Elucidating the Specificity Determinants of the AtxE2 Lasso Peptide Isopeptidase

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Lasso peptidase isopeptidase is an enzyme that specifically hydrolyzes the isopeptide bond of lasso peptides, rendering these peptides linear. To carry out a detailed structure-activity analysis of the lasso peptidase AtxE2 from Asticcaulis excentricus, we solved NMR structures of its substrates astexin-2 and astexin-3. Using in vitro enzyme assays, we show that the C-terminal tail portion of these peptides is dispensable with regards to isopeptidase activity. A collection of astexin-2 and astexin-3 variants with alanine substitutions at each position within the ring and the loop was constructed, and we showed that all of these peptides except for one were cleaved by the isopeptidase. Thus, much like the lasso peptide biosynthetic enzymes, lasso peptidase isopeptidase has broad substrate specificity. Quantitative analysis of the cleavage reactions indicated that alanine substitutions in loop positions of these peptides led to reduced cleavage, suggesting that the loop is serving as a recognition element for the isopeptidase.

Ribosomally synthesized and post-translationally modified peptides (RiPPs) (1) are a diverse set of natural products that are formed by the action of maturation enzymes on a linear peptide substrate. An emerging theme in the biosynthesis of RiPPs is that the maturation enzymes tend to have broad substrate specificity (2–8). Lasso peptides are a class of RiPPs, characterized by a threaded structure resembling a slipknot (9–11). The internal macrocycle is realized via a single isopeptide bond installed post-translationally by two maturation enzymes (12). While there is a quickly expanding literature about the lasso peptide maturation enzymes (12), there is little known about the catabolism of these molecules. Recently, we reported an enzyme, lasso peptidase isopeptidase, that specifically hydrolyzes the isopeptide bond of lasso peptides (Fig. 1A) (15). This enzyme, related to prolyl oligopeptidases, was found in the vicinity of a lasso peptide gene cluster in the freshwater α-proteobacterium Asticcaulis excentricus. This organism has two separate lasso peptide gene clusters, each with an associated isopeptidase gene (15, 16). There have been a large number of lasso peptides discovered in proteobacteria (11, 17), and an isopeptidase is commonly associated with such clusters (9).

We named the two isopeptidases in A. excentricus AtxE1 and AtxE2. The gene for AtxE1 is found next to the gene cluster that encodes for the biosynthesis of astexin-1, while the AtxE2 gene is located next to the gene cluster encoding astexins-2 and -3 (Fig. 1B) (15). We have previously characterized AtxE2 in vitro (15). This enzyme cleaves astexin-2 and astexin-3, but no cross-reactivity was observed between AtxE2 and astexin-1. Whereas astexin-2 and astexin-3 share relatively high sequence homology (identity at 13/24 positions), the astexin-1 sequence is more divergent (Fig. 1C). In addition, we have shown that, at least for astexin-2, the thermally unthreaded variant of the peptide is not a substrate for AtxE2. This suggests that the lasso peptidase isopeptidase enzyme recognizes the overall 3-dimensional fold of the peptide rather than a simple linear sequence epitope. Given these observations, we were interested in the substrate tolerance of AtxE2, and more broadly whether RiPP catabolic enzymes have the same promiscuity as RiPP maturation enzymes.

Here we have solved the NMR structure of a variant of astexin-2 with its four C-terminal amino acids removed (astexin-2 ΔC4) in water and solved a new structure of full-length astexin-3 in water. The structure of astexin-3 was previously determined in DMSO (15). In addition, we determined that the C-terminal tail portions of astexins-2 and -3 are dispensable with regards to isopeptidase activity. Finally, we carried out extensive alanine scanning mutagenesis on astexin-2 and -3 to determine the effect of these substitutions on the activity of both the maturation enzymes and the isopeptidase AtxE2. We show that both the maturation enzymes and catabolic enzyme AtxE2 are highly tolerant of different sequences.

Experimental Procedures

Materials—All cloning was performed using XL-1 Blue Escherichia coli. All expressions were performed using BL21 E. coli. Restriction enzymes were obtained from New England Biolabs. PicoMaxx DNA polymerase used for PCR was obtained from Agilent. Oligonucleotide primers used for cloning and mutagenesis were ordered from IDT.

Plasmid Construction—Previously we constructed plasmids pMM39 and pMM40 for the heterologous expression of...
astexin-3 and astexin-2, respectively (15). These plasmids are derived from pASK-75, which includes an anhydrotetracycline-inducible promoter (18). To simplify the cloning of precursor derived from pASK-75, which includes an anhydrotetracycline-pMM40 using the primers A21rep75-overlap forward and reverse and 3 fragments were joined using overlap PCR, cleaved with XbaI and EcoRI, and inserted back from pMM40 using the primers A21rep75-overlap forward and reverse and -3 Variants—
expression of astexin-2 and astexin-3 samples was dissolved in 95:5 H2O/D2O with the astexin-2 sample at 6.4 mg/ml (2.54 mM). COSY, TOCSY, NOESY, 13C-HSQC, and 15N-HSQC spectra were acquired for each peptide. Following a full proton resonance assignment based on intra and inter residue connectivity observed in the COSY, TOCSY, NOESY, and HSQC spectra, cross peak volumes from the 100 ms NOESY spectrum were integrated and calibrated using the r1/6 relation. Pseudoatoms were introduced in cases when a stereospecific assignment could not be made. The volumes associated with such peaks were scaled by the number of two-proton interactions that they represent. The average distance between prochiral β-methylene hydrogens was set to 2.11 Å as a reference using a modified version of the caliba macro in CYANA in which the r1/6 relation was enforced for all restraint types. Calibrated volumes were converted to upper distance restraints using CYANA and used as input to seven iterative cycles of NOE assignment and structure calculation (19, 20) allowing ambiguous assignment to vary. Ambiguous assignments were manually refined and the structure reannealed from 200 random starting conformers using a finalization set of restraints. The ensemble of structures was then aligned using the averaged coordinates of Cα carbons from residues 1 through 17 as template. The structures were energy-minimized in explicit solvent in GROMACS using a procedure described by Sprock et al. (21–24). Briefly, the peptide was placed in the simulation box and subsequently solvated with tip3p water. The system was simulated for 25 ps with cooling from 300 to 50 K.

Expression of AtxE2—AtxE2 used in the isopeptidase assays was expressed and purified as described previously (15). Briefly, BL21 cells harboring a pQE derivative with a C-terminally His-tagged gene for AtxE2 were grown and induced with IPTG at 37 °C. The cells were lysed via sonication, and the protein was purified on Ni-NTA resin per the manufacturer’s suggestions.

Heterologous Expression and Crude Purification of Astexin-2 and -3 Variants—For astexin-2 variants, cultures of cells containing plasmids for each variant were grown overnight in LB supplemented with 100 mg/liter ampicillin and used to inoculate 500 ml of M9 medium (3 g/liter Na2HPO4, 1.5 g/liter KH2PO4, 0.5 g/liter NH4Cl, 0.25 g/liter NaCl, 2 g/liter glucose, 1 mm MgSO4, and 500 μg/liter thiamine, supplemented with the

astexin-3 was produced at much higher levels, and the final yield of astexin-3 was 1.35 mg from 1.8 liters of culture.

NMR Studies—The purified astexin-2 and astexin-3 samples were dissolved in 95:5 H2O/D2O with the astexin-2 sample at a final concentration of 2.7 mg/ml (1.28 mM) and the astexin-3 sample at 6.4 mg/ml (2.54 mM). COSY, TOCSY, NOESY, 13C-HSQC, and 15N-HSQC spectra were acquired for each peptide. Following a full proton resonance assignment based on intra and inter residue connectivity observed in the COSY, TOCSY, NOESY, and HSQC spectra, cross peak volumes from the 100 ms NOESY spectrum were integrated and calibrated using the r1/6 relation. Pseudoatoms were introduced in cases when a stereospecific assignment could not be made. The volumes associated with such peaks were scaled by the number of two-proton interactions that they represent. The average distance between prochiral β-methylene hydrogens was set to 2.11 Å as a reference using a modified version of the caliba macro in CYANA in which the r1/6 relation was enforced for all restraint types. Calibrated volumes were converted to upper distance restraints using CYANA and used as input to seven iterative cycles of NOE assignment and structure calculation (19, 20) allowing ambiguous assignment to vary. Ambiguous assignments were manually refined and the structure reannealed from 200 random starting conformers using a finalization set of restraints. The ensemble of structures was then aligned using the averaged coordinates of Cα carbons from residues 1 through 17 as template. The structures were energy-minimized in explicit solvent in GROMACS using a procedure described by Sprock et al. (21–24). Briefly, the peptide was placed in the simulation box and subsequently solvated with tip3p water. The system was simulated for 25 ps with cooling from 300 to 50 K.

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Lasso Peptide Isopeptidase Specificity

20 amino acids (0.04 g/liter each) along with 100 mg/liter ampicillin) at OD600 0.02. The cultures were grown at 37 °C before inducing at OD600 0.2–0.3 with 200 μg/liter anhydrotetracycline (aTc) and incubation was continued at 37 °C. After 20 h, the cultures were spun down at 9,800 × g, and the pellets were washed with cold 1× PBS. Cells were then resuspended in 5 ml of methanol and lysed by vortexing with glass beads. After reaching homogeneity, the mixture was spun down at 22,700 × g for 10 min. The supernatant was collected and dried using a speed-vac. After the samples were dried, they were resuspended in 10 ml of ultrapure water and spun down at 22,700 × g to remove insoluble material. The remaining liquid was passed through a 0.2 μm filter and 9 ml were applied to Strata 1000 mg/ml C8 columns (Phenomenex), followed by a 20-ml wash with ultrapure water and elution with 10 ml of methanol. Samples were dried again using a speed-vac and resuspended in 100 μl of 50% acetonitrile in water (5000× concentrated relative to the initial culture volume) for additional studies. In the case of the astexin-2 tail truncation variants, the dried material after C8 extraction was resuspended in 500 μl to give an extract 1000× concentrated relative to the initial culture.

For the astexin-3 variants, a similar protocol was followed but with two changes. First, instead of growing at 37 °C after induction with aTc, the cells were grown at room temperature for the same amount of time to maximize the full-length form of the peptide. Second, the final extracts were resuspended in 500 μl of acetonitrile/water to make an extract 1000× concentrated relative to the initial culture.

The identity of each peptide was confirmed using MALDI mass spectrometry (MALDI-MS). The concentrated extract samples were diluted 40-fold in 50:50 acetonitrile/water and mixed 1:1 with a 2.5 mg/ml solution of α-cyano-4-hydroxycinnamic acid matrix prior to being spotted onto an Applied Biosystems (ABI) 384 Opti-TOF 123 mm × 81 mm SS plate. The samples were analyzed using an ABI 4800 MALDI-TOF-MS in positive ion mode to verify the correct product was present. For MALDI-MS on tail truncation variants, samples were diluted 80-fold, instead of 40-fold, prior to being mixed with 2.5 mg/ml solution of α-cyano-4-hydroxycinnamic acid matrix.

Measuring Production of Astexin-2 and -3 Variants in Extracts—For each of the alanine variants along with the wild-type for each peptide, 15 μl of the concentrated extract samples were injected onto a Zorbax 300-SC C18 analytical column (3.0 × 150 mm) and the following gradient at 0.75 ml/minute was applied: 10% acetonitrile for 1 min, 10–50% ACN over 19 min, 50–90% ACN over 5 min, 90% acetonitrile for 5 min, and 90–10% acetonitrile over 2 min. The traces were analyzed, and the area under the curve (AUC) corresponding to each of the variants was calculated. For astexin-2, the AUC was calculated for both the ∆C3 and ∆C4 peptide variants. For astexin-3, truncations are not observed, so the AUC was calculated for the full-length variant. Wild-type astexin-3, however, also contains a methionine at position 5, which becomes partially oxidized in the course of preparing the extract (15). Thus the AUC for peaks corresponding to the methionine sulfoxide species was also determined. Three separate cultures were processed for each variant to give error bars in the production level. For engineered astexin-3 tail truncation variants, the same procedure as above was followed with the following variations: 20 μl of the concentrated extract samples were injected onto the Zorbax 300-SC C18 analytical column.

In Vitro Isopeptidase Assays—For assays to assess cleavage by the isopeptidase AtxE2, varying amounts of concentrated extract samples of astexin-2 and astexin-3 variants in 50:50 acetonitrile/water were dried using a speed-vac. The dried material was reconstituted in 50 μl of 1× PBS pH 7.5 such that the peptide AUC was constant, resulting in concentrations of astexin-2 varying from 98.2 to 100.7 μM and concentrations of 84.1–86.7 μM for astexin-3. Purified AtxE2 enzyme was added to a final concentration of 233 nM. Reactions were incubated at 24 °C in a thermocycler (Bio-Rad DNAEngine) for 40 min before stopping the reaction by the addition of 950 μl water and application of the sample to a Strata 100 mg/ml C8 column (Phenomenex), washing with 2 ml of water, and eluting with 2 ml of methanol. The methanol extracts were dried and resuspended in 33 μl of 50% acetonitrile/water, followed by injection of 15 μl onto a Zorbax 300-SC C18 analytical column using the same gradient as described above to determine the extent of digestion by integrating the peaks corresponding to uncleaved (lasso) and cleaved (linear) peptide. The lasso and linear forms of the peptide often differ in retention time by at least 1 min under these conditions. To confirm cleavage, samples were diluted 40–200-fold, mixed with a 2.5 mg/ml solution of α-cyano-4-hydroxycinnamic acid matrix prior to being analyzed using MALDI-MS as described above. For assays on engineered astexin-2 and astexin-3 truncations, a variant of the above procedure was followed: 20 μl of the C5-purified extract was incubated with 620 nM AtxE2 for 3 h at 25 °C in a thermocycler before stopping and purifying the reaction in the same manner as the other variants.

Results

Heterologous Expression and Purification of Astexins-2 and -3—We have previously reported plasmids for the heterologous expression of astexins-2 and -3 in E. coli (15). In our previous studies, the peptides were produced at 37 °C in E. coli, leading to different patterns of C-terminal truncation. At 37 °C, astexin-3 is produced primarily as a full-length peptide, with some ∆C1 and ∆C2 variants at longer expression times. Astexin-2 is expressed at lower levels than astexin-3 and is isolated primarily as the ∆C3 and ∆C4 variants with little full-length product.

Since we were interested in maximizing peptide yield for NMR studies, we examined the expression of astexins-2 and -3 at room temperature. Cells that are shifted to room temperature after induction produce more full-length peptide than those that continue to grow at 37 °C. Expression at room temperature allows for nearly exclusive production of full-length astexin-3. Truncations of astexin-2 are observed even at room temperature, but a nearly equal amount of full-length astexin-2 is also produced. To obtain sufficient material for NMR analysis we expressed astexin-3 at room temperature and HPLC-purified the full-length species (supplemental Fig. S1A). We chose to express astexin-2 at 37 °C to maximize production of the ∆C3 and ∆C4 variants, and solved the structure of the ∆C4 variant (supplemental Fig. S1B).
Astexin-2 and Astexin-3: NMR Structures and Comparison to Astexin-1

Structural Analysis of Astexins-2 and -3 and Comparison to Astexin-1—NMR-derived models for astexin-1 in DMSO (16), astexin-1 ΔC4 in water (25), and astexin-3 in DMSO (15) have previously been published. We have also recently revisited the structure of full-length astexin-1 in aqueous solution, and this new structure (PDB file 2N68) is in good agreement with the structure previously published by Marahiel et al. (25). To date, the structure of astexin-2 has not been determined. A sample of astexin-2 ΔC4 was prepared in 95% H2O/D2O and COSY, TOCSY, NOESY, and C13-HSQC and N15-HSQC spectra were acquired. Well-dispersed signals in the amide region of the TOCSY and NOESY spectra allowed for a successful residue assignment. To permit a fair comparison of this structure to that of astexin-1 and astexin-3, a sample of astexin-3 was prepared in 95% H2O/D2O, and the same spectral set was acquired. Residue assignments for astexin-3 were similarly obtained. Supplemental Figs. S2–S5 show the TOCSY and NOESY spectra of the astexin-2 ΔC4 and astexin-3 peptides. Simulated annealing of the structures was carried out using CYANA (26), and energy minimization of the structures was realized using GROMACS with explicit water (21–24).

Ensembles consisting of the 20 lowest energy structures are presented in Fig. 2A for astexin-2 ΔC4 and Fig. 2B for astexin-3. The newly resolved astexin-2 ΔC4 model shows that the steric lock residues are amino acids 15 and 16 just as in astexin-3 (Fig. 2C). However, the steric lock residues of astexin-2 are Phe-15 and Arg-16 in contrast to astexin-3, in which the steric lock residues are Tyr-15 and Trp-16 (Fig. 2D) (15). Both astexin-2 and -3 have identical 5-residue loops with a conserved SVSGQ sequence. We calculated a backbone RMSD of 0.74 Å when aligning the first 17 residues of astexin-2 and astexin-3, showing that the overall fold of these two peptides is highly similar.

An overlay of the lowest energy structures of astexin-1, -2, and -3 from two different angles is shown in Fig. 3. Ring portions were used for alignment and are rendered as semi-transparent spheres to aid visualization. The comparison is striking in that it highlights the similarity between astexin-2 and -3 structures and their difference with astexin-1. The backbone of the rings of astexin-2 (blue) and -3 (green) are nearly planar, while the ring of astexin-1 (orange) has a kink at Pro-8 immediately preceding the isopeptide branch point at Asp-9. This sets the loop of astexin-1 in a different orientation, characterized by a sharp vertical rise that is nearly perpendicular to the plane of the ring. The loops of astexin-2 and -3 bend down first before rising and heading into the ring opening. This difference in loop structure can be attributed to the difference in loop size (4 aa versus 5 aa) and, to a lesser extent, the proline in position 8, the rigidity of which might help prime the ascent of the loop.

Effect of Astexin-2 and -3 Tail on Isopeptidase Activity—The presence of a lasso peptide isopeptidase specificity determinant in the tail portion of the astexins is not likely given that AtxE2 readily cleaves naturally occurring truncations of astexin-2 and -3 (15). To determine whether astexin-2 or -3 tail length had any effect on isopeptidase activity, we constructed genetically truncated variants of astexin-2 and astexin-3. For astexin-3, the ΔC1, ΔC2, ΔC4, ΔC5, and ΔC6 variants were all produced as judged by MALDI-MS (supplemental Fig. S6). However, HPLC analysis of cellular extracts containing these peptides revealed the expression levels of the peptides decreased with decreasing tail length. For the ΔC4, ΔC5, and ΔC6 constructs, no peptide signal was found on the HPLC using a UV detector (supplemental Fig. S7). For astexin-2, the ΔC3, ΔC4, ΔC5, and ΔC6 constructs were all produced as judged by MALDI-MS (supplemental Fig. S6). Though several of these truncated peptides were produced at levels too low to observe by HPLC, we could nonetheless determine whether these peptides were substrates for the isopeptidase using MALDI-MS. Under conditions in which the full-length astexin-2 and -3 peptides are completely present in...
cleaved (620 nM AtxE2 for 3 h), AtxE2 hydrolyzed each of the tail truncated peptides (supplemental Fig. S6). These data indicate that the tail regions of astexins-2 and -3 are dispensable with regards to the isopeptidase. However, the deletion of the tail regions greatly reduces the amount of lasso peptide produced suggesting that the tail likely plays a role in the maturation of astexins-2 and -3.

Astexins-2 and -3 Are Tolerant of Amino Acid Substitutions—
To further probe the specificity determinants of AtxE2, we systematically mutagenized residues 2–14 in astexin-2 and -3 to the amino acid alanine. The residues forming the macrocycle (G1 and D9) and the steric lock residues (residues 15 and 16) were not altered. Peptides with alanine substitutions at each of the ring positions (aa 2–8) were successfully produced for both astexin-2 and astexin-3 as judged by MALDI-MS (supplemental Figs. S8 and S9). In addition, we quantified the production of each of the alanine variants by HPLC (Fig. 4). Full-length, wild-type astexin-3 is produced with a yield of 1.83 mg/liter in our heterologous expression system. All of the astexin-3 variants were produced at levels of 20–90% of wild-type production (Fig. 4A), with the exception of the L8A variant of astexin-3 was not detected by HPLC, but was observed by MALDI-MS (supplemental Fig. S9). A, as in panel A, but for astexin-2 variants. Production of the L8A, V11A, and G13A variants of astexin-2 was too low to be observed by HPLC.

FIGURE 4. Quantitative assessment of astexin-2 and astexin-3 variant production by HPLC. A, astexin-3 variant production levels. The error bars in the bar graphs represent the standard deviation of three biological replicates. The relative production data is mapped onto a schematic of the ring and loop portions of astexins-3 in the accompanying scheme. Circles represent residues and are colored in a gradient from red (0–10% WT production) to green (>90% WT production). Line thickness of the circles represents absolute production level from 0 to 1.83 mg/liter of culture. Production of the L8A variant of astexin-3 was not detected by HPLC, but was observed by MALDI-MS (see supplemental Fig. S9). B, as in panel A, but for astexin-2 variants. Production of the L8A, V11A, and G13A variants of astexin-2 was too low to be observed by HPLC.

Lasso Peptide Isopeptidase Has Broad Substrate Specificity—
With the collection of alanine variants of astexins-2 and -3 in hand, we next turned our attention to the substrate specificity of the lasso peptide isopeptidase. We first carried out isopeptidase cleavage assays directly with cellular extracts while keeping the enzyme concentration (233 nM) and incubation time (3 h) constant. We analyzed these assays using MALDI-MS (supplemental Figs. S8 and S9). These data indicate that, just like the maturation enzymes, lasso peptide isopeptidase is highly tolerant of single amino acid substitutions in the peptide substrate. Our results show that alteration of the residue immediately preceding the isopeptide bond (Leu-8 in astexins-2 and -3) is strongly deleterious for production.

for the maturation enzymes as the L2A, Q4A, and Q6A variants are produced at higher levels than the wild-type peptide. This may be due to the fact that positions 2, 4, and 6 within the astexin-2 ring are on the less sterically congested side of the lasso peptide, away from the isopeptide bond (Fig. 4B). Astexins-2 and -3 share the same 5 aa loop sequence, SVSGQ. The V11A, G13A, and Q14A substitutions are deleterious for production of both astexin-2 and astexin-3. This finding suggests that the lasso maturation process involves recognition of specific amino acids in the loop region of the peptide. Like other lasso peptides (6, 25, 27–30), astexins-2 and -3 are highly tolerant of single amino acid substitutions. Our results show that alteration of the residue immediately preceding the isopeptide bond (Leu-8 in astexins-2 and -3) is strongly deleterious for production.

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astexin-2 and -3 were cleaved to a lesser extent than the wild-type peptides (Fig. 5). The exception to this trend is the P4A variant of astexin-3, which is cleaved more poorly than astexin-2 and astexin-3 was serving as a recognition element for the isopeptidase. Our work here indicates that this catalytic enzyme has broad substrate tolerance, a hallmark of RiPP biosynthetic enzymes. Analogous to what was observed with the maturation enzymes, alanine substitutions in the loop of astexin-3 led to decreased extents of cleavage of the peptide substrate. This finding suggests that the loop of the peptide may serve as a recognition element for the isopeptidase, an assertion that agrees with our previous data showing that unthreaded lasso peptides are not cleaved by lasso peptide isopeptidase. The broad specificity observed in RiPP biosynthetic enzymes and now in a RiPP catalytic enzyme may be a necessary feature of these enzymes. In an enzyme with a small molecule substrate, only the enzyme is subject to amino acid substitutions arising from mutations to the gene or translation errors. In the case of RiPPs, both the substrate and the enzyme are subject to such errors. Thus it is sensible that the enzymes that assemble (and disassemble) RiPPs have a degree of substrate promiscuity. In the case of lasso peptide isopeptidase, the biological role of the enzyme remains unknown. Based on the genomic context of the astexin biosynthesis genes and the isopeptidase gene, we have previously hypothesized that the astexins may be binding a cargo and that the isopeptidase releases that cargo. Another possibility is that the astexins are acting as a signaling molecule, and the isopeptidase allows for tight control of the levels of the astexins within cells. In either scenario, some substrate promiscuity in lasso peptide isopeptidase allows for proper function of the enzyme in the case that the substrate genes are mutated or mistranslated. Work to test this theory and more fully understand the catalytic activity of the isopeptidase is needed.

**Discussion**

In this study we set out to determine the sequence and structural characteristics that dictate the substrate specificity of a novel enzyme, lasso peptide isopeptidase. In order to generate a panel of substrates for these tests, we also investigated the tolerance of the lasso peptides astexin-2 and astexin-3 to alanine substitutions. As is the case with several lasso peptides, substitutions of the constituent amino acids with alanine were well-tolerated overall. The locations within astexins-2 and -3 that were least tolerant to alanine substitutions include the Leu-8 residue immediately preceding the isopeptide bond and three positions within the loop of the peptide. Since the lasso peptide loop (residues 10–14, SVSGQ for both astexin-2 and astexin-3) is converted from a linear epitope into the loop structure during lasso peptide maturation, it is conceivable that loop residues have an important role in the maturation process.

Lasso peptide isopeptidase is the first example of an enzyme that serves to catabolize a RiPP. Our work here indicates that this catalytic enzyme has broad substrate tolerance, a hallmark of RiPP biosynthetic enzymes. Analogous to what was observed with the maturation enzymes, alanine substitutions in the loop of astexin-3 led to decreased extents of cleavage of the peptide substrate. This finding suggests that the loop of the peptide may serve as a recognition element for the isopeptidase, an assertion that agrees with our previous data showing that unthreaded lasso peptides are not cleaved by lasso peptide isopeptidase. The broad specificity observed in RiPP biosynthetic enzymes and now in a RiPP catalytic enzyme may be a necessary feature of these enzymes. In an enzyme with a small molecule substrate, only the enzyme is subject to amino acid substitutions arising from mutations to the gene or translation errors. In the case of RiPPs, both the substrate and the enzyme are subject to such errors. Thus it is sensible that the enzymes that assemble (and disassemble) RiPPs have a degree of substrate promiscuity. In the case of lasso peptide isopeptidase, the biological role of the enzyme remains unknown. Based on the genomic context of the astexin biosynthesis genes and the isopeptidase gene, we have previously hypothesized that the astexins may be binding a cargo and that the isopeptidase releases that cargo. Another possibility is that the astexins are acting as a signaling molecule, and the isopeptidase allows for tight control of the levels of the astexins within cells. In either scenario, some substrate promiscuity in lasso peptide isopeptidase allows for proper function of the enzyme in the case that the substrate genes are mutated or mistranslated. Work to test this theory and more fully understand the catalytic activity of the isopeptidase is needed.
stand the biological function of the astexins and their isopeptidases is underway in our laboratory.

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