Zn-dependent bifunctional proteases are responsible for leader peptide processing of class III lanthipeptides

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Lanthipeptides are an important subfamily of ribosomally synthesized and posttranslationally modified peptides, and the removal of their N-terminal leader peptides by a designated protease(s) is a key step during maturation. Whereas proteases for class I and II lanthipeptides are well-characterized, the identity of the protease(s) responsible for class III leader processing remains unclear. Herein, we report that the class III lanthipeptide NAI-112 employs a bifunctional Zn-dependent protease, AplP, with both endo- and aminopeptidase activities to complete leader peptide removal, which is unprecedented in the biosynthesis of lanthipeptides. AplP displays a broad substrate scope in vitro by processing a number of class III leader peptides. Furthermore, our studies reveal that AplP-like proteases exist in the genomes of all class III lanthipeptide-producing strains but are usually located outside the biosynthetic gene clusters. Biochemical studies show that AplP-like proteases are universally responsible for the leader removal of the corresponding lanthipeptides. In addition, AplP-like proteases are phylogenetically correlated with aminopeptidase N from *Escherichia coli*, and might employ a single active site to catalyze both endo- and aminopeptidyl hydrolysis. These findings solve the long-standing question as to the mechanism of leader peptide processing during class III lanthipeptide biosynthesis, and pave the way for the production and bioengineering of this class of natural products.

lanthipeptide | biosynthesis | natural product | protease | ribosomal peptide

Aided by the advance of genome sequencing, ribosomally synthesized and posttranslationally modified peptides (RiPPs) have emerged as a major class of natural products over the past two decades (1, 2). The biosynthesis of RiPPs is initiated by the production of precursor peptides, which are typically composed of an N-terminal leader peptide and a C-terminal core peptide. Leader peptides are generally important as a recognition sequence for the corresponding biosynthetic machinery to install posttranslational modifications (PTMs) in core peptides. In many cases, leader peptides protect the producing organism by keeping the natural products inactive inside the host until secretion (3). As an important subfamily of RiPPs, lanthipeptides are distinguished by the presence of sulfur–to–β-carbon thioether cross-links named lanthionines (Lans) and methyllanthionines (MeLans). The installation of these thioether bridges is initiated by the dehydration of serine or threonine residues in the core peptides, followed by intramolecular Michael-type addition of cysteine thiols to the newly formed dehydroamino acids. Upon the completion of enzymatic modifications, proteolytic removal of the N-terminal leader peptides by proteases is required for lanthipeptide maturation (3, 4).

To date, four distinct classes of lanthipeptides have been characterized based on their biosynthetic machinery (*SI Appendix*, Fig. S1) (5, 6). While PTM enzymes are well-characterized for lanthipeptides, the mechanisms for their leader peptide removal are less explored. So far, two types of proteases have been characterized in class I and class II lanthipeptide biosynthesis (Fig. 1*A*). Typically, the leader cleavage of class I lanthipeptides is carried out by a subtilisin-like serine protease termed LanP (7–11), such as NisP and ElxP from the biosynthesis of nisin and epilancin 15X, respectively (12). For most class II lanthipeptides, leader removal is executed after a highly conserved Gly-Gly motif at the C terminus of leader peptides by a bifunctional $LanT_p$ enzyme, which contains an N-terminal papain-like cysteine protease domain and is also responsible for the export of the final products (13–16). A few class II lanthipeptides employ a combination of $LanT_p$ and LanP proteases to complete leader processing. For example, during lichenicidin biosynthesis, the leader peptide of modified LicA2 is first partially truncated by the C39 protease transporter $LicT_p$ after the double-Gly motif by generating NDVNPE-Licβ. Subsequently, an extracellular S8 protease, LicP, trims off the remaining six residues to produce the mature product (16).

Class III lanthipeptides are structurally characterized by triamino acid cross-links named labionin (Lab) (Fig. 1*B*) (17–22). Whereas labionin synthetases have been characterized (19, 20, 22–25), the mechanism of leader peptide removal for class III lanthipeptides remains unclear, posing a major obstacle to their production and bioengineering (21, 26). Unlike class I and class II lanthipeptides, a designated protease is missing from the biosynthetic gene clusters (BGCs) of most naturally produced class III lanthipeptides reported to date. A series of congeners containing leader overhangs of various lengths are often observed during the production of class III lanthipeptides, such as labyrinthopeptins and curvopeptins (Fig. 1*B*). It is therefore proposed

Significance

Lanthipeptides represent one of the largest classes of peptide natural products with a ribosomal origin, and their maturation requires a designated protease(s) to remove the N-terminal leader peptides after core peptide modifications are properly installed. For class III lanthipeptides, the identity of the protease(s) responsible for leader processing remains unclear, posing a major obstacle to their heterologous production and bioengineering. This study reveals that an unprecedented class of bifunctional Zn-dependent proteases is universally responsible for the leader removal of class III lanthipeptides, thereby clarifying the long-standing question regarding the biosynthesis of this emerging group of natural products. Our study should facilitate the discovery and bioengineering of class III lanthipeptides in the future.

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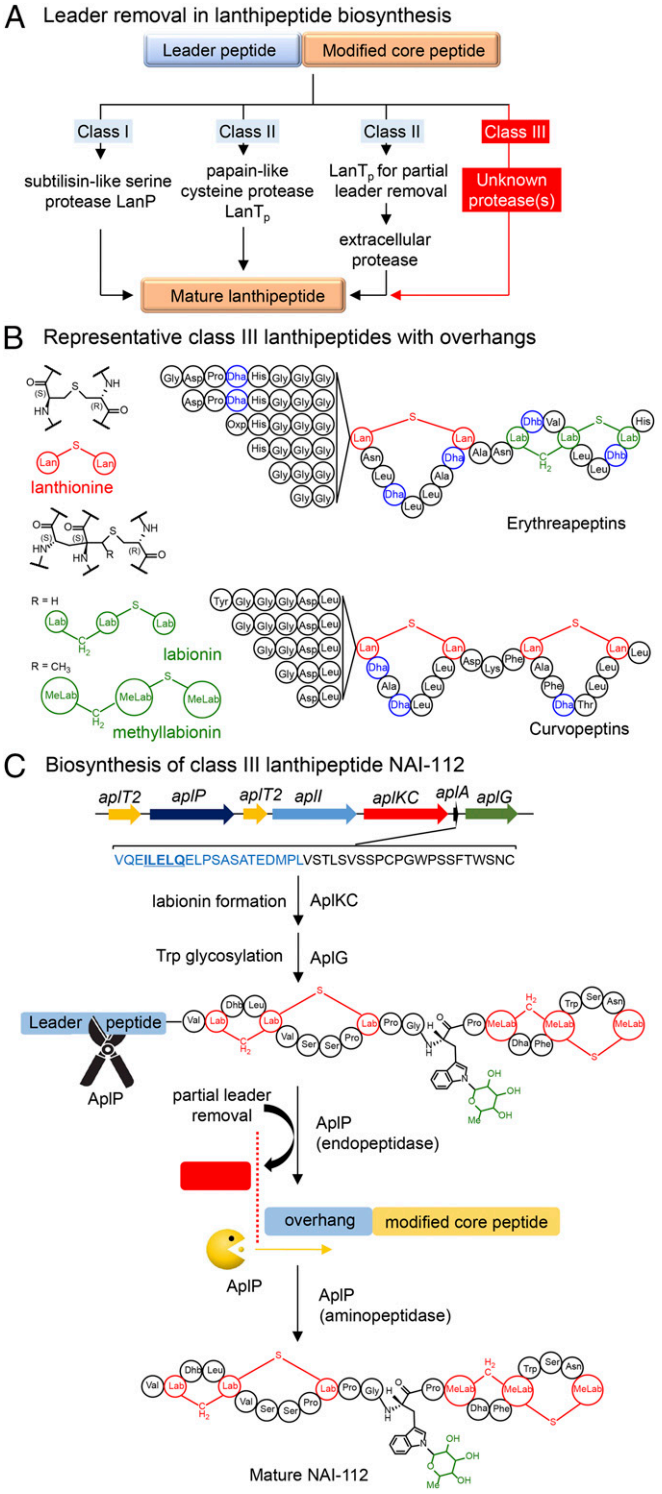

Fig. 1. Biosynthesis of class III lanthipeptide NAI-112 involves a Zn-dependent bifunctional protease, AplP. (*A*) Leader peptide removal of class I, class II, and class III lanthipeptides. (*B*) Structures of curvopeptins and labyrinthopeptins with overhangs. (*C*) Biosynthesis of the class III lanthipeptide NAI-112. The leader peptide of the precursor AplA is in blue, the core peptide is in black, and the conserved ILELQ motif in the leader sequence is underlined.

that after partial leader removal by an endopeptidase, successive degradation of the remaining overhang by an additional aminopeptidase(s) is required (21, 26). However, the identity and mechanism of these proteases are currently unknown.

Recently, Süssmuth and coworkers (27) reported the first examples of putative class III BGCs containing designated prolyl oligopeptidases (POPs), which are capable of cleaving the leader peptide of modified precursor peptides C-terminal of a Pro residue. However, it remains unclear whether POPs are the only proteases required for leader processing, since natural products from these putative BGCs have not been isolated and characterized. Furthermore, POPs are not found in the BGCs of many other class III lanthipeptides, and therefore might not be a common strategy. Thus, investigation of class III proteases is still an urgent need in understanding the key biosynthetic step of leader peptide removal.

NAI-112 is a recently discovered class III lanthipeptide produced by an *Actinoplanes* sp. strain with potent bioactivity against nociceptive pain (28). NAI-112 contains two labionin(Lab)/Methyllabionin(MeLab) motifs and a unique deoxyhexose N-linked to a tryptophan residue (Fig. 1*C* and *SI Appendix*, Fig. S2) (28). Along with the structural gene *aplA* (*Actinoplanes* lanthipeptide), two genes encoding posttranslational modification enzymes are found in the NAI-112 BGC: *aplKC*, encoding a putative class III lanthipeptide synthetase, and *aplG*, encoding a putative glycosyltransferase. Additional genes found in the BGC include *aplI*, *aplT1*, and *aplT2*, which encode putative ABC transporters with immunity and secretion functions. Interestingly, an *aplP* gene encoding a stand-alone protease, AplP, is also present in the NAI-112 BGC, suggesting a role during NAI-112 maturation (Fig. 1*C*) (29, 30). Herein, we report that AplP is a Zn-dependent bifunctional protease with both endo- and aminopeptidase activities for NAI-112 leader processing, representing an unprecedented class of proteases in the biosynthesis of lanthipeptides. AplP first cleaves the leader peptide after the highly conserved EL-Q motif as an endopeptidase, and subsequently removes the remaining residues successively through its aminopeptidase activity to generate mature NAI-112 (Fig. 1*C*). Furthermore, AplP-like proteases exist in the genomes of all class III lanthipeptide-producing organisms but are typically located outside class III BGCs. In vitro reconstitution of these proteases confirms their capability to process the leader peptides of the corresponding lanthipeptides. AplP-like proteases are phylogenetically correlated with aminopeptidase N from *Escherichia coli*, and might employ a single active site to catalyze both endo- and aminopeptidyl hydrolysis. Collectively, our studies reveal that an unprecedented class of AplP-like proteases represents a universal strategy for the leader processing of class III lanthipeptides.

Results

Reconstitution of NAI-112 Biosynthesis in *E. coli*. Our investigation was initiated by characterizing the modification enzymes in NAI-112 biosynthesis. Genes encoding AplA and AplKC were inserted into the multiple cloning site I of the pRSFDuet-1 and pACYCDuet-1 vectors, respectively, and coexpressed in *E. coli* BL21 (DE3) (*SI Appendix*, Fig. S3*A*). After purification by immobilized metal ion affinity chromatography and RP-HPLC, a peptide product with a mass corresponding to sixfold dehydrated His$_6$-tagged AplA was obtained, as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis (*SI Appendix*, Fig. S3*B*). Subsequent tandem MS analysis of this peptide product indicated the installation of two labionin rings in the core peptide (*SI Appendix*, Fig. S3*C*), thereby establishing AplKC as a bifunctional labionin synthetase that catalyzes both dehydration and cyclization. AplG is a putative GTB-type glycosyltransferase, and might be responsible for the deoxyhexose modification of Trp13. We then coexpressed His$_6$-AplA, AplKC, and AplG proteins in *E. coli* BL21 (DE3) (*SI Appendix*, Fig. S4*A*). The resulting peptide product has a mass increase of 146 Da compared with the AplKC-modified AplA, suggesting the installation of a deoxyhexose motif (*SI Appendix*, Fig. S4 *B* and *C*). Tandem MS analysis further locates the sugar modification at the Trp13

residue (*SI Appendix*, Fig. S4*D*). To determine the identity of the deoxyhexose modification in *E. coli*, authentic NAI-112– and AplKC-AplG–modified AplA peptide were hydrolyzed, derivatized, and subjected to GC-MS analysis. Results showed that the deoxyhexose in the modified AplA peptide was identical to that in authentic NAI-112 (*SI Appendix*, Fig. S5). This result implies that AplG is highly specific toward the sugar substrate, and an activated form of the corresponding deoxyhexose must be present in *E. coli*. To probe the order of PTMs during NAI-112 biosynthesis, we coexpressed AplG and His_6-AplA in *E. coli*. No glycosylation of the AplA peptide was observed, indicating that His_6-AplA peptide is not a substrate for AplG, and therefore labionin rings are essential for enzymatic recognition during glycosylation. Together, these results show that the biosynthesis of NAI-112 is initiated by the installation of labionin motifs by AplKC, which is followed by Trp glycosylation by AplG before the removal of the leader peptide.

Bifunctional Protease AplP Is Responsible for Leader Peptide Processing of NAI-112. The successful heterologous production of modified AplA peptides provided us the opportunity to investigate the function of AplP. Upon incubation with His_6-AplP heterologously produced from *E. coli*, AplKC-modified AplA was cleaved C-terminal of E-(I/L)-(L/Q) motifs (Fig. 2*A*), which is highly conserved among class III leader peptides and important for recognition by the modification enzymes (*SI Appendix*, Fig. S6) (24). S-A-(S/T) motifs appeared to be additional cleavage sites for AplP in the leader sequence. As a result, three pairs of leader and core peptide products, LP(−14)/CP(−13), LP(−8)/CP(−7), and LP(−6)/CP(−5), were generated after 4-h incubation (Fig. 2*A*). In addition, the unmodified precursor peptide His_6-AplA was cleaved by AplP with similar efficiency and primarily after E-(I/L)-(L/Q) and S-A-(S/T) motifs (*SI Appendix*, Fig. S7), indicating that the labionin structures have little impact on the enzymatic recognition by AplP. Thus, AplP acts as an endoprotease by cleaving both unmodified and modified AplA peptides, which is in contrast to the prolyl protease FlaP that specifically recognizes peptide substrates bearing labionin rings (27).

During the production of class III lanthipeptides, prolonged fermentation time usually leads to peptide products with minimal leader overhangs (21, 26), suggesting that overhang degradation by an aminopeptidase(s) might be a slow process. Therefore, we extended the in vitro incubation time of AplKC-modified AplA with AplP to 72 h. Gratifyingly, the AplKC-modified core peptide CP (NAI-112 aglycon) with complete leader removal was detected as a major product by LC-MS (Fig. 2*B*). Four peptide congeners, CP(−1) to CP(−4), as well as their derivatives with fivefold dehydration, were also identified (Fig. 2*B*). These results demonstrate that AplP is capable of removing the leader peptide completely, and strongly suggest that AplP removes leader overhangs stepwise as an aminopeptidase. To separate the endo- and aminopeptidolytic activities of AplP, peptide CP(−4) was prepared by AspN digestion of AplKC-modified AplA and subjected to AplP treatment. As expected, incubation of CP(−4) with AplP resulted in the generation of core peptides CP(−3) and CP as major products (*SI Appendix*, Fig. S8). To further characterize its aminopeptidase activity, AplP was incubated with amino acid *para*-nitroanilide (*p*NA)

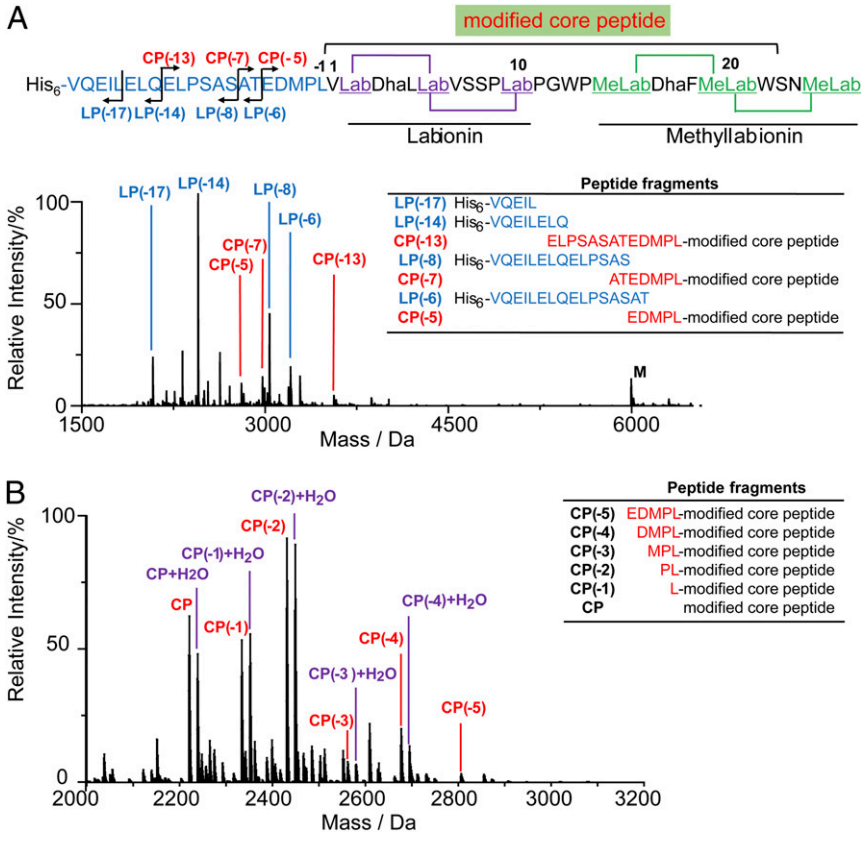

Fig. 2. AplP completely removed the leader peptide of AplKC-modified AplA through endo- and aminopeptidase activities. (*A*) AplP cleaved the leader peptide of AplKC-modified AplA as an endopeptidase after 4-h incubation. (*B*) An extended incubation time of 72 h resulted in complete removal of the AplA leader peptide. Conditions: 20 mM Tris buffer (pH 8.0), 100 μM AplKC-modified AplA, 10 μM AplP. CP, core peptide; LP, leader peptide. Numbers in parentheses represent the positions of cleavage sites. According to standard RiPP nomenclature, the first amino acid of the core peptide is designated as residue 1, while the last amino acid of the leader peptide is defined as residue (−1). Detailed MS data are provided in *SI Appendix*, Tables S1 and S2.

derivatives, which are typical aminopeptidase substrates, and the reactions were continuously monitored by UV-vis spectrometry (31). Results showed that AplP hydrolyzed L-Ala-*p*NA, L-Pro-*p*NA, and L-Leu-*p*NA efficiently by releasing *p*-nitroanilide (*SI Appendix*, Fig. S9). Bestatin, an aminopeptidase-specific inhibitor (32, 33), abolished the proteolytic activity of AplP toward L-Ala-*para*-nitroanilide, further establishing its identity as an aminopeptidase (*SI Appendix*, Fig. S10). Together, these results indicate that AplP is a class III lanthipeptide protease that completes leader removal through a two-step process by both endo- and aminopeptidase activities.

AplP Is a Zn-Dependent Protease. Bioinformatics analysis indicates that AplP belongs to the family of zinc-dependent M1-class metalloenzymes with a highly conserved zinc-binding motif, $H_{291}E_{292}XXH_{295}$-X_{18}-E_{314} (*SI Appendix*, Fig. S11). To evaluate the importance of this conserved motif for Zn binding in AplP, we generated an AplP-ΔZn mutant with four conserved amino acids (H291, E292, H295, and E314) replaced with Ala residues. The zinc content of wild-type AplP and AplP-ΔZn mutant was then analyzed by inductively coupled plasma mass spectrometry after extensive dialysis to remove loosely bound metal ions. Results showed that 0.7 equivalent of zinc was retained in the sample of wild-type AplP, whereas AplP-ΔZn contained less than 0.1 equivalent of zinc. Furthermore, mutations of Zn-binding residues completely abolished the endopeptidase activity of AplP toward AplKC-modified AplA peptide and significantly decreased its ability to hydrolyze L-Ala-*p*NA (*SI Appendix*, Fig. S10), indicating the zinc ions are important for both proteolytic activities. *o*-Phenanthroline, a metal-chelating compound, also displayed a significant inhibitory effect on L-Ala-*p*NA hydrolysis by AplP (*SI Appendix*, Fig. S10). Thus, AplP is a Zn-dependent bifunctional protease for the removal of class III leader peptides, which is unprecedented in the biosynthesis of lanthipeptides.

AplP Cleaves Leader Peptides of Other Class III Lanthipeptides. AplP cleaves AplA peptides at E-(I/L)-(L/Q) motifs, which is highly conserved in the leader sequences of class III lanthipeptides (*SI Appendix*, Fig. S6). This observation prompted us to investigate the potential of AplP as an enzymatic tool to remove leader peptides of other class III lanthipeptides in vitro. We chose AciA peptide, the precursor peptide of catenulipeptin, as a model compound and prepared modified AciA bearing two labionin rings in vitro following a procedure from the literature (20). Incubation of modified AciA with AplP in assay buffer for 3 h resulted in efficient cleavage at the conserved LLDLQ motif, and prolonged incubation ultimately generated core peptide $CP(3)_{AciA}$ with complete removal of the leader peptide (Fig. 3*A*). Similarly, the leader peptide of unmodified AciA was also cleaved efficiently by AplP (*SI Appendix*, Fig. S12), indicating that AplP is capable of processing the leader of another class III lanthipeptide. To examine the substrate tolerance of AplP, we synthesized peptides EryA-L, RamS-L, and FlaA-L, the leader peptides of erythreapeptin, SapB, and flavipeptin, respectively. Treatment of the three leader peptides with AplP resulted in efficient cleavage by generating a series of leader peptide fragments, as determined by MALDI-TOF MS analysis (Fig. 3 and *SI Appendix*, Fig. S13). Again, the conserved (L/V)-(L/F)-(D/E)-L-Q motifs were the primary cleavage sites. Together, these results demonstrate that AplP has a broad substrate scope toward class III leader peptides with no core peptide attached, which makes it a potential enzymatic tool for leader removal of class III lanthipeptides in vitro.

AplP-Like Proteases as a Universal Strategy for Leader Removal of Class III Lanthipeptides. The capability of AplP to process multiple class III leader peptides raises the possibility that the biosynthesis of these lanthipeptides employs AplP-like proteases as a universal strategy. Through genome mining, we found that all

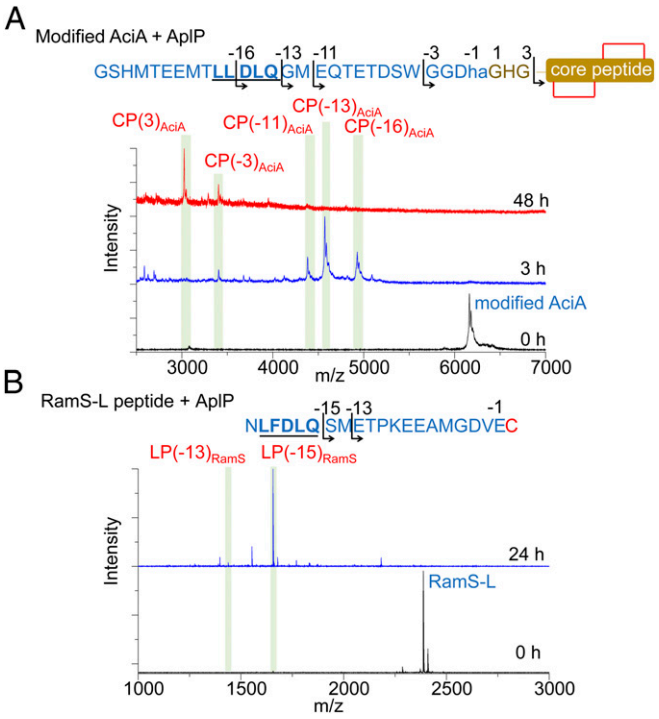

Fig. 3. AplP is able to process leader peptides of other class III lanthipeptides. (*A*) Modified AciA was cleaved by AplP. (*B*) RamS-L was cleaved by AplP. The conserved L(L/F)DLQ motif is in bold and underlined. Detailed MS data are included in *SI Appendix*, Fig. S13.

class III lanthipeptide-producing strains encode at least one AplP homolog in their genomes (*SI Appendix*, Table S3). For example, *Streptomyces coelicolor*, the SapB-producing strain, contains two AplP homologs, named SapP-1 and SapP-2, in its genome; *Saccharopolyspora erythraea* NRRL 2338, the erythreapeptin-producing strain, contains one AplP homolog, termed EryP. Three AplP-like proteases (AciP-1, AciP-2, and AciP-3) were discovered from the genome of the catenulipeptin-producing strain. All AplP homologs are putative Zn-dependent proteases containing the highly conserved HEXXH-X_{18}-E motif (*SI Appendix*, Fig. S14). The genes encoding these putative proteases are located far outside the class III BGCs, and are not associated with the biosynthetic gene clusters of any secondary metabolites after analyzing the gene contents 10 kbp up- and downstream (*SI Appendix*, Table S4).

To investigate their functions, we selected three AplP-like proteases, SapP-1, EryP, and AciP-1, and heterologously expressed them as N-terminal His_6-tagged fusion proteins. Upon incubation with SapP-1, RamS-L peptide was cleaved primarily at the conserved L-F-D-L-Q motif, generating leader fragments $LP(-17)_{RamS}$ and $LP(-14)_{RamS}$ (Fig. 4*A* and *SI Appendix*, Fig. S15), which indicates that SapP-1 indeed recognizes the leader peptide of SapB as a substrate. Extended incubation resulted in the fragments $LP(-13)_{RamS}$ and $LP(-12)_{RamS}$, suggesting that SapP-1 functioned as an aminopeptidase to trim off N-terminal amino acids of $LP(-14)_{RamS}$ after initial cleavage (Fig. 4*A* and *SI Appendix*, Fig. S15). Furthermore, a UV-vis spectrometric enzymatic assay showed that SapP-1 hydrolyzed L-Ala-*p*NA efficiently, consistent with its aminopeptidase activity (*SI Appendix*, Fig. S19*A*). Similarly, EryP and AciP-1 cleaved the EryA-L peptide and AciKC-modified AciA peptide, respectively, primarily at the conserved (L/V)-L-E-L-Q motif (*SI Appendix*, Figs. S16 and S17). In addition, both EryP and AciP-1 displayed high aminopeptidase activity toward L-Ala-*p*NA (*SI Appendix*, Fig. S19). These results indicate that AplP homologs discovered from the genomes of class III lanthipeptide-producing strains, although not

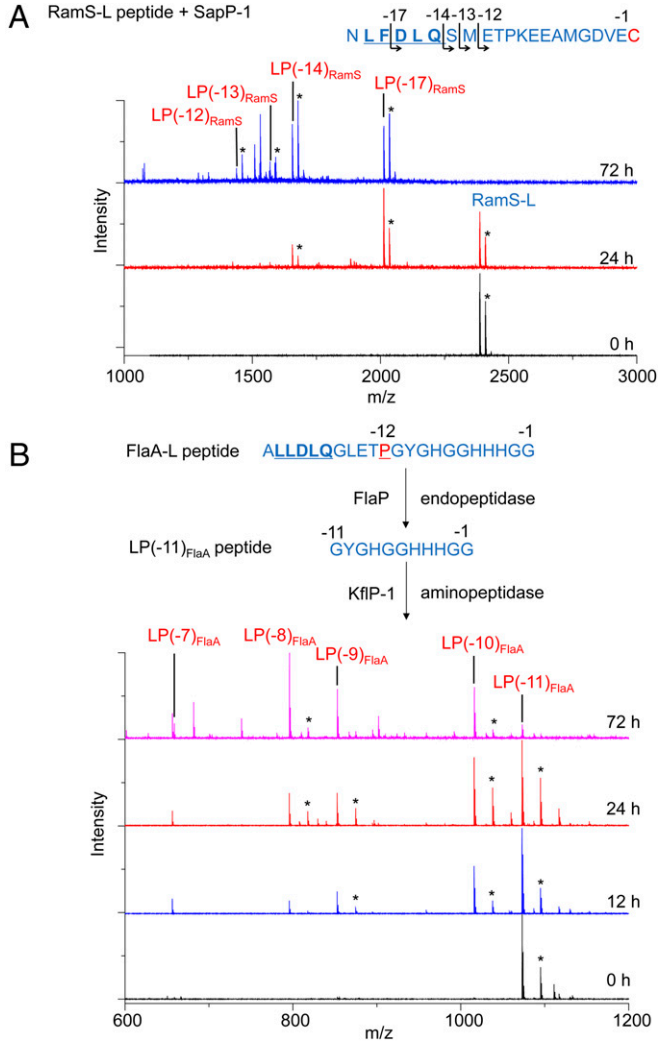

Fig. 4. AplP-like proteases are responsible for the leader processing of class III lanthipeptides. (*A*) SapP-1 cleaves RamS-L peptide, the leader peptide of SapB. (*B*) KlfP-1 truncated LP(−11)$_{FlaA}$ peptide progressively as an aminopeptidase. Asterisks in the MS spectra indicate the sodium adducts of corresponding peptide fragments.

included in the BGCs, are capable of processing the corresponding leader peptides.

The putative gene cluster of flavipeptin is of particular interest because it contains a prolyl oligopeptidase, FlaP, to partially cleave the leader peptide of modified FlaA (27). However, the removal of the remaining residues of the leader peptide remains unclear. By scanning the genome of *Kribbella flavida* DSM 17836, we discovered three AplP-like proteases, named KflP-1 to KflP-3, encoded distant from the *fla* gene cluster (*SI Appendix*, Table S5). We next attempted to express KflP-1 to KflP-3 heterologously from *E. coli*, and only KflP-1 was acquired as a soluble protein. Incubation of KflP-1 with FlaA-L, the leader peptide of FlaA, resulted in no cleavage after 48-h incubation (*SI Appendix*, Fig. S18*A*), suggesting that KflP-1 does not recognize FlaA-L for endopeptidyl hydrolysis. We further postulated that KflP-1 might function as an aminopeptidase to trim off the remaining overhang of the leader peptide after the initial cleavage by FlaP. LP(−11)$_{FlaA}$, the product of FlaP cleavage, was therefore synthesized and subjected to KflP-1 treatment. Indeed, KflP-1 was able to remove the N-terminal residues of LP(−11)$_{FlaA}$ successively as an aminopeptidase, as determined by LC-MS analysis (Fig. 4*B* and *SI Appendix*, Fig. S18*B*). Although the functions of KflP-1 and KflP-2 have not been determined yet, these results suggest that the biosynthesis of flavipeptin utilizes a combination of the POP FlaP and aminopeptidase KflP-1 to complete leader removal. Together, our studies show that AplP-like proteases are universally responsible for the leader processing of class III lanthipeptides.

Discussion

As a key step during maturation, the leader removal of class III lanthipeptides appears unique compared with class I/II compounds. It was proposed that an endopeptidase would first remove an N-terminal segment of the leader peptide, followed by the action of an aminopeptidase(s) that trims off the overhangs progressively until completion. The discovery of the prolyl oligopeptidase FlaP from the flavipeptin gene cluster supported such a proposal; however, the identity of an aminopeptidase(s) for the second stage of flavipeptin leader removal remained unknown (27). Our studies reveal that a group of zinc-dependent M1-class metalloproteases with both endo- and aminopeptidase activities is universally responsible for the leader removal of class III lanthipeptides. The involvement of this class of proteases is unprecedented in the biosynthesis of lanthipeptides, and even the entire family of RiPPs. As endopeptidases, AplP-like proteases favor the conserved E-(I/L)-(L/Q) motif in class III leader sequences as the cleavage site; as aminopeptidases, AplP-like proteases are highly tolerant toward leader peptides of various amino acid sequences and sizes.

Bioinformatics analysis reveals that AplP-like proteases are widespread in bacterial genomes containing putative class III lanthipeptide BGCs, and most of them are located far outside class III gene clusters (*SI Appendix*, Table S5). In addition, AplP-like proteases are also found in host strains for heterologous production of class III lanthipeptides (*SI Appendix*, Table S6), such as *Streptomyces lividans* TK24, which explains why class III leader peptides could be at least partially removed in these systems (26). Similar to class III lanthipeptides, class IV lanthipeptides often lack a designated gene encoding a protease in their BGCs, and products bearing leader overhangs of various lengths are isolated from the broth of producing strains (34, 35). Through genome mining, we discovered multiple AplP homologs from class IV lanthipeptide-producing strains *Streptomyces venezuelae* and *Streptomyces collinus* Tu 365 (*SI Appendix*, Table S7), suggesting that class IV might also employ AplP homologs for leader peptide removal. Furthermore, phylogenetic analysis shows that AplP-like proteases group into separate polyphyletic clades compared with class I and class II lanthipeptide proteases (*SI Appendix*, Fig. S20), suggesting that they have evolved independently for class III and class IV lanthipeptides. Interestingly, phylogenetic analysis reveals that AplP-like proteases are correlated with ePepN (*SI Appendix*, Fig. S21), which is the major aminopeptidase in *E. coli* involved in ATP-dependent downstream processing during cytosolic protein degradation (31, 36). ePepN shares ∼30% sequence similarity with AplP, including a thermolysin-like domain with a catalytic zinc-binding HEXXH-X$_{18}$-E motif (30). Although there are conflicting reports regarding whether ePepN possesses endopeptidase activities (31, 37), we observed that ePepN was able to cleave one out of three His$_6$-tagged class III peptide substrates under assay conditions (*SI Appendix*, Fig. S22), and the cleavage site is not at the conserved LLDLQ motif preferred by AplP-like proteases. Combined with previous reports, our data suggest that besides high aminopeptidase activity, ePepN possesses limited endopeptidase activity with a narrow substrate scope. These results agree with phylogenetic analysis that AplP-like proteases are phylogenetically correlated with ePepN but might have gained enhanced endopeptidase activities toward class III lanthipeptides during evolution. The crystal structure of ePepN is composed of four distinct domains with a single zinc-binding active site (30). A predicted structural model of AplP generated by

I-TASSER suggests that AplP is highly similar to ePepN in their domain organization and overall structure, including a single zinc-binding pocket (*SI Appendix*, Fig. S23) (36). Biochemical assays showed that mutations in the Zn-binding pocket abolished the functions of AplP as an endo- and aminopeptidase, suggesting that AplP-like proteases utilize one single active site for class III leader processing. Intriguingly, the genes for most AplP-like proteases are located outside class III BGCs, raising the possibility that AplP-like proteases still function as aminopeptidases for cytosolic protein degradation in bacteria.

In summary, we have discovered an unusual class of Zn-dependent bifunctional proteases as a universal strategy for leader peptide removal of class III lanthipeptides, thereby clarifying a long-standing question regarding the biosynthesis of this emerging group of natural products. The involvement of the M1 family of zinc metallopeptidases is unprecedented in the biosynthesis of lanthipeptides and even the entire family of RiPPs. Thus, our findings expand the scope of proteases for this class of peptide natural products and would facilitate their discovery by genome mining. Furthermore, this study opens the opportunity for the heterologous production and bioengineering of class III lanthipeptides in the future.

Methods and Materials

Detailed information on instrument settings, culture conditions, gene cloning, mutant construction, protein expression and purification, and bioinformatics analysis is provided in *SI Appendix*.

In Vitro Assay of AplP-Cleaving AplA Peptides. All digestion assays were performed in 20 mM Tris buffer at pH 8.0 in a 37 °C water bath. The final concentration of AplP or AplP-ΔZn mutant was 10 μM, and AplA peptides were at a concentration of 100 μM. Negative controls were performed by using the boiled enzyme or by omitting the AplP enzyme. For inhibitory assays, the final concentrations of *o*-phenanthroline and bestatin were 4 mM and 280 μM, respectively.

Kinetics Studies of the Hydrolytic Activity of AplP Toward Amino Acid-*p*NA. All hydrolytic activity assays were performed in 20 to 50 mM Tris buffer (pH 8.0) at 37 °C using a continuous UV-vis spectrometric assay monitored at 405 nm. The final concentration of AplP or AplP-ΔZn mutant was 10 μM, and amino acid-*p*NA derivatives were at 1 mM concentration. The final concentrations of *o*-phenanthroline and bestatin as inhibitors were 4 mM and 280 μM, respectively.

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