Discovery and Characterization of a Disulfide-Locked C₂-Symmetric Defensin Peptide

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Supporting Information

ABSTRACT: We report the discovery of HD5-CD, an unprecedented C₂-symmetric β-barrel-like covalent dimer of the cysteine-rich host-defense peptide human defensin 5 (HD5). Dimerization results from intermonomer disulfide exchange between the canonical α-defensin Cys₁₁⁻Cys₂⁰ (Cys³⁻Cys⁰) bonds located at the hydrophobic interface. This disulfide-locked dimeric assembly provides a new element of structural diversity for cysteine-rich peptides as well as increased protease resistance, broad-spectrum antimicrobial activity, and enhanced potency against the opportunistic human pathogen Acinetobacter baumannii.

Disulfide bonds provide structures and enable the biological functions of various naturally occurring and synthetic peptides.¹ Defensins are prominent examples of disulfide-linked peptides found in Nature.² These cysteine-rich host-defense peptides (2–5 kDa) are ribosomally synthesized by lower and higher eukaryotes, and participate in the innate immune response. In humans, oxidized defensins exhibit three structures (i.e., oxidized or reduced, various oligomeric states) that are physiologically relevant in various biological microenvironments, and precisely how these structures contribute to innate immunity or other biological phenomena, remains unclear. To address such questions in the context of the human gut, our laboratory is investigating human α-defensin 5 (HD5), which is abundantly produced by small intestinal Paneth cells and is an important contributor to mucosal immunity.²⁻⁴ This peptide exhibits broad-spectrum antimicrobial activity in vitro and may help control the composition of the resident gut microbiota.⁵⁻⁶ Here, we describe an appreciated facet of the molecular gymnastics displayed by defensins and report that HD5 has the propensity to assemble into a novel disulfide-locked C₂-symmetric covalent dimer. During an exploratory set of oxidative folding assays, 32-residue HD5 (Figure 1A,B) in its reduced (HD5ᵦ) or oxidized (HD5ᵦ) form was incubated at pH 7.0 (75 mM HEPES) under anaerobic conditions in buffers of varying redox potentials (−275 to −235 mV) defined by the GSH/GSSG redox couple. The presence of a new peak with a HPLC retention time of 13.1 min (HD5ᵦ, 20.0 min; HD5ᵦ, 15.0 min) was observed in the chromatogram of every sample (Figure 1C). This species constituted ca. 45% to 70% of the total HPLC peak area and was most abundantly formed from HD5ᵦ at −275 mV. LC-MS of the new peak (denaturing conditions) provided a deconvoluted m/z value of 7164.1, which is twice the m/z value of HD5ᵦ (m/z calc 3582.2) (Figure 1D), and thiol quantification supported no (0.55 ± 0.39) free Cys residues. Moreover, two ions corresponding to [M + SH]⁺ and [M + 7H]⁺ identified in the mass spectrum of the new species were not observed for HD5ᵦ (Supporting Information).

The paucity of other new HPLC peaks observed with peptide folding, which would be suggestive of other non-native disulfide-linked species or glutathione adducts, was striking. In total, this work suggested that anaerobic buffers containing the GSH/GSSG redox couple enable an unexpected HD5 congenor to predominate. We reasoned that the new species is a disulfide-linked covalent dimer (CD) of HD5 monomers with six disulfide bonds. We named this peptide HD5-CD. Under the anaerobic redox buffer conditions, HD5-CD formed irrespective of initial peptide concentration (5–100 µM) and in the presence of millimolar concentrations of NaCl (100 mM), MgCl₂ (10 mM), or CaCl₂ (10 mM). Using the same GSH/GSSG-containing buffer and aerobic conditions, HD5-CD also formed, albeit to a lesser degree. In total, the conditions and redox potentials that afford HD5-CD suggest that this species has the potential to exist in vivo. HD5-CD formation was attenuated when GuHCl (≥250 mM) was added to the buffer, which increased the amount of HD5ᵦ in the mixture. A standard folding protocol performed aerobically that affords HD5ᵦ in high yield employs a 10:1 GSH/GSSG ratio as well as ~2 M GuHCl. Evaluation of HPLC traces from prior HD5ᵦ preparations revealed that a peak with the HD5-CD retention time is often observed as a minor (<10%) product. Moreover, when studying oxidative folding of HD5ᵦ in the absence of GuHCl, a minor side product with a retention time of ~13.0 min was observed,⁸ which we now assign to HD5-CD. To further characterize HD5-CD, we established preparative-
scale folding conditions that afford milligram quantities of the CD in high purity (Supporting Information).

First, we conducted analytical ultracentrifugation (AUC) to ascertain the molecular weight of HD5-CD and probe its self-association properties. Sedimentation velocity experiments revealed that HD5-CD sedimented as a single species with a sedimentation coefficient of ca. 1.2 S and a diffusion coefficient of 13.6 F over a range of peptide concentrations (40–120 μM) and pH (2.0–8.0) (Supporting Information). Hydrodynamic modeling of HD5ox afforded a $S_{20,w}$ value of 0.69 S for the monomer determined by solution NMR (PDB 2LXZ) and 1.16 S for the noncovalent dimer determined by X-ray crystallography (PDB 1ZMP). The molecular weight of HD5-CD was determined to be 6985 Da with a 95% confidence interval of 6598–7330 Da from global fitting of sedimentation equilibrium data. In total, AUC confirmed the HD5-CD molecular weight and demonstrated that it does not form higher-order oligomers in aqueous solution (Figure 1E, Supporting Information), which contrasts the behavior of canonical α-defensins.

Next, we prepared $^{15}$N- and $^{15}$N,$^{13}$C-labeled HD5-CD for NMR spectroscopic investigations. The 2D $^1$H-$^{15}$N HSQC spectrum of $^{15}$N-HD5-CD contained only 24 peaks, which contrasted the 62 amide protons expected for HD5-CD. Greater than 90% of these peaks were visually assigned upon comparison with the $^1$H-$^{15}$N HSQC spectrum of HD5ox. This analysis suggested that HD5-CD adopts a symmetric structure where each protomer is similar in fold to the HD5ox monomer. $^{15}$N-$R_1$, $^{15}$N-$R_2$, and $^1$H-$^{15}$N heteronuclear NOE experiments confirmed that HD5-CD is a rigid, dimeric molecule under the NMR sample conditions (Supporting Information).

To solve the HD5-CD solution structure (Figure 2A), the standard suite of 3D triple-resonance experiments were performed on uniformly labeled $^{15}$N,$^{13}$C-HD5-CD using a nonuniform sampling protocol. The peptide was assigned to 93.5% completeness with the exception of the amide resonances of residues 11–16, the Hα of Thr17, and the Hα and Hβ of Glu14 (Supporting Information). To unambiguously identify NOEs at the dimer interface, we prepared a mixed-labeled sample by oxidative folding of a 1:1 molar ratio of $^{15}$N,$^{13}$C-HD5red and unlabeled HD5red. This procedure afforded a statistical mixture of uniformly labeled, mix-labeled, and unlabeled HD5-CD, which was employed in a 2D $^{13}$C-edited/$^{15}$N,$^{13}$C-filtered HSQC-NOESY (Figure 2B–D). In this experiment, diagonal NOE peaks, or NOEs originating from protons directly bonded to $^{13}$C-resonances, are coupled yielding two peaks offset from the chemical shift by the respective $J_{CH}$ constant (Figure 2B). In contrast, internuclear NOEs crosspeaks, in which the proton is bonded to a $^{12}$C-resonance, exhibit only a single crosspeak at the chemical shift. Numerous internuclear NOEs confirmed that HD5-CD possesses 2-fold rotational symmetry with Ile22 and Leu29 each packing against its symmetry mate in the other protomer, a feature that differs from the crystal structures of dimeric HD5ox (Supporting Information).

The HD5-CD solution structure reveals an unprecedented disulfide-locked $C_2$-symmetric β-sheet cylinder (Figure 2A). Each protomer within HD5-CD exhibits the canonical α-defensin fold composed of three antiparallel β-strands with a type 1 β-hairpin connecting β2 and β3 and a flexible loop between β1 and β2. The final collection of 20 lowest energy structures (Figure 2A) was calculated with explicit water refinement to obtain a heavy atom backbone RMSD of 0.46 Å. Each HD5-CD protomer aligns to the NMR solution structure of HD5ox (PDB 2LXZ) with a Ca RMSD of 1.36 Å. The HD5-CD disulfide topology is like that of α-defensins except that the Cys3–Cys20 (Cys10–Cys17, Figure 1B) bond is intermolecular. The intramolecular Arg6–Glu14 salt bridge of HD5ox is not observed in the HD5-CD solution structure. The NOE data indicate that the guanidino group of Arg6 is pointed...
toward, rather than away from, the β3 strand. Nevertheless, residues 11−16 appear to be in intermediate exchange on the NMR time scale (Supporting Information) and possess few inter-residue NOE restraints. Thus, it is possible that Glu14 can adopt a position consistent with formation of the canonical α-defensin salt bridge.

HD5-CD exhibits a hydrophobic interior and a hydrophilic exterior. The intermolecular disulfide bonds constrain the majority of hydrophobic residues within a 10-Å diameter β-barrel-like core. Polar moieties, including all 12 Arg residues, decorate the surface (Figure 2A). The proximity of the interprotomer β1 and β2 strands in HD5-CD provides a cylindrical array that is found to be unique among β-sheet structural families and most closely matches β-sandwich folds.

Canonical defensin scaffolds confer protease resistance and antimicrobial activity. Remarkably, HD5-CD exhibits greater stability than HD5ox (Supporting Information). HD5-CD is significantly more resistant to proteolysis, and no hydrolysis of HD5-CD was observed under the harsh double-digest conditions required for HD5ox degradation (≥10 μg/mL trypsin and chymotrypsin, 37 °C). We also found that HD5-CD is more difficult to chemically reduce than HD5ox.

The distribution of Arg residues gives HD5-CD an extensive cationic surface (Figure 2A) and we hypothesized that this feature would afford bactericidal activity. We compared the antimicrobial activity (AMA) of HD5-CD and HD5ox against 10 strains. Both peptides exhibit broad-spectrum AMA; however, some strains exhibit variable sensitivities to these peptides (Figure 3). The enhanced killing (ca. 104-fold reduction in cfu) of the opportunistic human pathogen Acinetobacter baumannii by HD5-CD relative to HD5ox is noteworthy. Multidrug resistant A. baumannii is a serious public health threat and cause of nosocomial infections with high mortality rates, and new therapeutic strategies to combat infections caused by this pathogen are needed.

In closing, HD5-CD provides, to the best of our knowledge, the first example of a disulfide-locked C2-symmetric defensin scaffold and expands the structural diversity of this peptide family. It will be interesting to ascertain whether other defensins form such covalent oligomers. The physiological relevance and practical utility of HD5-CD, as well as the design and characterization of related peptide scaffolds, also merit further investigation.

Figure 2. NMR solution structure of HD5-CD. (A) Overlay of the 20 lowest energy structures, ribbon diagrams, and structures showing disulfides and the hydrophilic residues. (B−D) 2D 13C-edited/15N,13C-filtered HSQC-NOESY indicating Ile22 packing and the intermolecular disulfide bonds (see text and Supporting Information).

Figure 3. Antimicrobial activity of HD5-CD and HD5ox (mean ± SD, n = 3).
Notes
The authors declare no competing financial interest.

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■ REFERENCES

(11) These residues are located in the flexible loop and are in intermediate exchange on the NMR time-scale.