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Self-Assembly of Catenanes from Lasso Peptides

Caitlin D. Allen[†] and A. James Link*[,†,‡]

[†]Departments of Chemical and Biological Engineering and [‡]Molecular Biology, Princeton University, Princeton, New Jersey 08544, United States

Supporting Information

ABSTRACT: Lasso peptides exist naturally in a threaded state as [1]rotaxanes, and we reasoned that lasso peptides cleaved in their loop region could serve as building blocks for catenanes. Mutagenesis of the lasso peptide microcin J25 (McJ25) with two cysteine residues followed by cleavage of the peptide with trypsin led to a [2]rotaxane structure that self-assembled into a [3]catenane and [4]catenanes at room temperature in aqueous solution. The [3]catenane represents the smallest ring size of a catenane composed solely of polypeptide segments. The NMR structure of the [3]catenane was determined, suggesting that burial of hydrophobic residues may be a driving force for assembly of the catenane structure.

Catenanes are mechanically interlocked molecules and can be constructed from small organic molecules[1,2] as well as biopolymers, both natural[3−9] and engineered.[10−13] The central challenge of synthesizing any catenane structure is establishing the threading between the catenated rings. There are two remarkable natural examples of covalently catenated proteins: the coat of the HK97 bacteriophage[9] and the citrate synthase enzyme from *Pyrobaculum aerophilum*.[6] There are also examples of engineered protein catenanes based on the bisecting U motif found in the tetramerization domain of p53.[12,13] We considered lasso peptides as a natural choice for a scaffold for the assembly of new peptide-based catenanes because these peptides already exist in a threaded state. Lasso peptides are defined by an N-terminal "ring" of 7−9 aa forged by an isopeptide bond (Figure 1A). The remaining C-terminal segment of ∼8−15 aa is threaded through the ring, forming a [1]rotaxane structure.[14,15] Previous work has shown that upon backbone scission of the "loop" region of the antimicrobial lasso peptide microcin J25 (McJ25) that the C-terminal tail remains locked in place within the 8 aa ring.[16] We reasoned that such a species would be an excellent starting material for the generation of polypeptide catenanes with much smaller ring sizes than previously described protein catenanes.

To date, there is no synthetic route to lasso peptides, so production of these peptides must be carried out in bacteria harboring a gene cluster with the lasso peptide maturation enzymes. In the case of McJ25, these maturation enzymes are found on a plasmid in some wild strains of *Escherichia coli*,[17] and heterologous systems in laboratory *E. coli* have also been developed.[18] The mutagenesis of McJ25 has been studied in depth, showing that all positions within the peptide can tolerate single amino acid substitutions[19] except for the isopeptide bond forming residues, Gly-1 and Glu-8, and Gly-2, found in the ring

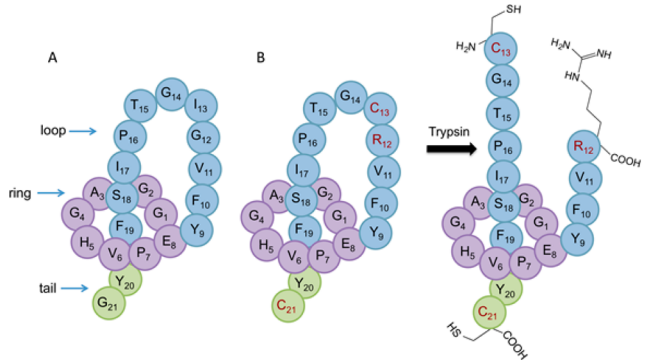

Figure 1. Structure and cleavage of McJ25. (A) Schematic of wild-type McJ25. (B) Sequence of McJ25 RCC with amino acid substitutions highlighted in red. When subjected to trypsin cleavage, the [2]rotaxane (right) is formed.

region of the peptide. Our group has previously screened saturation mutagenesis libraries of triple mutants of McJ25, showing that many triply substituted variants of McJ25 can also be produced.[20] To generate a starting material for catenanes, we introduced three amino acid substitutions into McJ25: G12R, I13C, and G21C (Figure 1B). This peptide could be cleaved by trypsin after the Arg residue, resulting in a [2]rotaxane structure with Cys residues at the N- and C-termini of the peptide thread (Figure 1B). Disulfide bonding between these Cys residues could result in a [2]catenane as well as more complex catenanes or rotaxanes formed by multimerization. Though the G21C substitution is reported to not be tolerated by the McJ25 scaffold,[19] we found that this triply substituted variant was successfully produced, albeit at lower yield than the wild-type peptide (2 mg/L culture vs ∼10 mg/L for wild-type McJ25, Figure S1). For the sake of brevity, we will refer to this peptide as McJ25 RCC.

Next we turned to trypsin cleavage of this peptide. There is precedent for cleavage of McJ25 in its loop region. Wild-type McJ25 can be cleaved by thermolysin between the F10 and V11 residues.[16] McJ25 has also been engineered to be susceptible to chymotrypsin by introducing aromatic amino acids into positions 12 and 13.[21] We found that McJ25 RCC was completely cleaved by trypsin within 24 h (Figure S2). When the trypsin cleavage reaction was carried out without reducing agents, we observed several new peaks in HPLC analysis of the reaction mixture, and three of these peaks continued to grow in over the course of 50 h, after which all of

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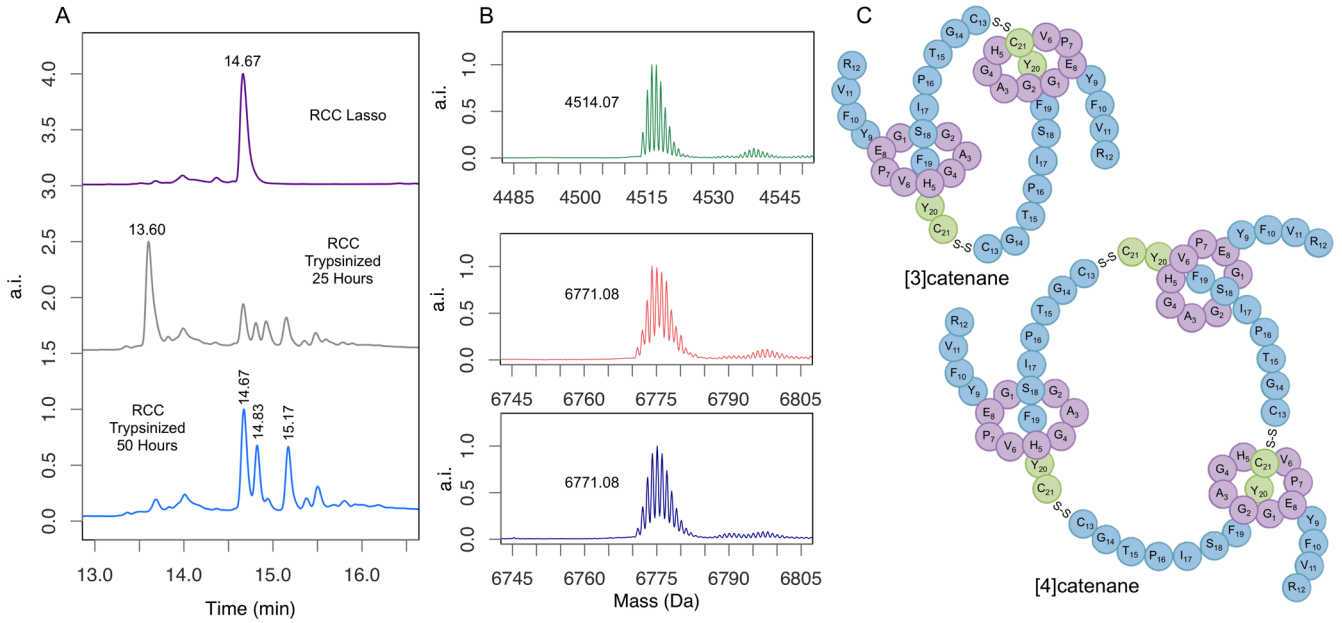

Figure 2. Assembly of [3] and [4]catenanes from MccJ25 RCC. (A) HPLC traces showing reaction of lasso peptide (top) with trypsin after 25 h (middle) and after 50 h (bottom). (B) Mass spectra of the three major peaks from 50 h reaction, where each peak was individually purified. Top is for the peak at retention time 14.67 min, middle is for the peak at 14.83 min, and bottom is for the peak at 15.17 min retention time. (C) Schematic of the [3] and [4]catenanes.

the cleaved lasso monomer was consumed (Figure 2A). There are three major new species that appear in the HPLC trace, and when treated with DTT, all of these peaks collapse back to the cleaved monomer species (Figure S3). Treatment of the same mixture with iodoacetamide results in no change in retention time of the major products, suggesting that each of these products is fully disulfide bonded (Figure S4) and thus are catenanes rather than linear rotaxane multimers.

Each of the three major products was purified using HPLC (Figure S5), and the purified peptides were analyzed using electrospray mass spectrometry. The earliest eluting peak (retention time 14.67 min) had a monoisotopic mass of 4514.02, corresponding to a doubly disulfide bonded dimer of the cleaved MccJ25 RCC peptide (Figure 2B). The two other major peaks (retention times 14.83 min and 15.17 min) both had monoisotopic masses consistent with the formation of a trimer of the lasso peptide with three disulfide bonds formed. This mass spectrometry data suggests that the cleaved MccJ25 RCC peptide oxidizes to form a linear [3]catenane and two different radial [4]catenanes (Figure 2C). The [3]catenane represents ~50% of the products formed, whereas the two [4]catenanes are made in roughly equal amounts. The [3]catenane major product consists of a center ring composed of two 9 aa peptides circularized by two disulfide bonds for a total of 58 atoms in the macrocycle. The outer rings of the [3]catenane are unchanged from the isopeptide-bonded ring in MccJ25 and thus are 26 atoms in length. This structure represents the smallest catenane formed from solely polypeptide building blocks. The two putative [4]catenanes consist of an 87-atom central ring and three 26-atom isopeptide-bonded rings. Perhaps the most remarkable aspect of these structures is that they self-assemble in water at ambient temperature with air as an oxidant.

The [3]catenane structure can potentially exist as two different isomers. The first isomer, represented in Figure 2C, has the Cys-13 residue of one lasso monomer connected to Cys-21 on the other monomer; a "head-to-tail" linkage. A head-to-head and tail-to-tail linkage is also possible, though likely sterically and energetically disfavored because this arrangement would place the isopeptide-bonded rings in close proximity (Figure S6). Likewise, there are two possible isomers of the [4]catenane (Figure S6), and we propose that both are being formed in our experiments, giving the two species with different retention times (Figure 2). The larger size of the center ring in the [4]catenane relative to the [3]catenane is more likely to allow head−head and tail−tail linkages.

Because the [3]catenane is the most abundant (and likely the most thermodynamically favorable) product in the reaction, we decided to undertake further experiments to probe its structure. Because intact MccJ25 has antimicrobial activity, the [3]-catenane was tested to determine whether that activity was retained using a spot-on-lawn assay against *Salmonella Newport*.[18] Wild-type MccJ25 and the uncleaved MccJ25 RCC peptide were also tested as controls. The [3]catenane exhibited no antimicrobial activity (Figure S7), suggesting further that the lasso structure had been converted into a new molecular entity. To analyze the orientation of the [3]catenane, we turned to a partial reduction experiment. Upon breaking a single disulfide bond, the head-to-tail linked [3]catenane would give only a single product. In contrast, partial reduction of the head-to-head/tail-to-tail isomer would give two different products (Figure S8). The HPLC trace of the products of [3]catenane treatment with reducing agent TCEP for 15 min (Figure S9) shows one peak corresponding to a singly reduced species, providing support for the notion that the [3]catenane is the head-to-tail isomer.

To build a model of the [3]catenane structure, we carried out TOCSY, NOESY, and ^{1}H-HSQC NMR experiments (Figures S10−S12). The ^{1}H-HSQC spectrum further confirmed the presence of the disulfide bond with the cysteine-13 β-carbon exhibiting a chemical shift of 38.1 ppm.[22] To assist in the identification of NOEs at the interface between the two cleaved

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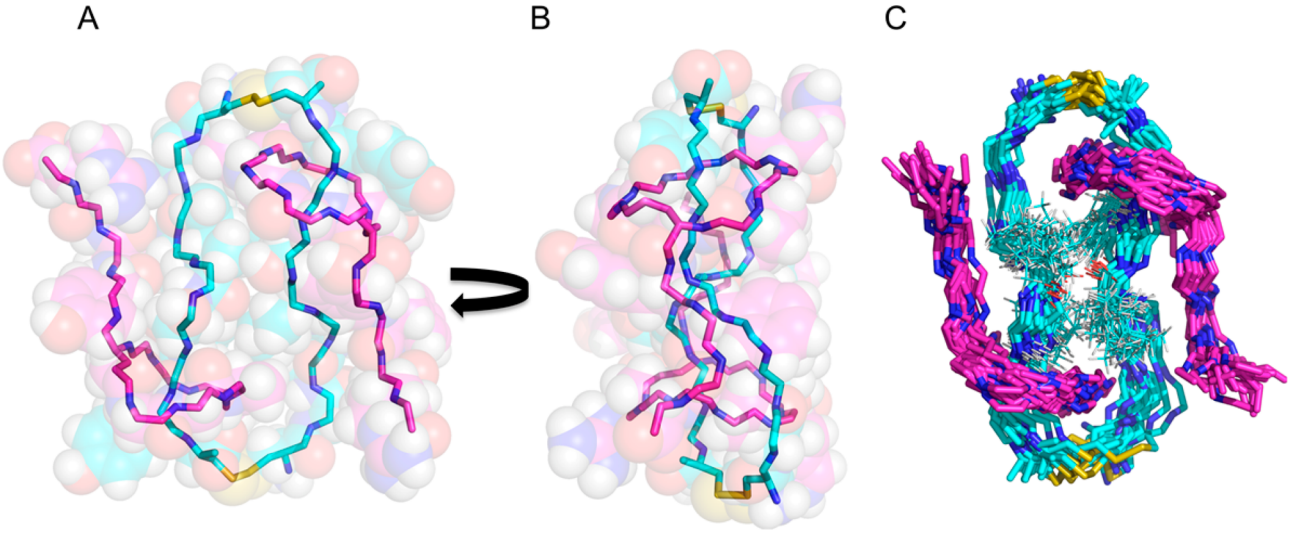

Figure 3. NMR structure of the [3]catenane. (A) Lowest energy structure of the [3]catenane showing the peptide backbone with the original MccJ25 rings in purple, and the newly formed central disulfide bonded ring in turquoise. (B) Rotation of panel A by approximately 90°. (C) The 20 lowest energy structures, where the Ile-17 and Phe-19 residues from both monomers are shown as lines, highlighting the intermonomer interactions identified in our NOEs.

lasso peptides, we turned to the software SymmDock[23,24] to generate potential models of the [3]catenane. A model of cleaved MccJ25 RCC was built in PyMol, and given as input to SymmDock to generate potential homodimers. Gratifyingly, 11/20 of the top models from SymmDock exhibited the head-to-tail orientation suggested by the partial reduction experiment described in the previous paragraph. The second highest-ranked model from SymmDock was explored further because it also places the cysteines in positions 13 and 21 in reasonably close proximity. This model revealed extensive interactions between Ile-17 of one monomer and Phe-19 of the other monomer at the interface of the lasso peptide dimer. In contrast, Ile-17 and Phe-19 do not interact appreciably in the monomer of MccJ25 in any of the three published structures.[25−27] Indeed, we observed peaks in the NOESY spectrum indicative of an interfacial interaction between the Ile-17 and Phe-19 residues (Figure S13, Table S3).

Nearly all residues were assigned in the TOCSY and NOESY spectra (Table S2), and the distance restraints were used to build a model of the [3]catenane using CYANA.[28,29] This structure was energy-minimized in GROMACS in explicit water[30,31] yielding the ensemble of structures presented in Figure 3. The structure suggests that the nonpolar Ile-17 and Phe-19 residues are forming a molten miniature hydrophobic core at the interface between the two lasso peptides, perhaps driving the self-assembly of the [3]catenane.

Here we report new [3]catenane and [4]catenane structures that self-assemble from cleaved lasso peptide building blocks under mild conditions: room temperature and atmospheric oxygen as an oxidant. To our knowledge, the [3]catenane represents the smallest ring size (58 atoms in the largest central ring) of a catenated structure forged solely from polypeptides. The smaller outer rings, at 28 atoms in length, are comparable in size to synthetic peptide rotaxanes using a proline−glycine repeat in the ring.[32] It is noteworthy that a [2]catenane could have been formed by disulfide bond formation between Cys-13 and Cys-21 within a single cleaved lasso peptide. This species was not observed, however, suggesting that this section of the peptide is too rigid or bulky to cyclize. A unique property of some catenanes is their ability to switch states under a stimulus.[33,34] The catenanes described here are not expected to exhibit any thermal switching due to the presence of the Phe-19 and Tyr-20 steric lock residues. These residues, especially Phe-19, can tolerate substitutions to smaller amino acids, opening up the possibility of engineering thermal switching in variants of the MccJ25-based catenane described here. Other lasso peptides, such as astexin-2 and variants of astexin-3,[35] are produced in a threaded form at room temperature, but unthread in minutes to hours upon heating to 95 °C. These properties make thermolabile lasso peptides excellent starting points for thermally switching peptide catenanes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b09454.

Detailed methods, supplementary figures and tables (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*ajlink@princeton.edu

Notes

The authors declare no competing financial interest.

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