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Leader Peptide Establishes Dehydration Order, Promotes Efficiency, and Ensures Fidelity During Lacticin 481 Biosynthesis

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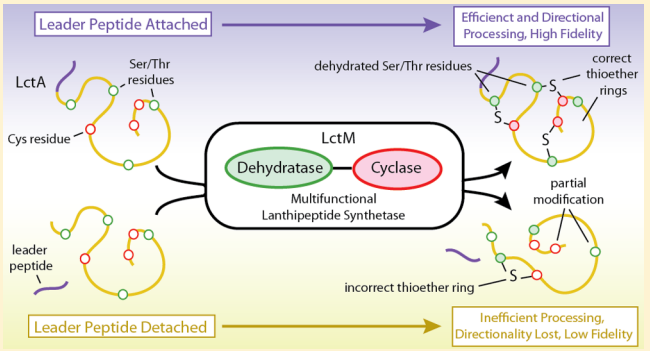
Supporting Information

ABSTRACT: The mechanisms by which lanthipeptide synthetases control the order in which they catalyze multiple chemical processes are poorly understood. The lacticin 481 synthetase (LctM) cleaves eight chemical bonds and forms six new chemical bonds in a controlled and ordered process. Two general mechanisms have been suggested for the temporal and spatial control of these transformations. In the spatial positioning model, leader peptide binding promotes certain reactions by establishing the spatial orientation of the substrate peptide relative to the synthetase active sites. In the intermediate structure model, the LctM-catalyzed dehydration and cyclization reactions that occur in two distinct active sites orchestrate the overall process by imparting a specific structure into the maturing peptide that facilitates the ensuing reaction. Using isotopically labeled LctA analogues with engineered lacticin 481 biosynthetic machinery and mass spectrometry analysis, we show here that the LctA leader peptide plays critical roles in establishing the modification order and enhancing the catalytic efficiency and fidelity of the synthetase. The data are most consistent with a mechanistic model for LctM where both spatial positioning and intermediate structure contribute to efficient biosynthesis.



INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a rapidly growing class of natural products with immense structural diversity both in terms of their modified amino acid building blocks and three-dimensional topologies.[1] This vast structural diversity, coupled with the ability to manipulate their structure by mutagenesis of the precursor peptides, makes RiPPs an exciting frontier in bioengineering. Most RiPPs contain post-translational modifications that are installed by enzymes that act at multiple sites within the precursor peptide. Interestingly, these iterative modifications are often carried out in a defined order. The order can result in *N*-to-*C* terminal directionality along the precursor peptide,[2,3] in *C*-to-*N* terminal directionality,[4−7] or in a process that is not directional but is still ordered.[7−9] The underlying molecular origins for the ordered processes are currently not known, but are expected to be important both for understanding the evolution of RiPP structures and for manipulating catalysis by RiPP biosynthetic enzymes.

Lanthipeptides are a class of RiPPs defined by the presence of lanthionine (Lan) and/or methyllanthionine residues (MeLan, Scheme 1).[10,11] Like most RiPPs, the lanthipeptide precursor peptide (LanA) is composed of an *N*-terminal leader peptide and a *C*-terminal core peptide where the modifications occur. The (Me)Lan moieties are formed in a two-step process involving dehydration of Ser and Thr residues to the corresponding dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, followed by conjugate addition of Cys thiols to the β-carbons of the dehydroamino acids to form thioether linkages (Scheme 1). For class II lanthipeptides, dehydration is achieved by phosphorylation of the side chain hydroxyl groups of Ser and Thr followed by elimination of the resulting phosphate esters.[12] The dehydration and cyclization reactions are catalyzed by a single enzyme (LanM) that contains *N*-terminal dehydration and *C*-terminal cyclization domains that both function iteratively at multiple positions in the core peptide.

Current hypotheses for what dictates modification order in lanthipeptides can be broadly broken down into two models: the spatial positioning model and the intermediate structure model (Scheme 2).[3,13,14] In the spatial positioning model, the leader peptide anchors the position of the core peptide relative to the dehydratase and cyclase active sites. In this model, the order of reactions is governed by the relative spatial orientation of the core peptide to the dehydratase and cyclase active sites of

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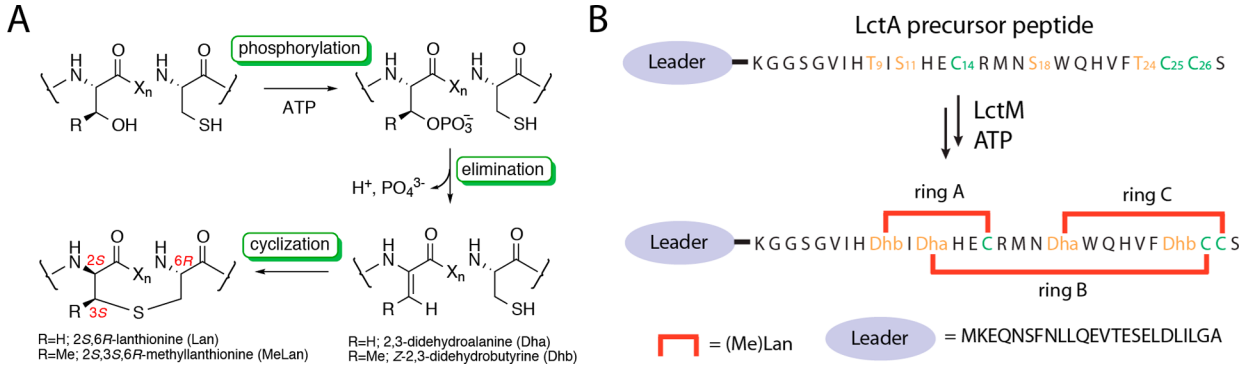
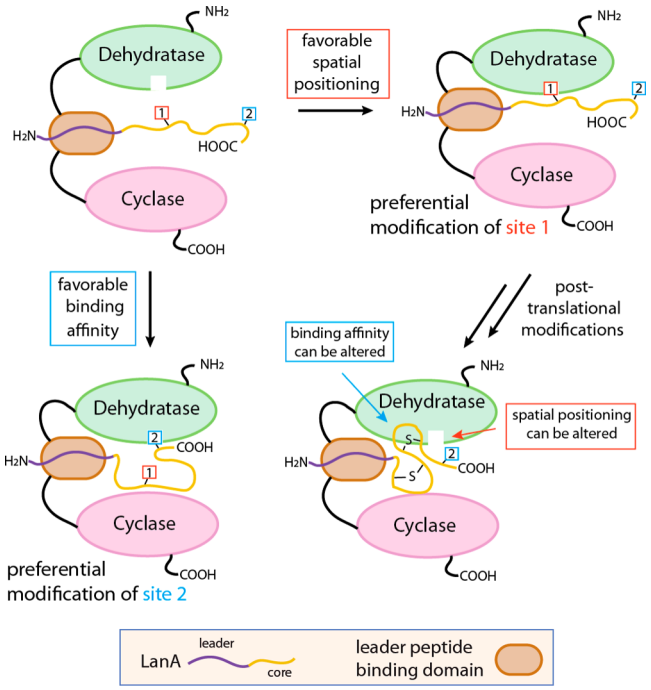
Scheme 1. Chemical Mechanism of LanM Enzymes (A) and Post-Translational Modifications Catalyzed by LctM During Maturation of LctA (B)



Scheme 2. Mechanistic Models for Directionality in LanM Biosynthesis[a]



[a]The order of post-translational modifications in class II lanthipeptide biosynthesis may depend on the relative spatial positioning of the modification and active sites and/or on the affinity of the active sites for local core peptide sequence/structure. Both the spatial positioning and binding affinity of a given site may change as a function of post-translational modification state. Similar proximity and affinity effects may be involved in determining directionality in the biosynthesis of other RiPPs.

the synthetase. The core peptide sites that are more accessible to the synthetase preferentially dock in the active sites and, hence, are preferentially modified. The spatial positioning model was originally invoked to explain the *N*- to C-terminal directionality observed in some class II lanthipeptide biosynthetic systems.[3] In this model, stochastic conformational sampling of the LanM/LanA Michaelis complex delivers the peptide sequence to the active sites, and the system would not necessarily need to evolve tight binding specificity for any given region of the core peptide in order to establish a defined order of biosynthetic events. As such, this model provides an attractive explanation for the inherently relaxed substrate specificity required by the LanM lanthipeptide synthetases and is in line with the observation that some class II lanthipeptide synthetases (including the subject of this study, LctM) modify a broad range of non-native peptide sequences.[15−22]

In the intermediate structure model, the changing structure of the core peptide as it matures into the final product impacts the binding affinity of the intermediate and/or the catalytic efficiency of the synthetase. This model was recently invoked to rationalize the differences in the kinetic properties of the highly substrate selective and catalytically efficient LanM enzyme, HalM2, and the highly substrate flexible and catalytically inefficient LanM enzyme, ProcM.[13] One of the most striking differences between these two enzymes is in the rates of the cyclization steps that generate the thioether rings. In the HalM2-catalyzed maturation of HalA2, the cyclization rates increase substantially as the reaction intermediates mature to the final form, suggesting that the extent of cyclization enhances the catalytic efficiency of the HalM2 cyclase domain. In the case of the ProcM-catalyzed modification of ProcA2.8, however, the first cyclization leads to a drastic reduction in the rates of downstream cyclization events, presumably due to a weakened interaction between the (now) structured core peptide and the ProcM cyclase active site. These observations are in line with the known substrate flexibility of ProcM, which installs a variety of diverse thioether ring topologies into its 30 natural substrate peptides,[23−25] and the highly specialized HalM2 enzyme that makes just a single product and does it much more efficiently than ProcM. Together, the ability of a particular post-translational modification to either enhance or diminish the rates of downstream events provides an attractive model to explain how most lanthipeptide synthetases guide the formation of specific final products, even when many different structural isomers are theoretically accessible. Indeed, a recent study has suggested that thioether topology in the ProcM/ProcA system is under kinetic control.[25]

In the present work, we attempt to shed light on the involvement of these mechanisms and the role of the leader peptide during the biosynthesis of lacticin 481, an antimicrobial lanthipeptide produced by *Lactococcus lactis* CNRZ 481.[26] The lacticin 481 synthetase, LctM, catalyzes the dehydration of four Ser/Thr residues and the formation of three (methyl)-lanthionine rings in its LctA precursor peptide (Scheme 1B).[27−29] Because of the interlocked ring topology of the final product[30] and the catalytic efficiency of the native enzyme, tandem mass spectrometry methods to determine the order of dehydration in lacticin 481 have proven technically challeng-

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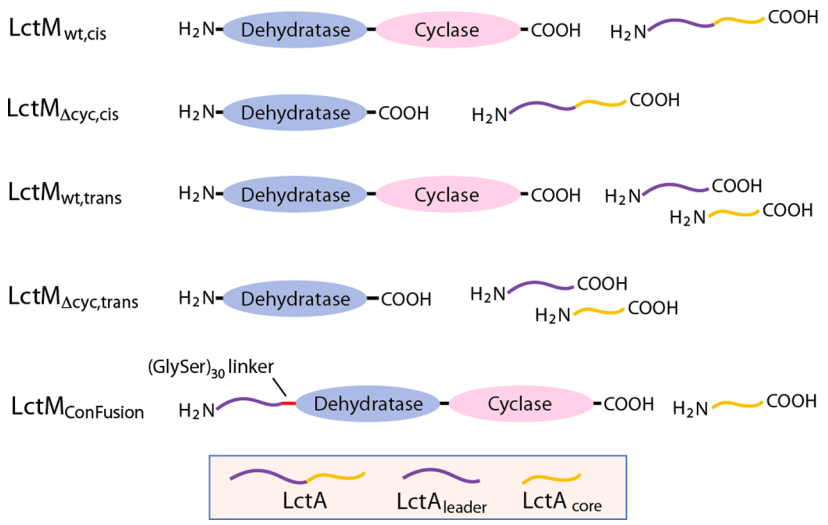

Figure 1. LctM/LctA biosynthetic systems employed in this study. In the $LctM_{wt,cis}$ system, the full-length LctM was incubated with the full-length LctA. The $LctM_{\Delta cyc}$ construct is composed of the *N*-terminal 658 amino acid residues of LctM, which constitutes the dehydration domain.[35] In the $LctM_{\Delta cyc,cis}$ system, the full-length LctA peptide was incubated with the $LctM_{\Delta cyc}$ enzyme. In the $LctM_{wt,trans}$ and $LctM_{\Delta cyc,trans}$ systems, the LctA leader and core peptides were supplied to the enzymes *in trans*. In the $LctM_{ConFusion}$ system, the LctA leader peptide (spanning residues 1−24, violet) was fused to the *N*-terminus of the LctM enzyme via a $(GlySer)_{30}$ linker (red).[32]

ing.[3,27] Thus, little is known about the maturation pathway of this lanthipeptide, which has several structurally closely related natural variants.[31]

Several recent technical advances have allowed us to reinvestigate the biosynthetic mechanism of LctM/LctA modification to address unanswered questions. First, Süssmuth and co-workers developed a technique for monitoring dehydration regiochemistry without the need for tandem mass spectrometry.[5] This method relies on the synthetic incorporation of a single α-deuterium-labeled Ser/Thr residue at a specific position in the substrate peptide.[5,7] The timing of the dehydration of the labeled Ser/Thr can then be elucidated by mass spectrometry because its dehydration involves the loss of 19 Da (rather than the 18 Da loss associated with dehydration of unlabeled Ser/Thr residues). Second, we recently engineered an LctM construct that is genetically fused to the LctA leader peptide in order to make a *con*stitutively active *fusion* enzyme ($LctM_{ConFusion}$) that catalyzes LctA dehydration without the need for exogenous leader peptide.[32] In addition, the dehydratase and cyclase activities of several LanM enzymes have been successfully split into two polypeptides,[25,33−35] an approach that we employ in this study in an attempt to decouple the LctM-catalyzed dehydration and cyclization reactions. Finally, we have recently developed mass-spectrometry- and computation-based approaches that enable detailed kinetic and structural characterization of lanthipeptide biosynthetic pathways.[13,36] Collectively, these new experimental developments have allowed us to directly test the effects of the LctA leader peptide and the LctM cyclization domain on the dehydration order and to propose models for LctM-catalyzed post-translational maturation of LctA.

RESULTS AND DISCUSSION

Overview of Experimental Approach. To study the role of the leader peptide in the LctM-catalyzed dehydration of LctA, we determined the modification order in several different assays (Figure 1). These systems included the wild type system, where the LctA leader peptide is attached to the LctA core peptide *in cis* ($LctM_{wt,cis}$), the $LctM_{ConFusion}$ system,[32] where the leader peptide is fused to the *N*-terminus of LctM, and the $LctM_{wt,trans}$ and $LctM_{\Delta cyc,trans}$ systems, where the LctA leader and core peptides are provided *in trans* to the wild type LctM ($LctM_{wt}$)[37] or to a variant enzyme with the cyclase domain deleted ($LctM_{\Delta cyc}$), respectively. Finally, assays were also conducted with the $LctM_{\Delta cyc}$ enzyme (residues 1−658) and full-length LctA (designated the $LctM_{\Delta cyc,cis}$ system). For the $LctM_{ConFusion}$, $LctM_{wt,trans}$, and $LctM_{\Delta cyc,trans}$ systems, Fmoc-based solid phase peptide synthesis was used to prepare core peptides in which Thr9, Ser11, Ser18, or Thr24 (the four LctA residues that are dehydrated) was individually replaced with [2,3,3-$^2H_3$]-Ser or [2,3-$^2H_2$]-Thr (Figure S1).[5,7] These synthetic peptides are hereafter referred to as $LctA_{core}$-dT9, -dS11, -dS18, and -dT24, respectively. The labeled peptides were incubated with each of the engineered catalytic systems; the reactions were quenched at desired time points, and the resulting reaction mixtures were analyzed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). For the $LctM_{wt,cis}$ and $LctM_{\Delta cyc,cis}$ systems, where the leader peptide is attached to the core peptide, expressed protein ligation was used to prepare site-specifically deuterated, full-length LctA analogues (Scheme S1).

Directionality When the Leader Peptide Is Attached to the Core Peptide. We first studied the dehydration directionality with wild type LctM and full-length LctA (i.e., the $LctM_{wt,cis}$ system). In a previous study,[3] an LctM mutant (Arg399Met) that is deficient in phosphate elimination[29] was used to phosphorylate an LctA mutant peptide that lacked Cys residues ($LctA_{\Delta Cys}$). This study showed that Thr9 and Ser11 were phosphorylated first, followed by Ser18, suggesting an *N*- to *C*-terminal directionality in the inferred dehydrations. However, since phosphorylated intermediates are not normally detected during turnover by the wild type enzyme, it was unclear whether the reaction of $LctM_{Arg399Met}$ with the $LctA_{\Delta Cys}$ peptide provided an accurate assessment of dehydration order in the wt system. Thus, we re-examined the dehydration order for the wt system using site-specifically deuterated, full-length LctA analogues. The poor solubility of LctA thwarted efforts to synthesize the full-length peptide containing deuterium labeled

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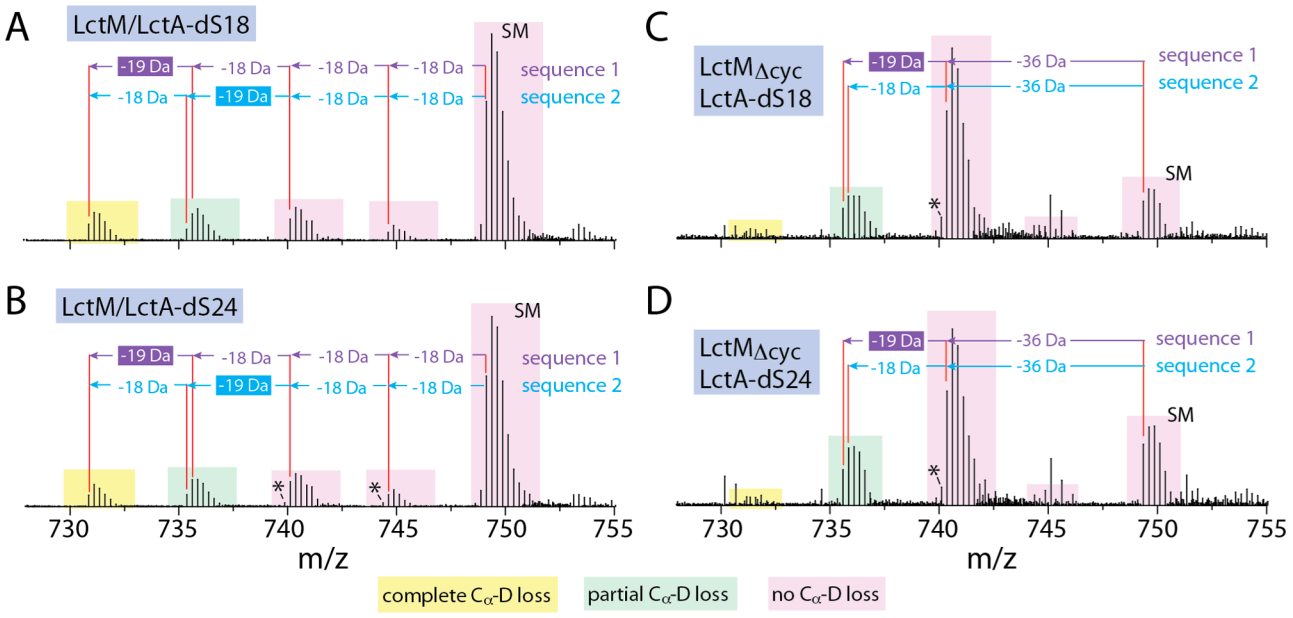

Figure 2. Dehydration order with full-length LctA peptides. Full-length LctA-dS18 and LctA-dS24 (5 μM) were incubated with either $LctM_{wt}$ (A and B) or $LctM_{\Delta cyc}$ (C and D) at enzyme/substrate ratios of 1:30 and 1:5, respectively. Reactions were quenched after 10 and 20 min, respectively, digested with LysN protease, and analyzed by ESI-MS (the $[M + 4H]^{4+}$ ions are shown). For both enzyme systems, the majority of the 2-fold dehydrated peptides retained the Cα deuterium at the labeled Ser residue as evidenced by their −36 Da mass shifts from the starting material. In contrast, a significant loss of Cα deuterium occurred during the 3rd and 4th dehydrations with both substrates in both enzyme systems. These data indicate that Thr9 and Ser11 are typically dehydrated before Ser18 and Ser24. Minor dehydration pathways that deviate from this general pattern are possible, and could contribute to the weak signals indicated with an asterisk in panels B–D. SM = starting material.

serine/threonine residues at each of the relevant positions in the core peptide. Instead, we employed an expressed protein ligation approach to prepare analogues in which either position Ser18 or Thr24 was replaced with [2,3,3-$^2H_3$]-serine (LctA-dS18 and -dS24, respectively, Scheme S1).[38] Because of the more facile chemical synthesis of [2,3,3-$^2H_3$]-serine, the native Thr24 residue in LctA was replaced with [2,3,3-$^2H_3$]-serine to make the LctA-dS24 analogue. Previous biochemical studies on the $LctM_{wt,cis}$ system[39] and additional assays performed in the current study on the $LctM_{ConFusion}$ system (Figure S11) showed that the T24S mutation does not significantly perturb LctM activity.

For a completely random process, where dehydration at none of the four sites in LctA is preferred, each labeled peptide would be expected to lose 25% of the deuterium label with each successive dehydration. This is clearly not observed with any of the LctM/LctA reaction systems investigated in this study. Instead, distinct patterns of deuterium loss were observed by mass spectrometry in the dehydration reactions of each site-specifically deuterated peptide. As illustrated in Figure 2, analysis of the reaction of LctM and full-length LctA labeled either at Ser18 or Ser24 shows that these two residues are typically the third and fourth residues to be dehydrated. These data are consistent with the *N*- to *C*-terminal directionality observed for the phosphorylation of the $LctA_{\Delta Cys}$ peptide by the $LctM_{Arg399Met}$ enzyme, and suggest that Thr9 and Ser11 are dehydrated prior to Ser18 and Ser24 along the major reaction path. We note that the fully dehydrated peptide, [M-4H_2O], was produced in substantial quantities within minutes in the $LctM_{wt,cis}$ reaction (Figure 3A).

To explore a potential role for the LctM cyclase domain in facilitating dehydration order, we also investigated the reaction of the $LctM_{\Delta cyc}$ enzyme with the full-length LctA peptides (i.e., the $LctM_{\Delta cyc,cis}$ system, Figure 1). Under the same conditions

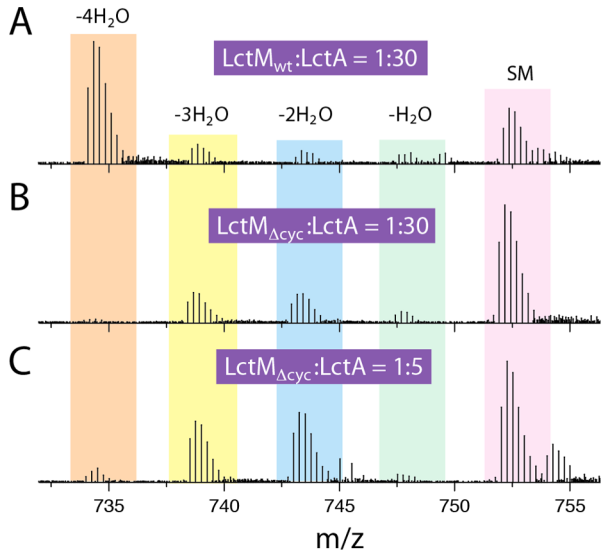

Figure 3. Relative dehydration efficiency of the $LctM_{wt}$ and $LctM_{\Delta cyc}$ enzymes with full-length LctA. All reactions had 5 μM LctA and either 0.165 μM (A and B) or 1 μM enzyme (C) and were incubated for 10 min prior to quenching, proteolysis with LysN to remove the leader peptide, and LC-ESI-MS analysis (the $[M + 4H]^{4+}$ ions are shown). Complete (4-fold) dehydration is more efficient with the wild type LctM (panel A), underscoring the important role of the LctM cyclase domain in driving complete dehydration of the LctA precursor peptide.

that were used for the $LctM_{wt,cis}$ system, the $LctM_{\Delta cyc}$ mutant clearly suffered from a drastic decrease in dehydration efficiency relative to the wt enzyme (Figure 3B). Thus, either the cyclase domain of LctM contributes significantly to productive LctA binding, or the installation of thioether rings by the functional

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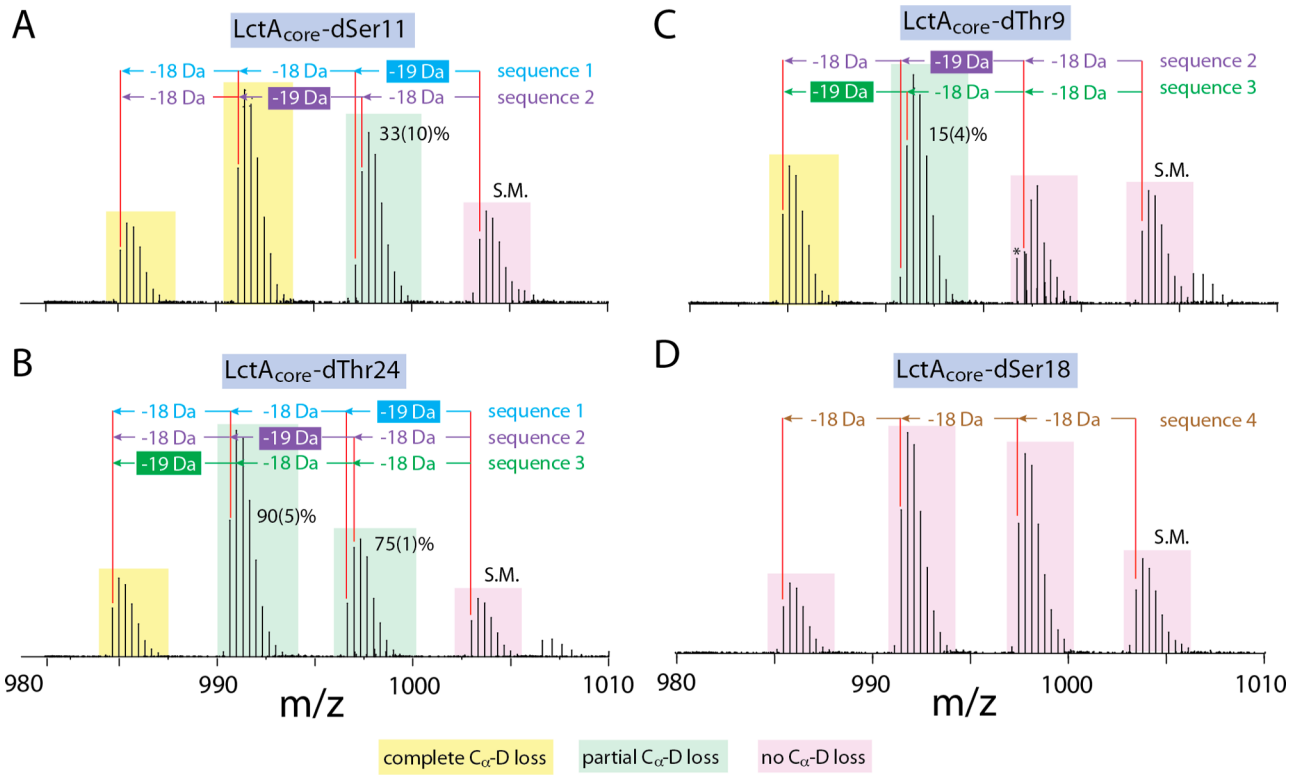

Figure 4. Dehydration in the LctM$_{ConFusion}$ system is ordered. LctM$_{ConFusion}$ (0.85 μM) was incubated with 25 μM LctA$_{core}$-dS11 (A), -dT24 (B), -dT9 (C), or -dS18 (D) under the reactions conditions given in the Supporting Methods, in the Supporting Information. The reactions were quenched after 5 min and analyzed by LC-ESI-MS, revealing a mixture of partially dehydrated intermediates (the $[M + 3H]^{3+}$ ions are shown). Dehydration of the labeled amino acid results in a characteristic mass shift of 19 Da, allowing the timing of dehydration at each site to be determined. For the LctA$_{core}$-dS11, -dT24, and -dT9 peptides, the deuterium can be lost in different dehydration steps, suggesting the presence of multiple dehydration pathways. The fourth and final dehydration occurs at Ser18 (see also Figure S2). For signals with partial loss of the deuterium label at Cα (shaded green), the percentage of Cα deuterium loss was estimated as described in Figure S5 (standard deviations are shown in parentheses). S.M. = starting material. A contaminant in panel C is labeled with an asterisk. Its mass is inconsistent with S.M. − 19 Da.

cyclase domain helps to drive LctA dehydration to completion. Intriguingly, when the dehydration order catalyzed by LctM$_{\Delta cyc}$ was assessed using the LctA-dS18 and -dS24 analogues, we again observed a strong preference for *N*- to *C*-terminal directionality, with the dehydrations of the labeled Ser18 and Ser24 residues not occurring until after Thr9 and Ser11 were dehydrated (Figure 2 and Figure S21). Thus, when provided with the full-length LctA peptide, the LctM$_{\Delta cyc}$ enzyme still catalyzes dehydrations in an order that mimics the LctM$_{wt,cis}$ system, but with much decreased efficiency.

LctA Dehydration Occurs by a Different Ordered Process When the Leader Peptide Is Detached from the Core Peptide. We next investigated the reaction systems in which the leader peptide is provided *in trans* or is attached to the enzyme. As depicted in Figure 4A for the LctM$_{ConFusion}$ reaction with LctA$_{core}$-dS11, a portion of the label was lost in the first dehydration, and the remainder of the label was lost in the second dehydration. Likewise, with the peptide labeled at Thr24, the majority of the label is lost in the first two dehydrations, with small amounts removed in the third dehydration (Figure 4B). With the LctA$_{core}$-dT9 peptide, no deuterium was lost in the first dehydration, and a small portion of the label was lost in the second dehydration; the majority of the deuterium was lost in the third dehydration (Figure 4C), suggesting that Thr9 is preferentially dehydrated third. Finally, with the peptide labeled at Ser18, none of the deuterium was lost until the final dehydration (Figure 4D and Figure S2). These results indicate that the dehydration sequence in the LctM$_{ConFusion}$ assay system is neither strict nor completely random and takes place by the following overall pattern: first Ser11/Thr24, followed by Thr9/Ser11/Thr24, then Thr9/Thr24, and finally Ser18. Clearly, multiple pathways for the dehydration of the LctA core peptide exist when LctM$_{ConFusion}$ reacts with LctA$_{core}$. Interestingly, very similar patterns of deuterium loss were observed when the leader and deuterated core peptides were provided *in trans* to the LctM$_{wt}$ or LctM$_{\Delta cyc}$ constructs (Figures S3 and S4, and Table S2). These data suggest similarities in the core peptide binding modes that lead to a similar sequence of dehydration in all three systems. In addition, the very similar results obtained with the LctM$_{\Delta cyc,trans}$ system when compared to the LctM$_{ConFusion}$ or LctM$_{wt,trans}$ systems again show that the LctM cyclase domain plays a negligible role in determining the dehydration sequence. Hence, the order of dehydration in these systems is not governed by cyclase-installed thioether rings. Importantly, the main dehydration pathway for all systems in which the leader peptide is not attached to the core peptide is quite different than what is observed in the wild type system.

As previously noted,[32,37] the reactions with the leader peptide provided *in trans* or attached to the enzyme were significantly less efficient than the reactions with full-length LctA, where the leader is attached to the core peptide. The wild type enzyme converts the full-length LctA substrate into the final product within approximately 60 min under the conditions

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employed (Figure S6). By comparison, even after 2 h under comparable conditions, complete 4-fold dehydration was minimal in the $LctM_{ConFusion}$, $LctM_{wt,trans}$, and $LctM_{\Delta cyc,trans}$ reactions (Figure S7). Clearly, the physical attachment of the LctA leader peptide to the LctA core peptide strongly influences not only the order, but also the efficiency of the LctM-catalyzed dehydration reactions.

These data are most consistent with a mechanistic model where the dehydration order in the wt system is driven primarily by the appropriate spatial positioning of the core peptide and dehydratase active site within the LctM:LctA Michaelis complex. The direct physical attachment of the leader peptide may help to anchor the core peptide relative to the dehydratase active site in a manner that leads to *N*- to *C*-terminal directionality. Consequently, when the physical constraint provided by the leader peptide is removed (as in the $LctM_{ConFusion}$, $LctM_{wt,trans}$, and $LctM_{\Delta cyc,trans}$ systems), the dehydration order changes. Moreover, the dehydration order in the $LctM_{ConFusion}$, $LctM_{wt,trans}$, and $LctM_{\Delta cyc,trans}$ systems changes in a very similar way, suggesting that the binding mode of the free core peptide to the dehydratase active site in these systems is similar and is independent of the presence of the cyclase domain. This binding mode is either not accessible or is not favorable when the leader peptide is attached to the core peptide. If this alternative binding mode is driven by molecular interactions between the core peptide amino acid sequence and active site, then the main dehydration pattern in the $LctM_{ConFusion}$, $LctM_{wt,trans}$, and $LctM_{\Delta cyc,trans}$ systems may reflect intrinsic binding affinity differences between the dehydratase active site and the different regions of the core peptide that flank the modification sites.

Cyclization Properties of the LctM Assay Systems. The observations discussed above led us to question to what extent the differences between the wt and engineered LctM/LctA systems are linked to the underlying (methyl)lanthionine architecture. Thus, we investigated the extent of cyclization in each catalytic system using *N*-ethylmaleimide (NEM) to alkylate free Cys thiol moieties, which allows otherwise isobaric cyclized and uncyclized species to be distinguished by mass spectrometry (Figure 5). With the wild type system, a substantial portion of the starting material is converted to the fully dehydrated and cyclized product ([M + Dhx + 3Lan]) within 5 min (Figure 5E), and the reaction is complete at 60 min (Figure S6). In contrast, for the $LctM_{ConFusion}$, $LctM_{wt,trans}$, and $LctM_{\Delta cyc,trans}$ systems, substantial portions of the total peptide remained partially modified, even after extended incubation times (Figure 5 and Figure S7). A substantial portion of the accumulated intermediates carry three NEM adducts (i.e., are not cyclized, Figure 5, high *m*/*z* ions) even though they are dehydrated at positions that are cyclized in lacticin 481; the fraction of dehydrated yet noncyclized intermediates is much larger than that seen for the $LctM_{wt,cis}$ system. Hence, similar to its effect on dehydration order and efficiency, the physical linkage of the leader and core peptides seems to greatly assist the rate of cyclization by LctM, hinting at some contribution of the spatial positioning model to efficient cyclization. The slow cyclization rates in the $LctM_{ConFusion}$, $LctM_{wt,trans}$, and $LctM_{\Delta cyc,trans}$ reactions also result in the formation of adducts of triscarboxyethyl phosphine (TCEP; present in the assay as a reductant) with nascent Dha residues (Figures S8 and S9), which further reduces the flux to the final product.

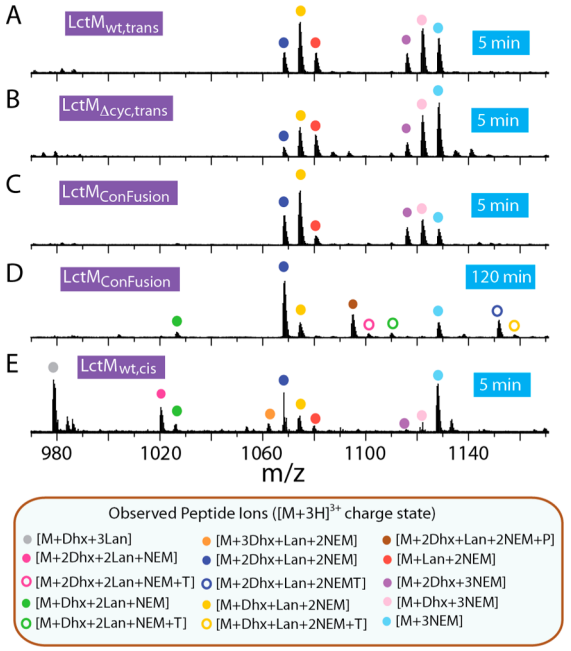

Figure 5. Electrospray ionization mass spectral comparison of reaction intermediates in the $LctM_{wt,trans}$ (A), $LctM_{\Delta cyc,trans}$ (B), $LctM_{ConFusion}$ (C and D), and $LctM_{wt,cis}$ (E) reactions. All reactions were performed at an enzyme:substrate ratio of 1:30 for the indicated length of time. The $LctM_{wt,trans}$, $LctM_{\Delta cyc,trans}$, and $LctM_{ConFusion}$ systems contained 0.85 μM enzyme and 25 μM of the $LctA_{core}$-dT24 peptide. The $LctM_{wt,trans}$ and $LctM_{\Delta cyc,trans}$ assays also contained 25 μM of the LctA leader peptide. The $LctM_{wt,cis}$ reaction contained 0.165 μM LctM and 5 μM LctA. After quenching, all samples were alkylated with NEM, leading to a +125.13 Da mass shift for each free Cys thiol present in the peptide. Lack of NEM alkylation indicates a cyclized Cys as described in a previous study.[13] The full-length LctA peptides from the $LctM_{wt,cis}$ reaction were digested with LysN to remove the leader peptide before MS analysis. Dhx = Dha or Dhb ([M − 18.01 Da] for H_2O or [M − 19.02 Da] for HDO); Lan = Lan or MeLan; P = HPO_3 ([M + 79.97 Da]); *T* = TCEP ([M + 250.06 Da]).

Remarkably, despite lacking the LctM cyclase domain, the $LctM_{\Delta cyc,trans}$ system showed nearly identical levels of cyclization as the $LctM_{ConFusion}$ and $LctM_{wt,trans}$ systems (Figure 5 and Figure S7). This observation is in line with a recent report by Sonomoto and co-workers,[33] who demonstrated cyclization activity similar to what is observed here using a truncated variant of NukM that shares 43% amino acid sequence identity with LctM and makes a structurally similar product. The observation that the thioether ring formation in the $LctM_{\Delta cyc,trans}$ reaction (and perhaps also in the $LctM_{ConFusion}$ and $LctM_{wt,trans}$ reactions) apparently does not absolutely require catalysis by the LctM cyclase domain can possibly be explained by a nonenzymatic event, potentially resulting in a loss of cyclization fidelity.

Indeed, evidence for the presence of a non-natural thioether topology for the first installed ring was obtained by careful analysis of the deuterium composition, and the chromatographic and fragmentation properties of the [M + Dhx + Lan] intermediate produced in the reaction of $LctM_{ConFusion}$ with the LctA-dS11 peptide (Figure 6). The extracted ion chromatogram for this species exhibits a time-dependent change from 12.8 to 12.6 min as the reaction progresses (Figure 6A), suggesting the presence of at least two [M + Dhx + Lan] isomers. The b_{20-24} fragment ions observed in the tandem mass

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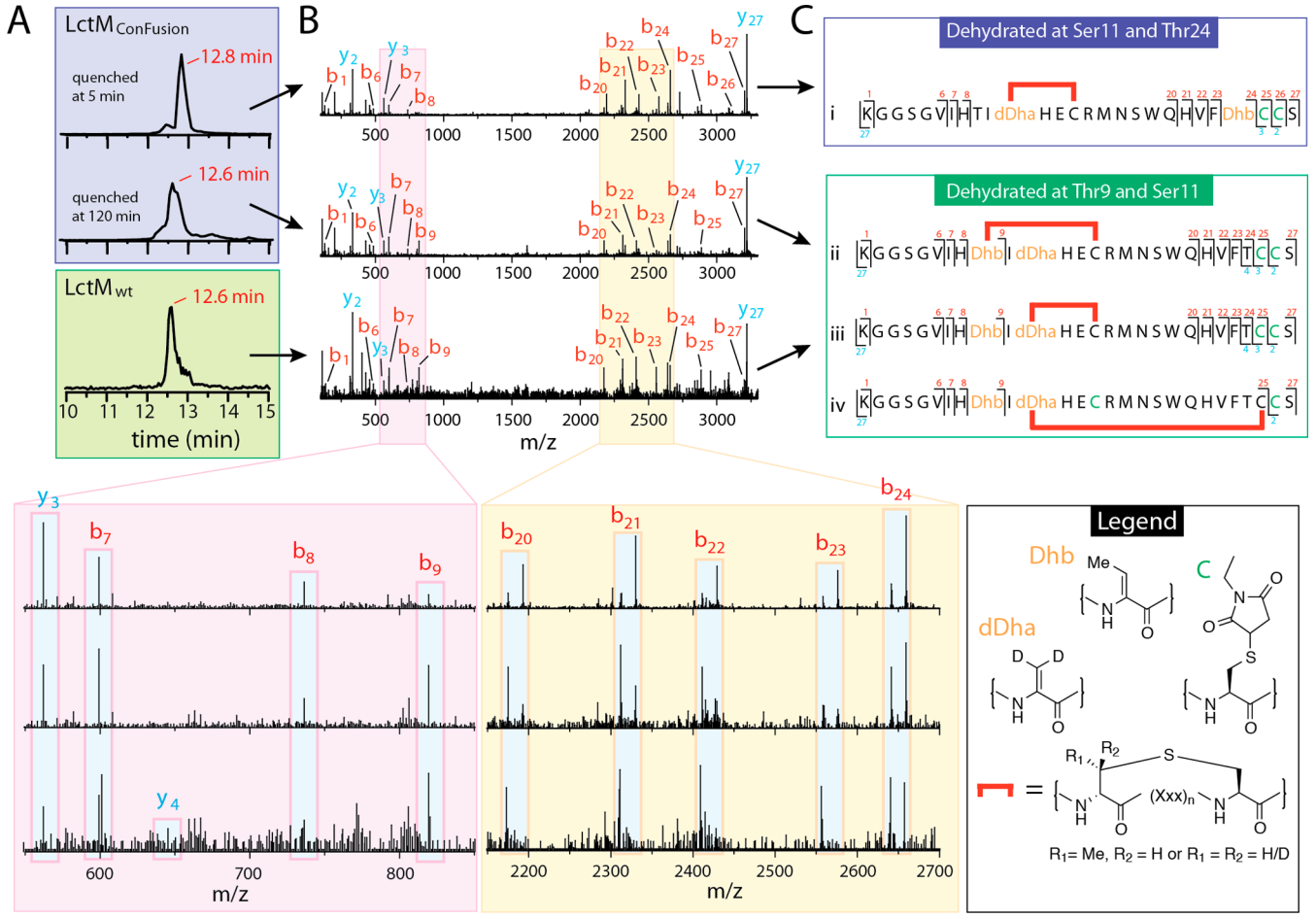

Figure 6. Structural analysis of the [M + Dhx + Lan] intermediate. Extracted ion chromatograms (A) and tandem mass spectra (B) of the [M + Dhx + Lan + 2NEM + 4H]$^{4+}$ ions derived from the reactions of LctM$_{ConFusion}$ with LctA$_{core}$-dS11 ([M + 4H]$_{obs}^{4+}$ = 806.109, [M + 4H]$_{calc}^{4+}$ = 806.115) and of LctM$_{wt}$ with full-length LctA ([M + 4H]$_{obs}^{4+}$ = 805.607, [M + 4H]$_{calc}^{4+}$ = 805.612, after LysN treatment). The insets to panel B highlight several critical fragment ions (discussed in the main text) that aided in the proposed assignment of the dehydration patterns and thioether topologies depicted in part C. Hypothetical structure enumeration and evaluation (Figure S12), tandem MS analysis of TCEP adducts (Figure S22), tandem MS of a lacticin 481 standard (Figure S9), and studies with other isotopically labeled peptides (Figures 2 and 4, and Figure S21) provide additional support for the structures proposed in part C.

spectra (Figure 6B) clearly show (via loss of the deuterium label) that the deuterated Ser11 residue has been dehydrated in both isomers, and that the two [M + Dhx + Lan] isomers differ in the regiochemistry of the second dehydration, with one isomer containing dehydroamino acids at Dha11 and Dhb24 and the second isomer containing Dhb9 and Dha11 residues. Regarding the Dha11/Dhb24 isomer, the observation of the doubly NEM-alkylated y_3 ion (Figure 6B) and the extensive fragmentation in the *C*-terminal region of the core peptide clearly suggests the presence of the *non-natural* Dha11-Cys14 lanthionine ring (Figure 6C, species i; note that the natural Dhb9-Cys14 ring cannot be formed in this species since Thr14 is not dehydrated). This non-natural Dha11-Cys14 lanthionine ring was also produced by the LctM$_{wt,trans}$ and LctM$_{\Delta cyc,trans}$ systems (Figure S20), further supporting the notion that the physical linkage of the core peptide to the leader peptide plays a critical role in controlling cyclization fidelity. The formation of an incorrect first ring, made possible by a different dehydration sequence, may also contribute to the overall decreased efficiency in all systems where the leader peptide is not attached to the core peptide. Because the first ring in the LctM$_{ConFusion}$, LctM$_{wt,trans}$, and LctM$_{\Delta cyc,trans}$ systems is cyclized incorrectly under the reaction conditions employed (see below how this outcome can change upon increasing the enzyme concentration), we did not attempt to characterize more extensively cyclized species formed under these reaction conditions, since they would not likely provide insights into catalysis by the LctM$_{wt,cis}$ system.

The non-natural ring may be installed by a *nonenzymatic* cyclization event that competes with the inefficient enzymatic cyclization in the LctM$_{ConFusion}$ system. Despite the existence of this side reaction, it is important to note that a fully modified, biologically active product can be produced in significant quantities in the LctM$_{ConFusion}$ reaction at *higher enzyme concentrations* (Figure S9), perhaps because the enzyme can more effectively compete with an incorrect, nonenzymatic cyclization event. A similar competition between correct enzymatic and incorrect nonenzymatic cyclization was reported for the cyclization catalyzed by NisC. For this system, increasing the enzyme concentration resulted in an increase in the fraction of correctly cyclized product.[40] The incorrect ring in the LctM$_{ConFusion}$ system can be formed because dehydration fidelity is lost and Thr9 is not dehydrated at early time points, enabling Cys14 to instead attack Dha11 to form the four-amino-acid Dha11-Cys14 ring. Previous nonenzymatic model studies have shown that attack on a Dha forming a small

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ring is a rather efficient process,[41] potentially because of the formation of a turn that stabilizes the intermediate enolate via intramolecular hydrogen bonding.[42,43] It is possible that incorrectly cyclized intermediates cannot be fully dehydrated by $LctM_{ConFusion}$, leading to the partially modified intermediates that persist in the reaction mixture (Figure S9A).

In contrast, there is no clear evidence for the presence of the non-natural Dha11-Cys14 topology in the $LctM_{wt,cis}$ reaction. Species i (Figure 6C) is not produced in the $LctM_{wt,cis}$ reaction, because the first two dehydrations in this system occur at Thr9 and Ser11 (Figure 2 and Figure S21). Instead, the chromatographic and fragmentation properties of the [M + Dhx + Lan] species produced in the $LctM_{wt,cis}$ reaction closely match the properties of the Dhb9/Dha11 [M + Dhx + Lan] isomer produced in the $LctM_{ConFusion}$ reaction (Figure 6). The collision-induced dissociation (CID) MS/MS spectra for the Dhb9/Dha11 [M + Dhx + Lan] isomers produced in the $LctM_{wt,cis}$ and $LctM_{ConFusion}$ reactions are characterized by alkylated Cys25 and Cys26 residues and by extensive fragmentation in the *C*-terminal region of the core peptide. These fragmentation data are consistent with one of several possible identities for the Dhb9/Dha11 [M + Dhx + Lan] species. The first, which we favor, is that it this species has the native Dhb9-Cys14 methyllanthionine ring (species ii). Indeed, we were able to trap species ii in the $LctM_{wt,cis}$ reaction by increasing the TCEP concentration in the assay mixture, which results in TCEP addition to Dha11 (Figure S22). The observed dehydrated b_9 ion at first glance appears inconsistent with species ii, but the same dehydrated b_9 ion is also observed in the CID fragmentation spectrum of an authentic lacticin 481 standard isolated from the *Lactococcus lactis* native producer, as well as in the fragmentation spectra derived from the fully modified, biologically active products of the $LctM_{ConFusion}$ and $LctM_{wt,cis}$ reactions (Figure S9). Hence, we believe that the b_9 ion results from fragmentation within the Dhb9-Cys14 methyllanthionine ring. While (methyl)lanthionines usually suppress fragmentation within the rings, such fragmentation has been observed previously with other lanthipeptides.[44−46] In support of this conclusion, we did not detect fragmentation within the (methyl)lanthionine rings of lacticin 481 when the peptide was subjected to fragmentation by electron transfer dissociation (Figure S24). A second possibility that we find less likely, but cannot fully exclude, is that the non-native Dha11-Cys14 lanthionine ring also forms in the $LctM_{wt,cis}$ reaction and that the observed fragmentation spectrum is derived from species iii (Figure 6C). It should be noted that the [M + Dhx + Lan] species produced in the $LctM_{wt,cis}$ reaction is a minor intermediate that does not accumulate to nearly the same extent as it does in the $LctM_{ConFusion}$ reaction (data not shown). Thus, even if the Dhb9/Dha11 [M + Dhx + Lan] intermediate produced by the $LctM_{wt,cis}$ system is indeed improperly cyclized, this incorrect topology would only be present as a small fraction of the final product. Consistent with this claim, we were unable to detect any time-dependent changes in the fragmentation properties of the [M + Dhx + 3Lan] final product that would have suggested a significant change in thioether topology as the $LctM_{wt,cis}$ reaction progresses (Figure S19). Finally, we cannot exclude the possibility that both species ii and iii (as well as other isomers such as species iv) are present in these reaction mixtures. Such a mixture of isomers may account for the asymmetric chromatographic peak shapes (Figure 6A) as well as the slow reactivity of a portion of the [M + Dhx + Lan] pool that persists in the $LctM_{ConFusion}$ reaction (Figure S9A).

CONCLUSIONS

The synthases involved in RiPP biosynthesis face the daunting task of converting linear precursor peptides into highly post-translationally modified peptides with defined structures. In order to achieve this remarkable feat, the iterative catalytic activities of the synthetase must be spatiotemporally controlled in a manner that facilitates formation of the proper final product, even when many potential structural isomers are possible. The mechanisms by which RiPP biosynthetic systems control biosynthetic fidelity have only recently come into focus, with the realization that these secondary metabolites are widespread in nature and possess a range of useful biological activities.[1] One of the key challenges in elucidating RiPP biosynthetic pathways is in deconvoluting the multiple reactions catalyzed by the iterative RiPP synthases. Toward this end, we combined in this study isotopically labeled peptides, engineered enzyme systems, and mass-spectrometry-based structural and kinetic analyses in an attempt to illuminate the key biosynthetic features of the class II lanthipeptide synthetase, LctM. Our studies demonstrate a role for the LctA leader peptide in controlling the directionality of dehydration, the fidelity of thioether ring biosynthesis, and the overall catalytic efficiency of the synthetase. All three properties are perturbed when the leader peptide is removed from the core peptide. The *N*- to *C*-terminal dehydration directionality and tight coupling of dehydrations and cyclization in the wt system suggests that the precise spatial arrangement of the core peptide and synthetase active sites (enforced by the leader peptide) is critical for LctA maturation. In addition, the observation that efficient 4-fold dehydration requires efficient cyclization suggests that the structure of the maturing intermediate also plays a role in catalytic efficiency. These studies show that the leader peptide is not merely an intramolecular allosteric element that activates the biosynthetic enzyme,[47] but that correct positioning of the core peptide by the leader peptide is critical for catalysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b00163.

Description of enzyme and peptide preparation, activity assays, mass spectrometric analysis, hypothetic structure evaluation, and additional results and discussion (PDF)

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Notes

The authors declare no competing financial interest.

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