectrum. (b) Cys C$\alpha$/C$\beta$ region from a 40-ms DARR spectrum. (c) Cys C$\alpha$/C$\beta$ region from a 0.8-ms CM5RR spectrum. (d–f) CO–C$\alpha$/C$\beta$ region: (d) 40 ms DARR spectrum, (e) 0.8 ms CM5RR spectrum, and (f) 1.5 ms CM5RR. The sign of the cross-peak intensities distinguishes the one-bond Gly CO–C$\alpha$ peaks (red, indicating negative) from the two-bond Cys CO–C$\beta$ peaks (blue, indicating positive).
which allowed the rest of the W27 spin system to be identified. For the unique glutamate E14 and glutamine Q23, the side-chain Cδ chemical shifts (181 ppm for E14 and 176 ppm for Q23) served as useful guides for identifying the chemical shifts of the other side-chain carbons.

The DQ-filtered 2D spectra confirmed the DARR assignment by exhibiting opposite intensities for one-bond and two-bond cross peaks. For example, Gly Cα and Cys Cβ, which both resonate at 40–45 ppm, are distinguished based on the positive intensities of the one-bond Gly Cα–CO peaks and the negative intensities of the two-bond Cys Cβ–CO peaks in the CM5RR spectra (Fig. 4c, e, and f). Similarly, while Q23 Cδ has similar chemical shifts to Arg Cγ in the DARR spectrum, in the DQ-filtered spectra, Q23 Cβ exhibits a positive one-bond Cα–Cβ cross peak, which is distinct from the negative two-bond Arg Cδ–Cγ cross peaks. By comparing various 2D 13C–13C spectra, we assigned all amino acid spin systems except for one Arg.

Next, we obtained sequence-specific assignment from inter-residue NCOCX and intra-residue NCACX 3D experiments. Figure 5 shows a representative strip of F2–F3 planes of the two 3D experiments for residues G18–Q23. We first identified the 15N chemical shift of residue i in the NCACX spectrum by comparing the 13C chemical shifts with those obtained from the 2D CC spectra. The 15N chemical shift of residue i+1 was then obtained from the NCOCX spectrum through its cross peaks with the 13C chemical shifts of residue i. The 15N plane of residue i+1 was then searched in the NCACX spectrum to identify the 13C chemical shifts of residue i+1.

A2, A9, T19, and C30 are clear starting points for assigning a consecutive segment of residues. The N-terminal residue A2 has a distinct 15N chemical shift of 41.7 ppm, which is folded to 106.9 ppm. Residue A9 has cross peaks with P8, which has characteristic 13C chemical shifts. Residue T19 has characteristic Cα and Cβ chemical shifts.

The four Ala residues, which are well resolved in the 2D CC spectra, were sequentially assigned in the 3D spectra. For example, A9 was assigned based on its correlation with the unique P8 and with C10, and A12 was assigned based on its correlation with the well-defined I11 and G13. For the three Gly residues, G24 was identified by inter-residue correlations Q23N–G24Cα and R25N–G24Cα in the 3D spectra and in the 100-ms DARR spectrum, while G18 was assigned based on the T19N–G18Cα correlations in the 3D spectrum.

The six Cys residues were resolved and assigned based on their cross peaks with their neighboring residues in the NCOCX spectrum. For example, C3 has characteristic cross peaks with A2 Cβ and Cα, and C5 has cross peaks with the Y4 spin system. Interestingly, C5 exhibits two Cβ chemical shifts differing by 0.9 ppm, suggesting the presence of two conformations at this site. C10 has a Cβ chemical shift of 33.4 ppm (on the TMS scale), which is slightly low for an oxidized cysteine. However, its assignment was corroborated by multiple sequential C10–A9 cross peaks and I11–C10 cross peaks. For example, C10N–A9Cα–A9Cα/Cβ cross peaks were identified in the 3D NCOCX spectrum, and sequential C10Cβ–A9Cβ, C10Cα–A9Cβ, and A9Cα–C10Cβ α cross peaks were also detected in the 100-ms DARR spectrum (Supplementary Fig. S2).

The complete 13C and 15N isotropic chemical shifts of HNP-1 are summarized in Table 1. Among the six cysteines, the C10–C30 pair has relatively upfield Cβ chemical shifts. To further verify that C10

![Fig. 5. F2–F3 strips of 3D NCACX (blue) and NCOCX (red) spectra for residues G18 to Q23, illustrating sequential resonance assignment. The strip width for the F2 dimension is 2.3 ppm. The F1 15N chemical shifts for the strips are indicated on the right end of each strip.](image-url)
and C30 are indeed disulfide-bonded, we compared the Cα and Cβ chemical shifts of all HNP-1 cysteines with literature statistical analyses of the dependence of cysteine 13C chemical shifts on the oxidation state and conformation. 43,44 On the DSS scale, the oxidized cysteines have Cβ chemical shifts of 33–51 ppm and Cα chemical shifts of 50–61 ppm. When the HNP-1 cysteine chemical shifts are also referenced to DSS (thus increasing the 13C chemical shifts in Table 1 by 1.7 ppm), we find that all six cysteines, including C10 and C30, lie in the oxidized range (Supplementary Fig. 3), thus confirming the disulfide-bonded nature of all three pairs.

Backbone dihedral angles and verification by dipolar correlation experiments

The 13C and 15N chemical shifts allowed us to determine the backbone (ϕ, ψ) torsion angles of HNP-1 using the well-known empirical relation between N, Cα, Cβ, and CO chemical shifts and protein secondary structure. 45 We used the TALOS program 16 to predict the (ϕ, ψ) angles and compared them with the values found from the crystal structure of HNP-3 3 [Protein Data Bank (PDB) code: 1DFN]. The chemical-shift-constrained torsion angles indicate three well-defined β-strands, β1, β2, and β3, separated by a type II β-turn at A12–G13 and a type I′ β-turn at Q23–G24 (Fig. 6). These secondary-structure motifs are consistent with those of the HNP-3 structure, although the boundaries of the loop or turn segments between β-strands differ slightly between HNP-3 and HNP-1.

Table 1. 13C and 15N chemical shifts of microcrystalline HNP-1 without lipids

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The 13C chemical shifts are referenced to TMS. The 13C frequencies in this table should be increased by 1.7 ppm to convert to DSS-referenced chemical shifts. The 15N chemical shifts are referenced to liquid ammonia.

a The partner cysteine involved in the disulfide bond is indicated in the parentheses.

Fig. 6. HNP-1 (ϕ, ψ) torsion angles determined from SSNMR 13C and 15N chemical shifts (filled squares). The torsion angles of HNP-3 are shown for comparison (open squares and ribbons) (PDB accession code: 1DFN). Residues with significantly different torsion angles from the crystal structure values of HNP-3 are shaded.
Among the 28 ($\phi$, $\psi$) pairs predicted by TALOS, excluding the terminal A2 and C31, 24 $\phi$ angles and 24 $\psi$ angles agree well with the crystal structure values within the experimental uncertainty. Residues with large dihedral angle discrepancies are P8, C10, Y17, G18, and Y22 (Fig. 6 and Supplementary Table 1). Both P8 and C10 lie in the loop between $\beta$1 and $\beta$2 strands and might be expected to be dynamically disordered. However, the HNP-3 crystal structure shows well-defined positions for these residues, with $B$-factors not higher than the average. Direct measurement of C–H order parameters of the HNP-1 sample by NMR (see Fig. 11 below) also indicates that the loop region in HNP-1 is not dynamic.

To verify the torsion angle differences between TALOS and the crystal structure, we conducted the HNCH experiment to directly measure the $\phi$ angles of C10 and Y22. The experiment measures the $\phi_{11}$ angle (defined as $H^\alpha-N-C^\alpha-H^\beta$, which is related to $\phi$ by $\phi_{11}+60^\circ=\phi$) by correlating the N–H and C$^\alpha$–H$^\beta$ dipolar couplings36,47 and is particularly sensitive to the $\beta$-sheet conformation, which has $\phi_{11}$ angles around $180^\circ$. To maximize the angular resolution, we implemented the N–H dipolar dephasing at the middle of the rotor period. Simulations have shown that the HNCH dipolar dephasing at the middle of the rotor period is the most sensitive to $\phi_{11}$ angle differences.48

Figure 7c shows the C$^\alpha$ region of the $^{13}$C spectra with 0 and half a rotor period of HNCH evolution. The peaks were assigned based on the 2D and 3D spectra above. Although single-site resolution is not available, most sites have consistent $\phi$ angles between the TALOS prediction and the crystal structure within $\pm 10^\circ$ (Supplementary Table 1). The only uncertain residues are C10 and Y22; thus, their $\phi_{11}$ angles can be extracted from the intensities. Specifically, the C10 C$^\alpha$ signal at 50.8 ppm overlaps with only one residue, A28, whose $\phi_{11}$ angle is $\sim 145^\circ \pm 5^\circ$, as indicated by both TALOS and the crystal structure (Fig. 7b). Thus, the calculated HNCH curve for 50.8 ppm is very sensitive to the C10 $\phi_{11}$ angle (Fig. 7d). The experimental intensity ($0.53$) is much more consistent with the TALOS prediction of a $\beta$-sheet-like $\phi_{11}$ angle of about $180^\circ$ rather than a low value of about $-130^\circ$ (Supplementary Fig. 4). Similarly, the measured HNCH intensity for the 54.5-ppm peak, which is a composite of Y22 C$^\alpha$ with five other C$^\alpha$ sites, is more consistent with the TALOS prediction than with the crystal structure, although the distinction between the two possibilities is smaller for this peak due to more resonances overlapping at this position.

**Fig. 7.** Direct measurement of $\phi$ torsion angles by HNCH. (a) Aliphatic region of the $^{13}$C spectrum. (b) C$^\alpha$ region (44–64 ppm) of the $^{13}$C spectrum, indicating the assignment of the resolved peaks and the $\phi_{11}$ torsion angles ($\phi_{11}+60^\circ=\phi$) of each residue. For most residues, the $\phi_{11}$ angles agree between TALOS and the crystal structure within $\pm 10^\circ$. However, for C10 and Y22, significant deviations exist. (c) Measured HNCH spectra with dipolar evolution time of 0 ($S_0$) and half a rotor period ($S$, shown in blue). The measured $S/S_0$ values are indicated along with calculated values. The 50.8-ppm peak has an $S/S_0$ value that is consistent with the chemical-shift-derived $\phi$ angles and not the HNP-3 value. (d) Calculated HNCH curves for the 50.8-ppm C10/A28 C$^\alpha$ peak. The A28 $\phi_{11}$ angle is fixed while the C10 $\phi_{11}$ angle is either 173$^\circ$ or 132$^\circ$. The measured HNCH intensity at the middle of the rotor period is consistent with the TALOS $\phi_{11}$ angle but not the crystal structure value.
For P8, both $\phi$ and $\psi$ angles differ between the chemical shift prediction and the crystal structure (Supplementary Table 1). The lack of H$_N$ at Pro precludes direct measurement of its $\phi$ angle. In principle, the $\psi$ angle can be measured using the NCCN technique. At present, the protein amount is insufficient for this experiment, which requires $^{13}$C double-quantum filtration in addition to $^{13}$C–$^{15}$N REDOR. Thus, future experiments are necessary to clarify the conformational discrepancy at P8. Nevertheless, based on distance constraints involving this loop, we hypothesize that the difference may be real instead of due to inaccuracy of the NMR structure determination (see below).

The torsion angle difference for Y17–G18 is also noteworthy. TALOS predicted a relatively ideal $\beta$-strand $\psi$ angle for Y17, whereas the crystal structure gives an unusual $\psi$ angle near 0°. On the other hand, the TALOS Y17 torsion angles have relatively large uncertainties ($\Delta \phi = 23^\circ$, $\Delta \psi = 20^\circ$) (Supplementary Table 1). Among the 10 pairs of torsion angles predicted, 7 pairs lie in the $\beta$-sheet region while 3 pairs lie near the $\alpha$-helical region, with $\psi$ angles of about $-20^\circ$, which are similar to the crystal structure $\psi$ value. Thus, the ensemble of NMR torsion angles for Y17 encompasses the crystal structure value.

For G18, chemical shifts predicted a $\phi$ angle of $-120^\circ$ whereas the crystal structure shows a $\phi$ angle near 180°. Since glycine residues have two H$_N$ protons, the HNCH experiment is not ideal for measuring their $\phi$ angles. The TALOS uncertainties for G18 are $\Delta \phi = 35^\circ$ and $\Delta \psi = 23^\circ$ (Supplementary Table 1), and three of the predicted pairs of angles lie in the right-handed $\alpha$-helix region of the Ramachandran diagram. Thus, the conformation of this residue is not well predicted from the chemical shifts. On the other hand, G18 is strictly conserved in all human defensins. Based on comparisons of HNP-4, HD5, and HD6 crystal structures, it has been suggested that G18 may act as a hinge for the G18–F29 $\beta$-hairpin, which has significantly different orientations in various HNPs. Thus, it remains a possibility that the G18 torsion angle difference between the NMR and crystal structures may reflect real conformational variations at this residue.

**Inter-residue distances**

To constrain the 3D fold of HNP-1, we measured distances between sequential and non-sequential residues using DARR experiments with long mixing times. Two spin diffusion mixing times, 100 ms and 200 ms, were chosen, which resulted in larger numbers of cross peaks that deteriorated the spectral resolution (Fig. 8). To bypass this problem, we used a comparative strategy to identify the long-distance correlation peaks. For each inter-residue cross peak, we first found all possible assignments that agree with the peak position to ±0.2 ppm. We then measured the distances of these inter-residue pairs in the HNP-3 crystal structure and selected the assignment with the shortest distance. For these inter-residue cross peaks, since distances between

![Fig. 8. 2D $^{13}$C–$^{13}$C DARR spectra of HNP-1 with the following mixing times: (a) 40 ms, (b) 100 ms, and (c) 200 ms. In (a), all peaks are assigned. In (b) and (c), only inter-residue and intra-residue multiple-bond correlations not observed in (a) are assigned. Red, non-sequential inter-residue correlations; blue, sequential correlations; black, intra-residue multiple-bond correlations.](image-url)
sequential residues are usually the shortest, where ambiguity arises, we gave preferences to sequential distances over medium- or long-range distances.

In this way, we identified 40 inter-residue CC correlations, among which 26 were sequential contacts, 1 was medium range (i–i(+4)), and 13 were long-range contacts (i–i+j > 4). Non-sequential correlations were observed for residues A2, Y4, R6, I7, C10, E14, R16, I21, L26, W27, F29, and C30 (Table 2). Six long-range correlation peaks were detected between β1 and β3 strands, and six contacts were detected between β2 and β3 strands. These inter-strand correlations provided important restraints to the 3D fold of the protein.

We also measured a 2D 13C–15N correlation spectrum to obtain additional inter-residue distance restraints (Fig. 9). The Gly region was the best resolved and provided many useful sequential and long-range cross peaks. P8 also gave well-resolved cross peaks due to its unique imine 15N chemical shift. The 110- to 130-ppm region is more congested; thus, we only assigned the intensity maxima and gave them tentative assignments in the same fashion as the CC constraints. The non-sequential NN contacts are included Table 2.

Since the above distance extraction strategy used the HNP-3 structure as partial input, potential differences of HNP-1 from HNP-3 can only be discerned based on missing correlations for HNP-1, and mis-assignment cannot be ruled out. To extract distance restraints in a de novo fashion, without input from the HNP-3 structure, we carried out further 3D 13C–13C correlation experiments, which removed the problem of resonance overlap.

As we report in a separate publication, the 3D CCC spectrum to obtain additional inter-residue distance restraints (Fig. 9). The Gly region was the best in the above SSNMR experiments, we calculated the HNP-1 structure using the Xplor-NIH program. We assigned the distance restraints to specific ranges based on the mixing times for the first appearance of cross peaks that correspond to well-
defined secondary structures (Table 3). Sequential Cα–Cα distances are fixed by the peptide plane geometry to be 3.8 Å and were thus placed in the range of 3.5–4.1 Å. Sequential N–N distances depend on the ψ torsion angle and fall within the range of 2.5–3.6 Å. All inter-residue peaks first observed in the 100-ms 13C–13C DARR spectrum were assigned to the range 2.5–5.4 Å, those first appearing in the 200-ms DARR spectrum were assigned to 2.5–6.3 Å, and those inter-residue peaks appearing in the 2D CHHC spectra were assigned to 2.5–7.5 Å. In addition, the few inter-residue correlations observed in the 40-ms 13C–13C DARR spectrum were assigned to the range 2.5–4.8 Å. All non-sequential 15N–15N contacts were placed in the 3.0- to 6.0-Å range.

In total, we obtained 60 inter-residue distance restraints, 56 (α, β) torsion angles, and 3 disulfide bond restraints and subjected them to structure calculation by Xplor-NIH. The 10 lowest-energy structures out of 200 calculated structures (Fig. 10a) cluster closely. Compared to the crystal structure, the average NMR structure has a backbone atomic root-mean-square deviation (RMSD) of 2.7 Å and a heavy-atom RMSD of 3.8 Å. Figure 10 compares the NMR structure ensemble with the X-ray structure of HNP-3. The NMR structure shows three well-defined β-strands connected by a long loop between the β1 and β2 strands and a tight turn between the β2 and β3 strands. Both the HNP-1 NMR structure and the HNP-3 crystal structure have the same topology: the β3 strand is inserted between the β1 and β2 strands. Beyond these similarities, we found two main differences: the β1–β2 loop has a very different orientation to the rest of the protein in the two structures, and the β2 strand has a different twist and orientation. The origins of these differences will be discussed below.

To ascertain whether large-amplitude motion exists in the HNP-1 backbone, we measured the 1H–13C one-bond dipolar couplings using a 2D LGCP experiment (Fig. 11). The spectrum shows that most backbone Cα sites have rigid-limit couplings of ~12.5 kHz after scaling by the homonuclear decoupling scaling factor. The only exception is the signal at 56 ppm, which shows a splitting of 9.8 kHz. This value corresponds to a smaller C–H order parameter of 0.78, consistent with moderate-amplitude dynamics of some of the Cα sites, including I7, Y17, and F29.

Discussion

HNP-1 conformational homogeneity and SSNMR structural quality

The microcrystalline HNP-1 samples are well ordered on the nanometer and micrometer scale. The 13C linewidths of 0.4–0.8 ppm and 15N linewidths 1.0–1.5 ppm are overall comparable to those observed in other microcrystalline proteins50 and are better than some amyloid proteins.31 The 15N linewidths are particularly sensitive to the conformational heterogeneity. Compared to one of the well-studied globular proteins, ubiquitin,30 whose

| Table 3. Structure calculation summary of HNP-1 |
|-----------------|-----------------|
| Restraints      |                 |
| Total inter-residue CC and NN restraints | 60 |
| Sequential (i–j = 1) | 36 |
| Medium range (1<|i–j|<4) | 4 |
| Long range (i–j >4) | 20 |
| β1-β3          | 6 |
| β2-β3          | 6 |
| β1-β2          | 1 |
| CC Restraints restraints in the class 2.5–4.8 Å | 4 |
| CC restraints in the class 2.5–5.4 Å | 18 |
| CC restraints in the class 2.5–6.3 Å | 5 |
| CC restraints in the class 2.5–8.0 Å | 7 |
| NN restraints in the class 2.5–4.0 Å | 10 |
| NN restraints in the class 3.0–6.0 Å | 10 |
| Total (α, β) torsional angle restraints | 56 |
| RMSD of 10 lowest-energy structures from the crystal structure (Å) | 2.7 |
| Backbone atoms | 2.7 |
| Heavy atoms | 3.8 |
| Eglobal (kcal mol⁻¹) | 264 |

Fig. 10. SSNMR structures of HNP-1 compared with the X-ray crystal structure of HNP-3. (a) Ten minimum energy SSNMR structures of HNP-1. (b) Average structure of (a). (c) Crystal structure of HNP-3. The disulfide bonds are shown in orange. (d) Comparison of the β1–β2 loop conformation between the crystal structure (red), the current SSNMR structure (blue), and the NMR structure with the additional distance restraints from the 3D CCC experiment (green).
15N linewidths were reported as 0.3–0.5 ppm, the microcrystalline HNP-1 still has residual conformational heterogeneity. However, since the eventual goal is to study HNP-1 in the lipid membrane, whose thermal disorder generally promotes conformational heterogeneity in small proteins, it is not essential to produce HNP-1 with the highest conformational homogeneity.

Variable-temperature 1D 13C and 15N spectra showed quantitatively similar intensities and linewidths in the range of 293–253 K (Supplementary Fig. 5), indicating that the HNP-1 backbone is largely immobilized at room temperature and only small-amplitude segmental motions exist. This observation is supported by the 2D LG-CP spectrum, which exhibits near rigid-limit Cα–Hα dipolar couplings for most sites. The persistence of narrow linewidths at mild low temperatures is consistent with the behavior of other microcrystalline proteins but contrasts with that of membrane peptides, which manifest gel-phase-induced line broadening in the same temperature range.

The quality of the HNP-1 structure can be compared with the SSNMR structures of other recently studied globular proteins, such as the α-spectrin SH3 domain,25 ubiquitin,26 and GB1.51 In terms of agreement with the crystal structure, the SH3 domain and HNP-1 are comparable: the SH3 domain NMR structure has a backbone RMSD of 2.6 Å from the crystal structure for the β-strand segments, while the HNP-1 NMR structure has an all-segment backbone RMSD of 2.7 Å from the crystal structure of HNP-3. However, this similarity belies the difference that a larger number of restraints were obtained (n = 292) for SH3, which corresponded to ~5 restraints per residue, and all constraints were independently extracted from NMR. In comparison, 116 restraints were obtained for HNP-1; thus, about 4 restraints were available per residue. The HNP-1 structure constraints also contain a smaller number of medium- and long-range distances (n = 24) compared to SH3 (170 non-sequential restraints).

For ubiquitin, structure determination using a combination of uniformly 13C-labeled protein and 2-13C selectively labeled protein resulted in 336 inter-residue contacts for the 76-residue protein,50 among which 149 were sequential, 74 were medium range, and 113 were long range. Combined with 122 torsion angle restraints, the high-resolution SSNMR structure had an ~1Å RMSD for secondary-structure elements compared to the crystal structure. For the 56-residue GB1, a very high resolution NMR structure was obtained from 888 unique distance restraints and dihedral angles, resulting in a backbone RMSD of 1.32 Å from one of the crystal structures.51

Conformation of the inter-strand β1–β2 loop and comparison with other human α-defensins

Compared to the structures of other human α-defensins,2 the HNP-1 NMR structure has the same overall fold, with three antiparallel β-strands arranged into a β-sheet. A tight β-hairpin connects β2 and β3 strands while a loop connects the β1 and β2 strands, so that the N- and C-termini lie close together. This structural fold separates the charged and hydrophobic regions spatially and, thus, may facilitate the insertion of the protein into the phospholipid bilayer with the hydrophobic region buried in the membrane interior while the charged region interacting with the polar surface of the bilayer and with water.3

Within the context of this general similarity, the SSNMR structure of HNP-1 shows two main differences from the crystal structures of various HNPs. First, the β2 strand in the NMR structure has less orientation change between the N- and C-terminal half compared to the crystal structure (Fig.
10b and c), and the strand twist is also less significant. We attribute these differences largely to insufficient distance restraints between the β2 strand and the rest of the protein (nine distances for seven residues). When the much larger number of inter-residue distances restraints from the 3D CCC experiment were used for structure calculation, the β2 strand was found to have a more similar twist and orientation to those of the HNP-3 structure. Thus, there appears to be no real conformational difference in the β2 strand of HNP-1 and HNP-3.

The second, more important, difference is the conformation of the loop between residues I7 and R15, which controls the relative orientation of the β1 and β2 strands. In the HNP-3 structure, the loop is bent at P8—A9 and protrudes significantly from the rest of the protein, while in the HNP-1 structure, the P8—A9 segment is extended and the loop is tugged closer to the β2 strand (Fig. 10d). Several lines of evidence suggest that these are real conformational differences between HNP-1 and HNP-3, rather than artifacts due to insufficient constraints of the NMR structure. The structural inputs for the loop region include the chemical-shift-based torsion angles (Supplementary Table 1) and 11 inter-residue distances (Table 2). The P8 and C10 torsion angles were the main factor that determined the presence or absence of a turn at this position. Although we have not directly measured the P8 ψ torsion angle due to sensitivity limitations, the fact that the C10 φ angle is confirmed by HNCH experiments to have a regular β-strand value suggests that there is a significant possibility that the P8–C10 segment in HNP-1 does not adopt a turn conformation as in HNP-3. Second, 11 inter-residue distances established close contacts between the N-terminal region of the loop (residues I7–P8) and the β3 strand (residues L26–W27), between residue C10 and residues G13–E14 within the loop, and between the end of the loop (residues E14–R15) and the β3 strand (residues F29–C30) (Table 2). Thus, this segment has a reasonable number of distance restraints. When the larger number of independently assigned restraints from 3D CCC experiments was inputted for structure calculation, the loop conformational difference largely remained (Fig. 10d), supporting the hypothesis that the conformational difference is real.

It is noteworthy that a previous 1H solution NMR structure of HNP-1 also concluded a different loop orientation.52 Similar to the current result, the solution structure showed the HNP-1 loop to be well defined by itself but that its relation to the rest of the protein was different from that of HNP-3. Moreover, when the HNP-1 solution structure was compared with the structures of the related rabbit defensins NP-2 and NP-5, it was revealed that the loop conformation also differs among these three proteins. Thus, the solution NMR study suggested that the β1–β2 loop conformation may be highly sequence-specific and may play a systematic role in regulating the activities of different α-defensins.

What might be the origin of the loop conformation difference between HNP-1 and HNP-3? It cannot result from fast motion, since the 2D LG-CP spectrum (Fig. 11) shows nearly rigid-limit C–H dipolar couplings and 13C variable-temperature spectra (Supplementary Fig. 5) also indicate little temperature dependence. Thus, this conformational difference must be attributed to intrinsic structural differences caused either by the single-residue change at the N-terminus or by external environmental factors such as the ionic content, hydration level, and intermolecular packing. Since the loop conformation difference was also seen in the solution NMR structure52 where there was no crystal packing effects or hydration problems, the single-residue difference at the N-terminus is likely the main reason for the loop conformational variability. The N-terminus residue is Ala in HNP-1, Asp in HNP-3, and absent in HNP-2, which thus starts its sequence with Cys. Thus, HNP-3 is more polar than HNP-1 or HNP-2 at the N-terminus. Correspondingly, the antimicrobial activities of HNP-1 and HNP-2 are similar but the HNP-3 activity is distinct.2 A recent study of HNP-1–HNP-3 against a panel of six bacteria of both Gram-positive and Gram-negative origins showed that the average LD50 of HNP-2 is 1.10±0.25 times that of HNP-1,8 indicating very similar potencies, while the average LD50 of HNP-3 is 2.20±1.10 that of HNP-1, indicating that HNP-3 is about 2-fold less active than HNP-1.5 Molecular dynamics simulations comparing the HNP-1–HNP-3 structures suggested that Asp2 in HNP-3 may facilitate electrostatic interactions between the two monomers of the dimer, thus making the basket-shaped dimer more compact for HNP-3 than for HNP-1 and HNP-2.53 This difference may in turn cause distinct interactions of HNPs with the lipid membrane.

Whatever the exact molecular reason for the loop conformation difference, given the tight structural constraints imposed by the disulfide bonds and the dominant β-strand motifs for these proteins, one would expect that it is precisely the non-strand residues, without backbone hydrogen bonds, that should modulate the interactions of these proteins with the lipid membrane and tune their antimicrobial activities. Indeed, preliminary spectra of membrane-bound HNP-1 containing site-specific isotopic labels showed that A12 in the loop undergoes an interesting chemical shift change between a more helical conformation and the conformation seen in the microcrystalline state (Supplementary Fig. 6). These data suggest that the β1–β2 loop conformation is sensitive to the environment, in addition to the sequence. Further comparative structural studies are necessary to fully understand the structure–activity relationship of the HNPs and elucidate the functionally relevant conformation of the β1–β2 loop. SSNMR experiments that determine the conformation, orientation, and depth of insertion of HNP-1 in lipid bilayers will be crucial for this purpose.
Materials and Methods

HNP-1 expression and microcrystalline sample preparation

Recombinant HNP-1, whose amino acid sequence is shown in Fig. 1, was obtained as a cleavage product from its precursor protein, proHNP1, which was expressed as a glutathione S-transferase fusion protein in *Escherichia coli* and folded. Uniformly $^{13}$C, $^{15}$N-enriched media Spectra 9 (Cambridge Isotope Laboratories) was used to label the protein. Briefly, the fusion protein glutathione S-transferase–proHNP1 was expressed in *E. coli* BL21 with IPTG induction. The insoluble inclusion bodies were denatured by 8 M urea and then folded in 2 M urea, 3 mM cysteine, and 0.3 mM cysteine. The folded fusion protein was dialyzed in a pH 7 buffer containing 20 mM Tris–HCl, 150 mM NaCl, and 1.5 mM CaCl$_2$. The fusion protein was then cleaved by thrombin, producing proHNP1, which was purified by reversed-phase HPLC. ProHNP1 was further cleaved by cyanogen bromide to yield the correctly folded HNP-1. The crude HNP-1 was purified by reversed-phase HPLC and analyzed by electrospray ionization mass spectrometry to confirm the mass (3634 Da). The yield of HNP-1 was ~3 mg/l culture. Antimicrobial assays confirmed the activity of the protein. For example, 100% killing of *Staphylococcus aureus* is reached at 64 μg/ml HNP-1.

Microcrystalline HNP-1 was precipitated from a PEG 400 solution containing 30 mM cacodylate and 60 mM Li$_2$SO$_4$ at pH 6.5. The PEG 400 solution was added quickly to a protein stock solution to reach a final PEG 400 concentration of 30 wt%. Two protein stock solutions, at concentrations of 30 mg/ml and 36 mg/ml, were used. The protein precipitants were centrifuged in a sealed rotor containing ~4 mg protein and the other in a 2.5-mm MAS rotor containing ~4 mg protein and the other in a 2.5-mm MAS rotor (~2.7 mg protein), were used in this study.

SSNMR spectroscopy

Most SSNMR experiments were carried out on a Bruker AVANCE-600 (14.1 T) spectrometer (Karlsruhe, Germany) using triple-resonance MAS probes. Most spectra were measured at 253–268 K under MAS frequencies of 8–15 kHz. 2D CMRSR spectra were acquired on a 900-MHz NMR spectrometer at the Harvard/MIT Center for Magnetic Resonance. The 900-MHz spectra were measured on a 2.5-mm MAS probe under 20 kHz MAS. Typical radio-frequency pulse lengths were 3.5 μs for $^{13}$C, 6.0 μs for $^{15}$N, and 2.5–4.0 μs for $^1$H. $^1$H decoupling fields were typically 70 kHz on the 4-mm rotor sample and 100 kHz on the 2.5-mm rotor sample. $^{13}$C chemical shifts were referenced externally to the $^1$H-Gly $^{13}$CO signal at 176.49 ppm on the TMS scale, and $^{15}$N chemical shifts were referenced to the $^{15}$N signal of N-acetylvaline at 122.0 ppm on the liquid ammonia scale. The TMS scale differs from the DSS scale by 1.7 ppm; thus, all $^{13}$C chemical shift values reported here should be increased by 1.7 ppm before comparing with DSS-referenced solution NMR chemical shifts. For torsion angle prediction by the TALOS software, we converted the TMS-based chemical shifts to DSS-referenced values.

2D $^{13}$C–$^{13}$C DARR experiments were acquired under 8 kHz MAS on the 4-mm rotor sample and 10–15 kHz on the 2.5-mm rotor sample. Four spectra were acquired with mixing times of 20 ms, 40 ms, 100 ms, and 200 ms. For the 4-mm rotor sample, the maximum evolution time for the indirect dimension was 5.6 ms, corresponding to 300 t$_1$ points. The number of scans per ms slice was 96 and the recycle delay was 2 s, giving an experimental time of ~16 h per 2D spectrum. For the 2.5-mm rotor sample, the maximum $^{13}$C evolution time was increased to 7.2–7.4 ms due to the better homogeneity and spectral resolution of the sample. A total of 380 t$_1$ points were measured for the 20-ms and 40-ms experiments, and 580 t$_1$ points were measured for the 100-ms and 200-ms mixing times. The experimental time was 18–26 h per 2D spectrum.

Sequence-specific $^{13}$C and $^{15}$N resonance assignment was carried out by 3D NCOCX and NCACX experiments under 8 kHz MAS at 268 K. Magnetization transfer between $^{15}$N and $^1$CO or $^{15}$N and $^{13}$C was achieved using band-selective SPECIFIC-CP. In the intra-residue NCA CX experiment, the $^1$C magnetization was selected by a 17-kHz $^{15}$N spin-lock field and a 25-kHz $^{13}$C spin lock field on resonance with $^1$C. In the inter-residue NCOCX experiment, the spin-lock fields were 27 kHz for $^{15}$N and 30 kHz for $^{13}$C on resonance with $^1$C. In the latter case, since the CO offset was 18 kHz, the effective field of the CO resonances was ~35 kHz, thus satisfying the sideband matching condition with $^{15}$N. After SPECIFIC-CP, $^{13}$C–$^{15}$N magnetization transfer occurred during a DARR mixing time, which was 40 ms for NCACX and 60 ms for NCOCX.

For the NCOCX experiment, the $^{15}$N spectral width was 4 kHz and the maximum t$_1$ evolution time was 4.75 ms, corresponding to 38 t$_1$ points. The $^{13}$CO spectral width was 2 kHz and the number of t$_1$ points was 22, giving a maximum t$_1$ of 5.25 ms. The experimental time was about 2.5 days. For the NCA CX experiment, the $^{15}$N spectral width was the same as that of NCOCX, while the $^{13}$C–$^{15}$N correlation peaks. The indirect dimension has a spectral width of 16 kHz and an acquisition time of 6.88 ms, corresponding to 220 t$_1$ points. The number of scan per t$_1$ slice was 144 and the recycle delay was 1.5 s, giving an experimental time of 40 h.

The N–H doubled HNCH experiment to determine $\phi$ torsion angles was carried out on the 2.5-mm rotor sample under 8 kHz MAS at 263 K. $^1$H homonuclear decoupling during the N–H and C–H evolution periods were achieved using the PSLG sequence. The $^{13}$C peaks of the chemical shift dimension were assigned based on the 2D and 3D correlation spectra. Although resonance overlap was unavoidable, most residues have consistent $\phi$ angles between the TALOS and the crystal structure results, thus allowing the residues of interest to be measured. For sensitivity reasons, only the intensities at 0 and half a rotor period of dipolar evolution times were measured. Simulations (Supplementary Fig. 4) indicate that the intensity at half a rotor period is the most sensitive to $\phi$ angle variations.

Structure calculation

The $^{13}$C$_{a}$, $^{13}$C$_{b}$, $^{13}$C$_{g}$, and $^{15}$N chemical shifts were inputted into the TALOS program to obtain ($\phi$, $\psi$)
torsion angles. Structure calculations were performed using the simulated annealing protocol of the Xplor-NIH program. Input constraints included 60 torsion angles, 9 distances associated with the disulfide bonds, 46 distances between sequential residues, and 24 non-sequential inter-residue distances. An ensemble of 200 structures was first calculated by performing molecular dynamics at 3500 K for 40 ps, followed by slow cooling from 3500 K to 25 K in 12.5 K steps. At each temperature, 0.4 ps of dynamics was performed using a soft square nuclear Overhauser enhancement potential. Each structure was then refined with the same protocol but with 10 ps of initial annealing using a hard square potential with the \( k_{B} T \) force constant held at 30 kcal. After refinement, the 10 lowest-energy structures were chosen to represent the final structure of HNP-1. All structures were visualized in Chimera (UCSF).

Accession numbers

The HNP-1 SSNMR structure coordinates have been deposited in the PDB (2KHT). The chemical shifts and distance restraints have been deposited in the Biological Magnetic Resonance Bank (accession number: rcsb101138).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.01.030

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