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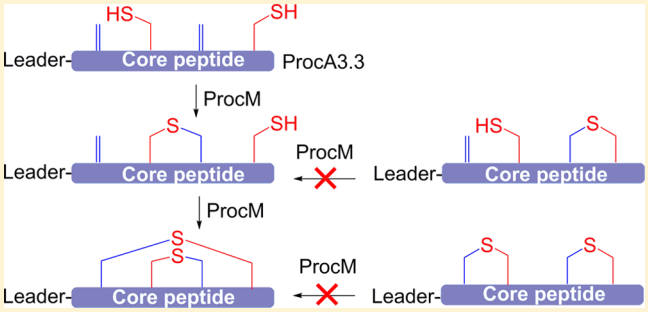
Product Formation by the Promiscuous Lanthipeptide Synthetase ProcM is under Kinetic Control

Yi Yu,[†] Subha Mukherjee,[‡] and Wilfred A. van der Donk*[,†,‡,§]

[†]Department of Biochemistry, [‡]Department of Chemistry, and [§]Howard Hughes Medical Institute, University of Illinois at Urbana−Champaign, 600 S. Mathews Ave. Urbana, Illinois 61801, United States

Supporting Information

ABSTRACT: Lanthipeptides are natural products that belong to the family of ribosomally synthesized and post-translationally modified peptides (RiPPs). They contain characteristic lanthionine (Lan) or methyllanthionine (MeLan) structures that contribute to their diverse biological activities. Despite its structurally diverse set of 30 substrates, the highly substrate-tolerant lanthipeptide synthetase ProcM is shown to display high selectivity for formation of a single product from selected substrates. Mutation of the active site zinc ligands to alanine or the unique zinc ligand Cys971 to histidine resulted in a decrease of the cyclization rate, especially for the second cyclization of the substrates ProcA1.1, ProcA2.8, and ProcA3.3. Surprisingly, for ProcA3.3 these mutations also altered the regioselectivity of cyclization resulting in a new major product. ProcM was not able to correct the ring topology of incorrectly cyclized intermediates and products, suggesting that thermodynamic control is not operational. Collectively, the data in this study suggest that the high regioselectivity of product formation is governed by the selectivity of the initially formed ring.

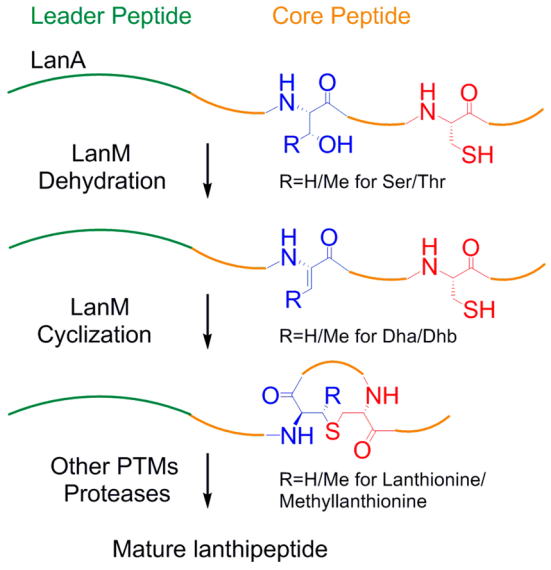


INTRODUCTION

The term lanthipeptides was introduced for lanthionine-containing peptides,[1] which are a group of peptide-derived biomolecules that are ribosomally synthesized and post-translationally modified to their mature forms.[2−4] The precursor peptides (generically termed LanAs) contain an N-terminal leader region in addition to a C-terminal core peptide that is processed to the mature compound. The modifications involve the dehydration of select serine and threonine residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively, followed by intramolecular conjugate additions of cysteines to the dehydro amino acids, resulting in extensively cross-linked polycyclic structures. After complete modification of the precursor peptide, the N-terminal leader sequence is removed by a protease to release the mature peptide (Scheme 1).

ProcM is a member of the class II bifunctional lanthipeptide synthetases (generically termed LanMs). The enzyme was recently discovered in the planktonic marine cyanobacterium *Prochlorococcus* MIT9313 and carries out both dehydration and cyclization reactions.[5] The genome of *Prochlorococcus* MIT9313 contains only one *lanM* gene encoding ProcM but 30 *lanA* genes encoding precursor peptides.[5,6] The ProcA peptides have a high level of conservation in the N-terminal leader region and hypervariability in the C-terminal core region (Scheme S1). The activity of ProcM has been confirmed for all 18 precursor peptides investigated thus far,[5−7] and it is highly likely that all 30 ProcAs are substrates of ProcM. Similar class II systems with a single enzyme and multiple, diverse substrate peptides are also found in other cyanobacteria.[6] In contrast, most LanM enzymes that have been investigated have only a single substrate.[8]

Scheme 1. Overview of Class II Lanthipeptide Biosynthesis[a]



[a]The canonical post-translational modifications are shown.

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The molecular details that allow this high substrate tolerance are largely unknown. Previous mechanistic studies on ProcM[9,10] and NMR analysis of purified prochlorosin products[7] support enzymatic formation of all the thioether rings and showed that specific structures were generated for several prochlorosins. However, whether ProcM forms only one product for each substrate or whether multiple products were formed and only one isomer was purified and structurally characterized remains unclear. Indeed, cyclization of substrates that contain multiple cysteines and dehydro amino acids could result in products with alternative ring topologies (e.g., Scheme S2). Using liquid chromatography electrospray ionization mass spectrometry (ESI-LC-MS), we show here that indeed ProcM is highly selective for the formation of one product from each substrate peptide, despite their highly diverse sequences (Scheme S1). These remarkable observations prompted further investigations of the factors that contribute to both substrate tolerance and product selectivity.

The C-terminal domains of LanM enzymes have low but detectable sequence identity with class I lanthipeptide cyclases (generically termed LanCs) including the conserved residues that are important for cyclization based on mutagenesis[11] and structural[12] studies with the prototype NisC. For NisC, these residues include one histidine and two cysteines that serve as ligands to an active site zinc ion. These residues are also conserved in the cyclization domain of LanM enzymes,[13] and their importance has been experimentally confirmed in the case of the lacticin 481 synthetase LctM.[14] Surprisingly, a sequence alignment of ProcM with other LanM and LanC proteins (Scheme S3) demonstrates that ProcM has three cysteines as the predicted zinc ligands. ProcM and its close homologues with three zinc-binding cysteines form a distinct clade in the phylogenetic tree of class II LanM synthetases,[15] and generally (but not always) are associated with multiple substrate peptides with diverse sequences.[6] As demonstrated in previous studies of other enzymes and model complexes, adding an additional sulfur-based ligand increases the reactivity of thiolates bound to a zinc ion.[16−22] Thus, the three-cysteine ligand set may be responsible for the promiscuous catalytic ability of ProcM and its homologues. Herein, the importance of these active site residues was investigated by mutagenesis, and the cyclization activity and regioselectivity of ring formation were determined. The role of the unique cysteine in the ProcM clade (Cys971 in ProcM) was assessed for the first time, which provides important information to understand the unusual substrate tolerance and selectivity.

To probe how ProcM can display both catalytic promiscuity and high specificity, several possible explanations were tested in this study. One possibility involves a highly organized order of the dehydration and cyclization reactions. To examine this hypothesis, the cyclization process was studied in isolation using dehydrated substrate and only the ProcM cyclase domain. A second recently suggested hypothesis to explain the properties of ProcM involves thermodynamic control of ring formation resulting in the accumulation of the most stable product. This hypothesis was born out of the observation that both NisC and the class II haloduracin synthetase HalM2 were able to efficiently catalyze retro-Michael additions on their products.[23] Thermodynamic control not only requires that the enzymes reversibly catalyze the Michael additions found in their products but also correct alternate cyclization patterns. This hypothesis was tested by incubation of ProcM with peptides containing incorrect rings and by searching for incorrect intermediates formed during catalysis by ProcM. A third hypothesis involves kinetic control of ring formation, with the earlier cyclization events determining the regioselectivity of later cyclizations. Our investigations on ProcM reported here support this last model, providing important new insights into the enzymatic mechanism of this unique clade of substrate-tolerant class II lanthipeptide synthetases.

RESULTS

The Paradoxical Catalytic Promiscuity and High Selectivity of ProcM. For most of the lanthipeptides discovered to date, the thioether rings are installed by nucleophilic attack of Cys onto Dha or Dhb residues located N-terminal to the Cys. But for prochlorosins, the thioether rings are formed by the addition of Cys residues to Dha or Dhb that are localized either N- or C-terminal to the Cys.[5] Five ProcA precursor peptides (ProcA1.1-G−1E, ProcA2.8, ProcA2.11, ProcA3.2, and ProcA3.3) were chosen for investigation out of the 30 possible substrates to provide a representative set of different ring topologies, ring size, and direction of cyclization (Figure 1). For Pcn1.1 (prochlorosin formed from ProcA1.1) and Pcn2.8, structures with two non-overlapping rings are formed, and for Pcn2.11, Pcn3.2, and Pcn3.3, structures with overlapping rings are generated.[5] The precursor peptides of these five prochlorosins containing an N-terminal hexa-histidine tag were co-expressed with ProcM in *Escherichia coli*, and the modified peptides were purified by Ni^{2+} affinity chromatography and digested with endoproteases to remove all or most residues of the leader peptides (see Supporting Information). Samples were treated with iodoacetamide (IAA) to test for any partially cyclized products by reaction with free thiols (Figure S1), and the resulting peptides were analyzed by ESI-LC-MS. For all five peptides, the extracted ion chromatograms of fully dehydrated and cyclized products showed formation of one major product (Figure 1); any products with alternative ring patterns were only present in <5% of the major product. Tandem ESI-MS confirmed that the structures of the products were consistent with those determined in previous reports (Figure S2). The presence of y″9, y″8, and y″5 ions in the tandem MS spectrum for ProcA1.1 G−1E and y″9, b39, and y″8 ions for ProcA2.8 indicates these peptides contain two non-overlapping rings, whereas the absence of any y″12 through y″3 ions for ProcA3.3 strongly suggests that overlapping structures are present. These findings are also in full agreement with the structures determined by NMR spectroscopy.[7] Hence, the data in Figure 1 confirm that ProcM is both remarkably tolerant with respect to its substrates and at the same time curiously specific in its product formation.

The Three Zinc-Binding Cysteines are Important for Cyclization. Unlike other lanthipeptide cyclases that utilize two Cys and one His as zinc ligands, ProcM contains a Cys instead of a His at position 971, suggesting that it likely uses three Cys residues (Cys924, Cys970, and Cys971) for binding of the active site zinc ion. To confirm that these three Cys residues are important for zinc binding and for the cyclization activity of ProcM, a series of individual mutants were generated (C924A, C970A, C971A, and C971H). The zinc content of wild-type (WT) ProcM and mutant proteins was measured by a spectrophotometric assay using the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR).[24] The proteins were expressed in *E. coli* and then analyzed after purification and exhaustive dialysis in chelex-treated buffer. The resulting samples were treated with guanidine hydrochloride and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to oxidize reactive cysteines to

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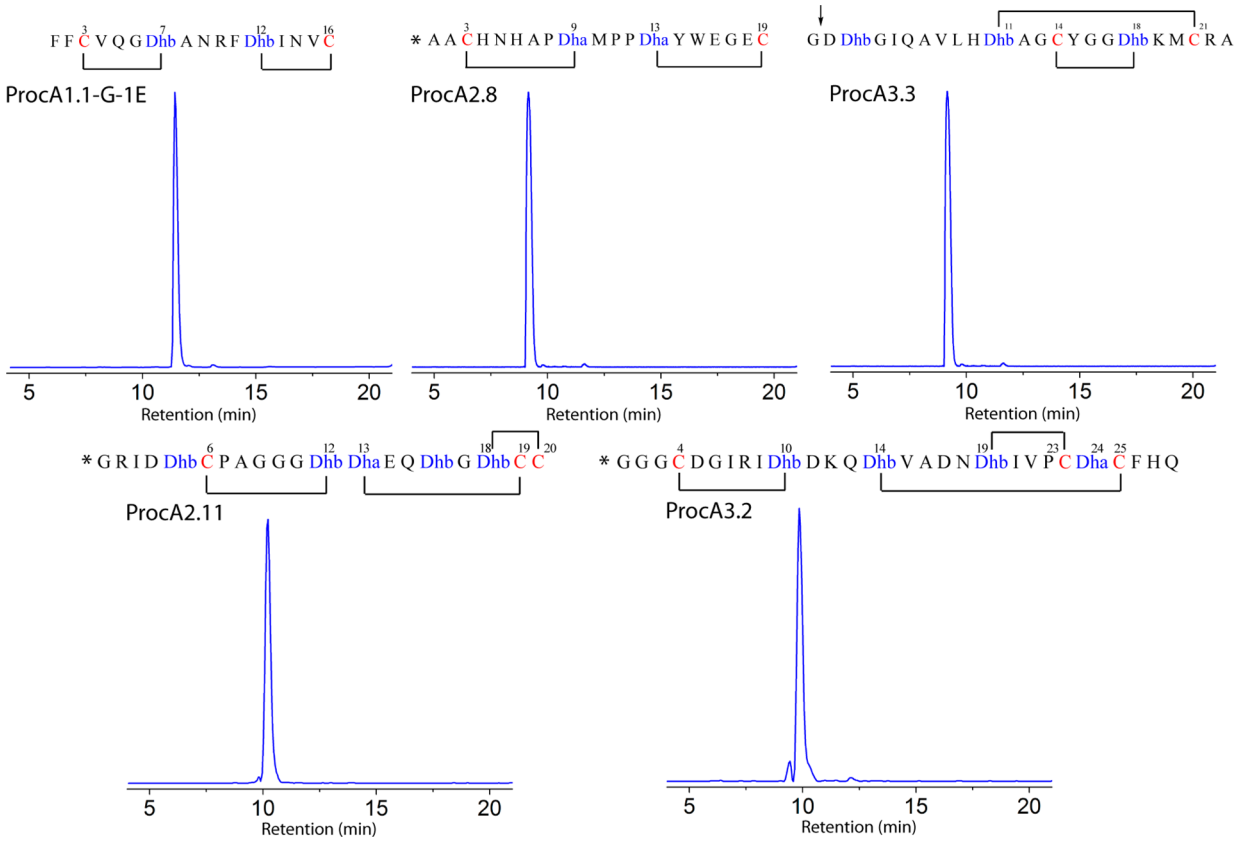

Figure 1. Extracted ion chromatograms (EICs) of the ESI-LC-MS experiments showing the fully dehydrated core-containing peptide ions from ProcA peptides co-expressed with ProcM, and subsequently digested with endoproteases and treated with IAA. ProcA1.1-G−1E and ProcA3.2 were digested by GluC, ProcA2.11 by LysC, and ProcA2.8 and ProcA3.3 by AspN. The star symbol represents a short peptide sequence remaining from the leader peptide after treatment with the commercial proteases: ProcA2.8 (* = DELEGVAGG), ProcA2.11 (* = EDLNSHRQTLSEDELESVAGG), and ProcA3.2 (* = GVAGG). Arrow symbol stands for the site of AspN cleavage in the ProcA3.3 core peptide.

disulfides as previously described.[25] The orange $Zn(PAR)_2$ complex was formed after the addition of PAR and was quantified by the absorbance at 500 nm. WT ProcM and ProcM-C971H contained nearly stoichiometric quantities of zinc, while the relative zinc content for ProcM-C924A and ProcM-C970A was decreased by about 50% (Table S1). ProcM-C971A precipitated during the dialysis treatment in two independent attempts, thus zinc content could not be determined for this mutant.

The dehydration activities of WT ProcM and mutants were studied by *in vitro* enzymatic assays on the substrate ProcA2.8 followed by endoprotease LysC removal of most of the leader peptide and analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). As anticipated since the mutations are in the cyclization domain, the dehydration activities were not affected by these zinc ligand mutations (Figure S3). To study the cyclization activities, IAA was added to the LysC cleavage reaction to detect free cysteines in the peptides. With ProcA2.8 as substrate, all four Cys mutants showed a significant decrease in the cyclization activity compared to the WT enzyme, with the majority of the products formed by the mutant enzymes carrying one IAA adduct, which indicated one ring was not formed (Figure 2). Considering about half of these mutants are apoprotein based on the zinc content analysis, these reactions were also performed with supplemented zinc or with four times the amount of enzyme, but similar results were observed. The partially cyclized peptides were analyzed by ESI-LC-MS of LysC-digested samples, which showed that it was the Cys3-Dha9 ring in ProcA2.8 that was formed slower for all four mutants (Figures S4 and S5 and Table S2). Collectively, these experiments demonstrate that having three Cys ligands is important for the cyclization activity of ProcM.

Product Distribution and Cyclization Rate of WT ProcM and ProcM Mutants. Incubation of ProcA1.1 or ProcA2.8 with WT ProcM or ProcM-C971H *in vitro* resulted in formation of the same products (Figures S4−S6). The rate of cyclization with these two substrates was studied *in vitro* by quenching the reaction at different time points. With WT ProcM, the first cyclization of Cys16 to Dhb12 in ProcA1.1 was almost completed in 30 min under the assay conditions used (0.2 μM enzyme, 10 μM substrate), and the second cyclization of Cys3 to Dhb7 was almost completed in 3 h (Figures 3 and S6 and Table S3). With ProcM-C971H, the first cyclization of Cys16 to Dhb12 was nearly completed in 1 h, but the second cyclization was much slower, with <10% fully cyclized product observed at 3 h (Figure 3), as calculated by the ion counts for signals corresponding to the fully modified peptide and peptides with IAA adducts in the mass spectrum. The enzymatic assay with ProcA2.8 showed similar results. For WT ProcM, the first cyclization of Cys19 to Dha13 was completed in <15 min under the assay conditions used, and the second cyclization of Cys3 to Dha9 was almost completed in 1 h (Figure S7). With ProcM-C971H, the first cyclization of Cys19 to Dha13 was also completed in <15 min, but the rate of the second cyclization was decreased significantly, with <30% fully cyclized product observed after 90 min. By increasing the concentration of ProcM-C971H to 30 μM (with 50 μM peptide),

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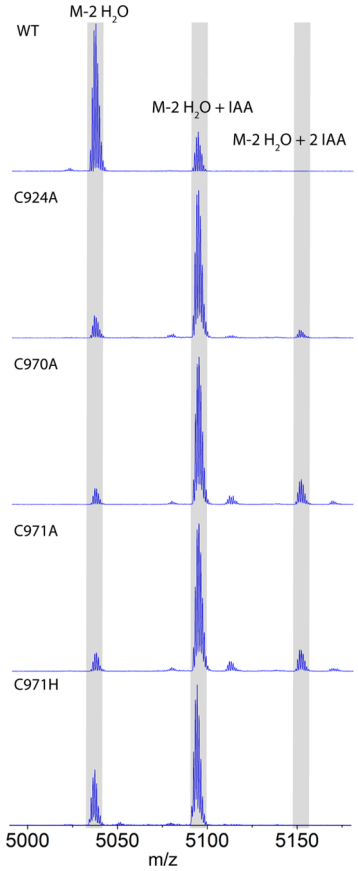

Figure 2. MALDI-TOF mass spectra of 25 μM ProcA2.8 peptide modified by 2 μM WT ProcM, ProcM-C924A, ProcM-C970A, ProcM-C971A, and ProcM-C971H *in vitro* for 1 h and subsequent digestion by LysC and treatment with IAA.

the ratio of fully cyclized to partially cyclized ProcA2.8 after 1 h was increased (Figure S8). This experiment shows that ProcM-C971H was able to catalyze the second cyclization, although the rate was considerably slower compared to WT enzyme.

Surprisingly, for one of the investigated ProcAs, ProcA3.3, co-expresssion with ProcM-C971H in *E. coli* generated two different final products in contrast to WT ProcM, which made one specific product (peptide **1**, Figure 4A). The major product generated by ProcM-C971H (peptide **2**) was demonstrated by tandem ESI-MS to contain a novel non-overlapping ring topology (Figure 4B). ProcA3.3 was also incubated with ProcM-C971H and the other mutants *in vitro* for 3 h, and the product distribution was analyzed by ESI-LC-MS. The ring patterns of each product and intermediate were determined by tandem ESI-MS and verified by comparison to standards (Figure 5, for the generation of the standards, see the Supporting Information). Tandem mass spectra of each product and intermediate are shown in Figures 4B and S9, and Table S4 lists the calculated and observed masses of each ion in the tandem MS analyses. For all mutants, peptide **2** was the major final product and peptide **5** was the major intermediate that leads to the formation of peptide **2** (Figures 5 and S10).

Given the interesting observation of a switch in product distribution with ProcA3.3, we also examined the time dependence of processing. Incubation of ProcA3.3 with WT ProcM resulted in near completion of the first cyclization of Cys14 to Dha18 in 15 min under the assay conditions, whereas the second cyclization of Cys21 to Dha11 took up to 90 min to complete (Figure S11). For ProcM-C971H, the first cyclization of Cys21

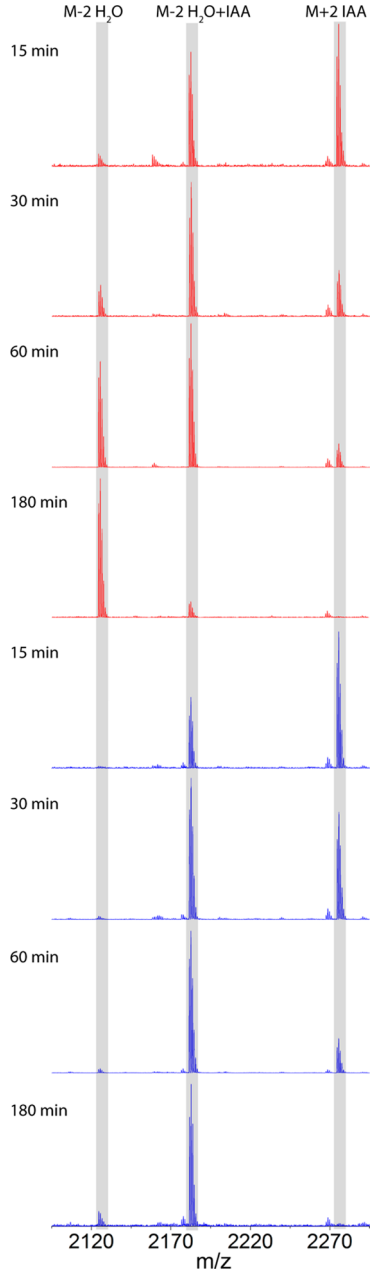

Figure 3. MALDI-TOF mass spectra of 10 μM ProcA1.1 modified by 0.2 μM WT ProcM (red) and ProcM-C971H (blue) for 15, 30, 60, and 180 min and subsequent digestion by GluC and treatment with IAA.

to Dha18 was nearly completed in 90 min, but the second cyclization of Cys14 to Dha11 was much slower, with <10% fully cyclized product observed at 90 min (Figure S11).

To determine the intrinsic regioselectivity of ring formation, non-enzymatic cyclization of ProcA3.3 was studied. First, the two cysteines in ProcA3.3 were linked by a disulfide bridge using oxidized glutathione, which resulted in the expected mass loss of 2 Da. The oxidized ProcA3.3 was dehydrated by ProcM *in vitro*, and the 3-fold dehydrated product was purified. Reduction by TCEP treatment shifted the mass of the peptide by 2 Da (Figure S12). Upon TCEP reduction, the non-enzymatic cyclization was investigated at the same pH of the enzymatic assay (pH 8) for 3 h. Very little cyclization occurred compared to the enzymatic reactions, illustrating that the mutants still accelerated the cyclization process (Figure 5). The regioselectivity of

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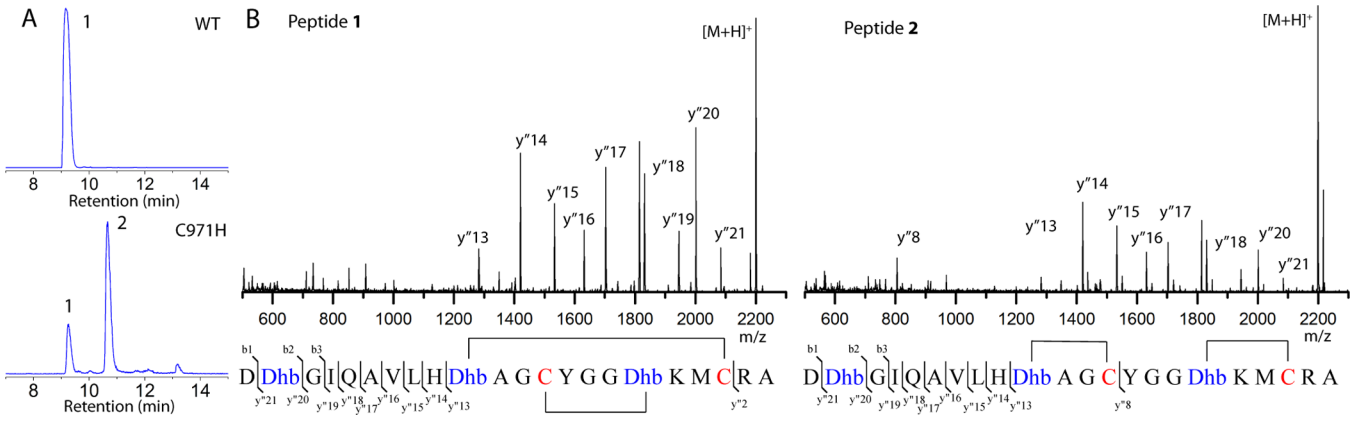

Figure 4. (A) EICs of the ESI-LC-MS experiments showing the 3-fold dehydrated and cyclized Δ1 core peptide ion (Δ1 because the first Gly is removed by AspN) produced by ProcA3.3 co-expression with WT ProcM or ProcM-C971H and subsequent digestion with AspN and treatment with IAA. (B) Tandem ESI-MS of peptides **1** and **2**. For calculated and observed masses, see Table S4.

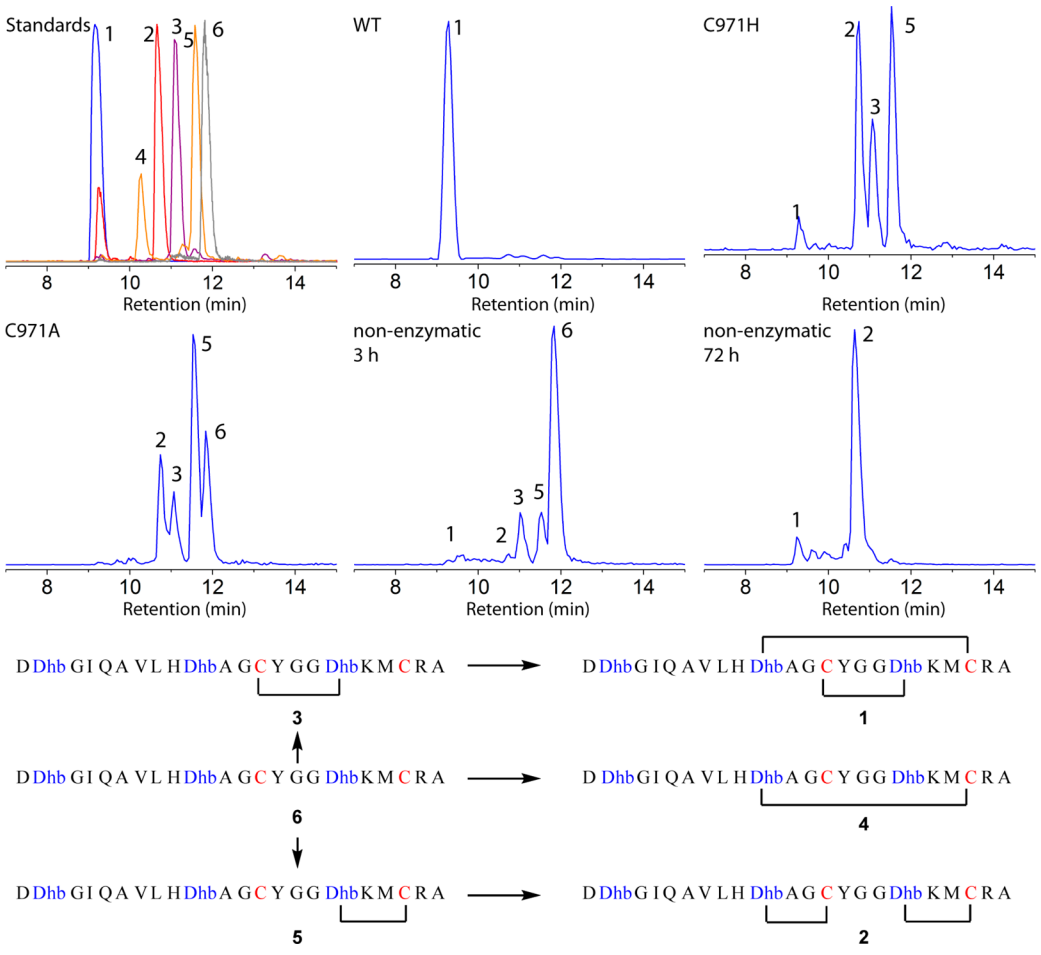

Figure 5. EICs of the ESI-LC-MS experiments showing the 3-fold dehydrated Δ1 core peptide ion obtained by AspN digestion of 25 μM ProcA3.3 incubated with 2 μM WT ProcM, ProcM-C971A, and ProcM C971H for 3 h. Standards: **1** (blue), ProcA3.3 co-expressed with WT ProcM; **1** and **2** (red), ProcA3.3 co-expressed with ProcM-C971H; **3** (purple), semisynthetic ProcA3.3 containing a ring between Cys14 and Dhb18 (see Supporting Information); **4** and **5** (orange), semisynthetic ProcA3.3 with Cys14 protected that was modified by WT ProcM and subsequently deprotected (see Supporting Information); and **6** (gray), 3-fold dehydrated ProcA3.3. Non-enzymatic: dehydrated ProcA3.3 incubated with reaction buffer at pH 8 for 3 or 72 h. All peptides were digested by AspN to remove the leader peptide.

non-enzymatic cyclization was very similar to that of the zinc ligand mutants, with the predominant products being peptide **2** and intermediate **5** that leads to peptide **2** (Figure 5).

The Order of Dehydration and Cyclization Does Not Determine the Ring Topology. It has been reported for nisin, haloduracin, and curvopeptin that the dehydration and cyclization reactions are alternating processes during biosynthesis.[10,26−28] Thus, a possible explanation for the unusual selectivity of the ring formation in the structurally diverse prochlorosins is that the ring patterns are determined by the

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order of dehydration and cyclization. The order of dehydration has been established in a previous study for ProcA2.8 and ProcA3.3.[9] To study cyclization in isolation, the putative cyclase domains of WT ProcM and ProcM-C971H were obtained by expressing the C-terminal domains spanning residues 655-1068 (ProcM-655-1068 and ProcM-C971H-655-1068). The cyclization reaction catalyzed by ProcM-655-1068 was studied with dehydrated ProcA3.3 that was generated as described in the previous section. As shown in Figure 6, incubation of WT

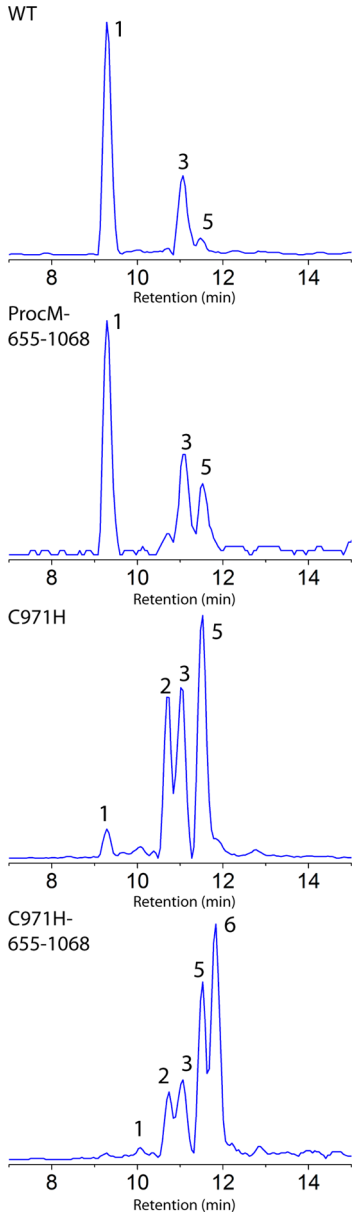

Figure 6. EICs of the ESI-LC-MS experiments showing the Δ1 core peptide ions obtained by AspN digestion of dehydrated ProcA3.3 incubated with WT ProcM, ProcM-655-1068, ProcM-C971H, and ProcM-C971H-655-1068.

ProcM-655-1068 with fully dehydrated ProcA3.3 resulted in a similar product distribution as with the WT enzyme at a similar extent of conversion, strongly favoring peptide **1** and intermediate **3** leading to peptide **1**. Conversely, incubation of ProcM-C971H-655-1068 with fully dehydrated ProcA3.3 resulted in preference for intermediate **5** leading to peptide **2**. These results are not consistent with a tight regulation of the order of dehydration and cyclization as a determining factor for the selectivity for certain ring patterns in prochlorosins, since decoupling of the dehydration reaction from the cyclization events gave very similar product distributions compared to the selectivities observed with the full length proteins. Interestingly, cyclization of fully dehydrated substrate by ProcM-655-1068 required the leader peptide (Figure S13), suggesting that the cyclase domain may contain a leader peptide binding site. This suggestion is also consistent with the observed binding of the substrate NisA to the stand-alone class I cyclase NisC,[29,30] which has sequence homology with the cyclase domain of ProcM.

Thermodynamic Control Involving Reversible Ring Formation Is Not Observed. One attractive explanation for the catalytic selectivity of ProcM is thermodynamic control over the selectivity of ring formation. This model requires reversibility of the cyclization reaction to allow the generation of the most stable product. Indeed, reversible ring formation has recently been observed for the haloduracin synthetase HalM2 and the nisin cyclase NisC.[23] Thermodynamic control not only requires ring opening of the rings found in the final product but also ring opening of energetically less favored "incorrectly" cyclized products and intermediates. At present, such activity has not been investigated for any lanthipeptide. To investigate this model for ProcM, three different experiments were carried out. First, the non-physiological product **2** was obtained by co-expressing ProcA3.3 with ProcM-C971H, and the peptide **2** was subjected to the *in vitro* enzymatic assay with WT ProcM. Conversion of peptide **2** to peptide **1** was not observed (Figure 7A). Furthermore, ProcM-C971H did not convert peptide **1** to peptide **2** either (Figure 7B). However, it is possible that the final cyclized

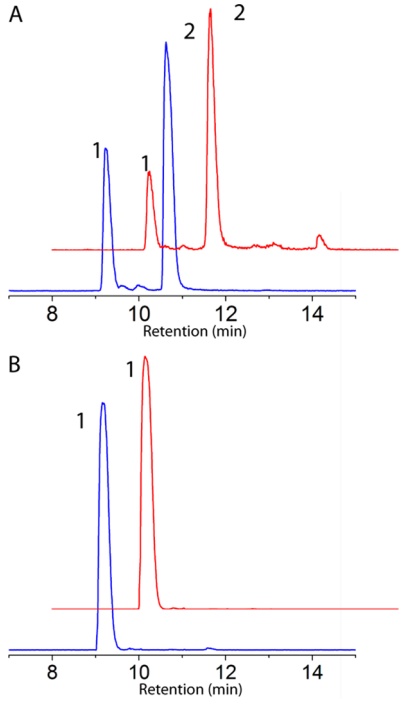

Figure 7. (A) EICs of the ESI- LC-MS experiments showing the Δ1 core peptide ions from ProcA3.3 co-expressed with ProcM-C971H (red) and subsequently treated with WT ProcM (blue). (B) EICs of the ESI-LC-MS experiments showing the Δ1 core peptide ion from ProcA3.3 co-expressed with WT ProcM (red) and subsequently treated with ProcM-C971H (blue). All peptides were digested by AspN and treated with IAA.

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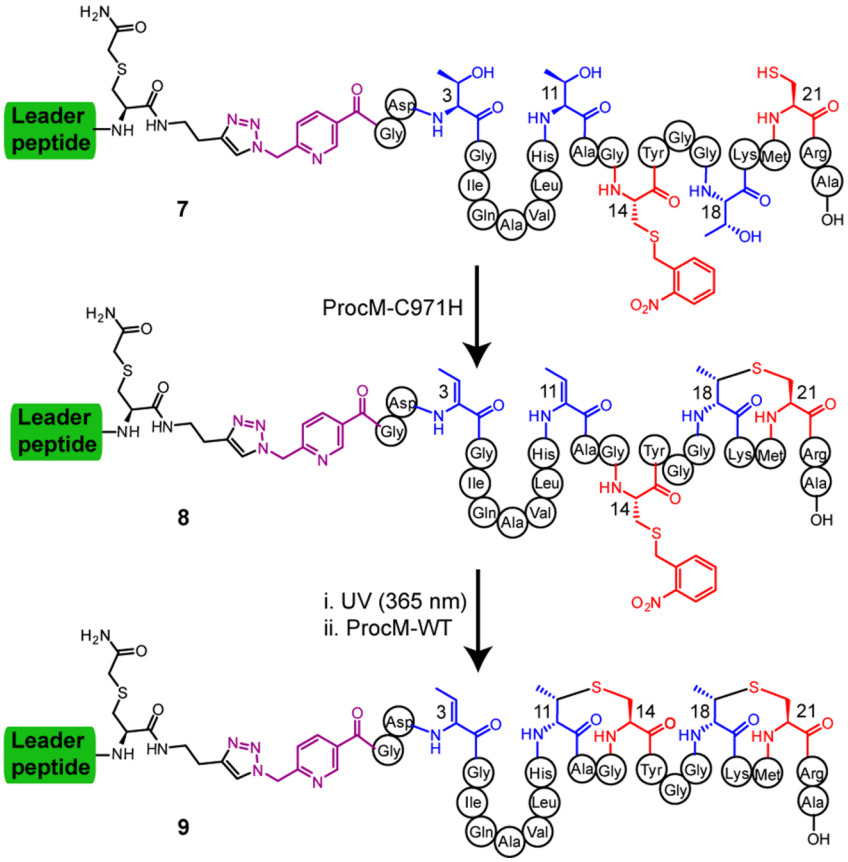

Figure 8. Scheme showing the approach used to determine if WT ProcM can correct non-native ring topology in an intermediate. A ProcA3.3 semisynthetic analog with Cys14 protected with a photolabile protecting group was modified by ProcM-C971H to generate the non-native MeLan cross-link between Cys21 and Dhb18. After UV-mediated deprotection of Cys14, treatment with WT ProcM generated a thioether cross-link between Cys14 and Dhb11, thus showing no evidence of correction of the initially formed ring to generate the native overlapping ring topology.

peptides were not accessible to the active site of the enzyme. Indeed, for all substrates investigated in this study, the second cyclization event was very slow, possibly reflecting decreasing affinity of the enzyme for the peptide as more rings are installed. Therefore, we also investigated potential reversibility on an incorrectly cyclized intermediate. A ProcA3.3 derivative with a Cys21-Dhb18 ring was generated by orthogonal protection of Cys14 using methodology previously described (Figure 8).[9] Incubation of the intermediate with WT ProcM did not result in conversion of this "wrong intermediate" to the correct product with overlapping rings, but instead a product with non-overlapping rings was formed (Figures 8 and S14). In other words, once the incorrect first ring was installed, ProcM proceeded to the incorrect product. As a final test of potential thermodynamic control, the *in vitro* enzymatic assay of WT ProcM and ProcA3.3 was quenched at different time points and analyzed by ESI-LC-MS to identify the intermediates (Figure S15). At all time points, peptides **1** and **3** were detected at significant levels, whereas incorrect intermediate peptide **5** was only observed at very low levels (possibly from background non-enzymatic cyclization). Collectively, these results argue against thermodynamic control of product formation for ProcM.

DISCUSSION

In this study, we demonstrated that, curiously, the substrate promiscuity of ProcM does not come with a cost of reduced product selectivity as for all the peptides investigated, the enzyme makes essentially single products, with only very minor amounts, if any, of alternative ring topologies observed. We investigated several possibilities for the determinants of the observed selectivity for certain ring topologies. In a previous study, we ruled out that the high selectivity for the final ring topology is a consequence of non-enzymatic processes. [9] Here we again show that non-enzymatic cyclization is much slower than the enzymatic process and that the regioselectivity for non-enzymatic cyclization of ProcA3.3 is different than that observed with WT ProcM. Hence, the enzyme must exert a specific influence on the regiochemistry of cyclization and is not simply accelerating formation of a product that is already favored by the substrate. In this work we ruled out that the selectivity is caused by tight control between the dehydration and cyclization activities or by thermodynamic control. A very recent study illustrated that two lantibiotic cyclization processes involved in the biosynthesis of the class I lantibiotic nisin and the class II lantibiotic haloduracin are reversible when the cyclization enzymes NisC and HalM2, respectively, were exposed to their final correctly cyclized products.[23] ProcM on the other hand appears much less reactive toward its products. All attempts to detect products of retro-Michael processes using the same methodology as used for NisC and HalM2,[23] whether with the native ProcM products or incorrectly cyclized products or intermediates, have been unsuccessful. No ring-opened products were detected with ProcM (~5% estimated detection limit), whereas NisC and HalM2 generated substantial amounts of peptides in which up to five and four rings were opened, respectively.[23] These observations may be explained by the very

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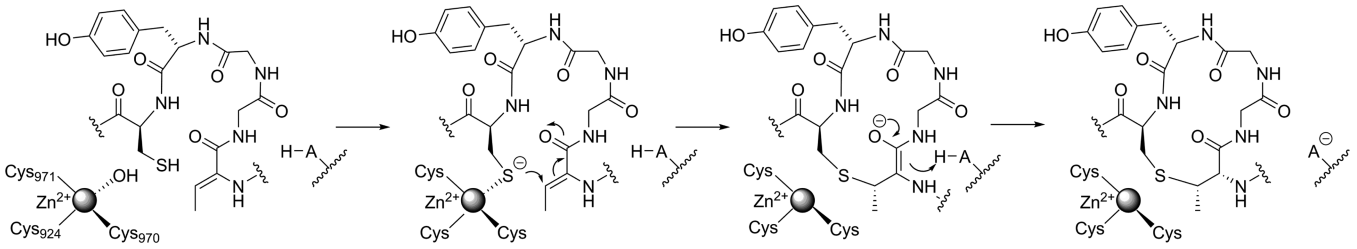
Scheme 2. Proposed Mechanism for the Cyclization Reaction Catalyzed by ProcM Illustrated for the Formation of the Ring in Involving Cys14 and Dhb18 of ProcA3.3[a]



[a]A-H is an active site acid that protonates the enolate. A recent study suggests it is a conserved His[23].

different kinetic profiles of the enzymes.[10] The reactions that HalM2 catalyzes on its substrate HalA2 become progressively faster, whereas the opposite is observed here and in a previous study[10] for ProcM. A well-known problem in feeding intermediates to enzymes is that they cannot always access the catalytically competent form of their cognate enzyme, for instance if such an intermediate is normally processed further while still bound in the enzyme active site. However, ProcM is not processive in its catalysis with WT substrates as shown here and in a previous study,[10] and hence this explanation for the lack of reversibility does not hold. Instead, we believe that the difference between HalM2/NisC and ProcM is the result of poor recognition of cyclized intermediates by the latter. We have suggested that the HalA2 peptide becomes increasingly complementary to the HalM2 active site as the post-translational modifications take place, whereas ProcM, which makes 30 structurally very different products, cannot be complementary in shape and/or electrostatics to all its products.[10] Thus, the fully processed HalA2 peptide likely interacts much more favorably with HalM2 than the processed ProcA peptides interact with ProcM, explaining the observed reversibility for HalM2 and the apparent lack of reversibility for ProcM.

If neither the inherent reactivity nor the thermodynamics of the products or tight coupling between dehydration and cyclization events determine the outcome of ProcM catalyzed cyclization reactions, what might govern the high observed regioselectivities? One possibility is that the enzyme-catalyzed selectivity for formation of the first ring determines the overall outcome. The mature products of ProcA1.1, 2.8, and 3.3 all have two rings in their final products but with different ring sizes and topologies. With all three peptides, ProcM forms the first ring much faster than the second ring. Since we demonstrated that ProcM does not open up any formed rings, kinetic control over the regioselectivity of formation of the first ring determines the final ring topology for these peptides. The regioselectivity of the formation of that first ring is enzyme controlled because for ProcA3.3, non-enzymatic cyclization is both much slower and results in a different first ring compared to ProcM.

Zn^{2+} is often used in enzymes that catalyze alkylation of thiols, and it has been noted that these enzymes typically have a net negatively charged zinc site in their substrate-bound form (i.e., more than two negatively charged ligands from enzyme + substrate).[19] In addition, the negatively charged ligands in these enzymes typically are predominantly thiolates. It has been suggested that this ligand environment might either serve to facilitate substrate thiolate dissociation to release the nucleophile or to increase the reactivity of the Zn-bound thiolate of the substrate in an associative process.[19] Although studies on model complexes have shed some light onto this question,[16,20−22] at present, it is not clear whether the enzymes use a dissociative or associative mechanism. Therefore, the exact nature of substrate activation in lanthipeptide cyclases is not yet clear, but this study is in agreement with the notion that an increase in thiolate ligands increases the enzyme's activity.[15,16,19] Interestingly, all the zinc ligand mutants that perturb the three Cys ligand set (four Cys after binding of the substrate, Scheme 2) display the same general regioselectivity as the non-enzymatic reaction. All these mutants also have attenuated cyclization activity. Hence, it appears that the unique three-cysteine ligand set increases the reactivity of the zinc site and that this increased reactivity can overcome the inherent selectivity of the substrate. How this is achieved for a very diverse set of substrates is not clear and likely involves substrate-enzyme interactions that await structural information on ProcM and its complexes with the substrates. What is clear is that the high reactivity afforded by the three-cysteine ligand set may be required for full cyclization of the prochlorosins at a reasonable rate, since the second cyclization was very slow for all the mutant enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, synthetic procedures, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*vddonk@illinois.edu

Notes

The authors declare no competing financial interest.

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