

Distributive and Directional Behavior of Lantibiotic Synthetases Revealed by High-Resolution Tandem Mass Spectrometry

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Abstract: The lantibiotic synthetases LctM and HalM2 are bifunctional enzymes that catalyze both the dehydration of serine and threonine residues and the Michael-type additions of cysteine residues to the resulting dehydroamino acids in their substrate peptides. Using Fourier transform mass spectrometry to analyze these activities *in vitro*, the dehydration is shown to take place by a distributive mechanism, with build-up of intermediates observed in electrospray mass spectra. The cyclization activity of HalM2 was monitored through alkylation of free cysteines in intermediates, providing access to the regioselectivity of lanthionine ring formation using high-resolution tandem mass spectrometry. HalM2 is shown to catalyze the cyclization process in a largely N- to C-terminal directional fashion, forming a total of four lanthionine rings in its HalA2 substrate. These studies advance a model for lantibiotic production where substrate binding *via* an N-terminal leader results in dehydration and cyclization on similar time scales and with a high, though not strict, propensity for N-to-C directionality.

Introduction

Lantibiotics are post-translationally modified polycyclic antimicrobial peptides of varying structure.¹ The best-known family member is nisin, which has been used for nearly four decades in the food industry to combat food-borne pathogens. During biosynthesis of class II lantibiotics, the bifunctional LanM enzymes install the characteristic lanthionine and methylanthionine thioether cross-links that have given these compounds their name. The activities of the LanM enzymes involved in the biosynthesis of lactacin 481 (LctM)² and haloduracin (HalM1 and HalM2)³ have been reconstituted *in vitro*. Studies on these enzymes have demonstrated that they catalyze the dehydration of serine (Ser) and threonine (Thr) residues in their peptide substrates to generate the corresponding dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, as well as the ensuing Michael-type addition of cysteines to the dehydroamino acids to generate the thioether cross-links. LctM dehydrates four Ser/Thr residues in its substrate LctA and subsequently generates three thioether cross-links, whereas HalM2 dehydrates seven Ser/Thr residues and installs four (methyl)lanthionines in its substrate HalA2 (Figure 1). All modifications occur on the C-terminal structural region of the substrate peptide, whereas

the N-terminal leader peptide is not modified. The timing and order in which these fascinating enzymes carry out these multiple modifications is currently unknown. In this contribution, we used Fourier transform mass spectrometry to address these questions for both LctM and HalM2 synthetases, using methodology analogous to a prior study of the ribosomally encoded precursor to the thiazole/oxazole-containing antibiotic, microcin B17.⁴ However, because cyclization during lantibiotic biosynthesis does not result in a change in mass, and because the rings in many lantibiotics are intertwined, answering these questions for lantibiotic biosynthesis is technically more demanding. The findings reported in this work have important mechanistic implications and ultimately may help the manipulation of these impressive catalysts for lantibiotic engineering.

Results

Kinetic Time Course of Phosphorylation of LctA by LctM. Previous work on LctM has shown that the serines and threonines in the structural region targeted for dehydration by LanM enzymes are first phosphorylated in an ATP/Mg²⁺-dependent mechanism,^{5,6} prior to an elimination step that may or may not occur in the same active site. In addition, prior studies have demonstrated that at relatively high substrate concentrations (*i.e.*, just below the solubility limit of $\sim 1 \mu\text{M}$), the LctA substrate is converted into the 4-fold dehydrated product without accumulation of intermediates. Recent data

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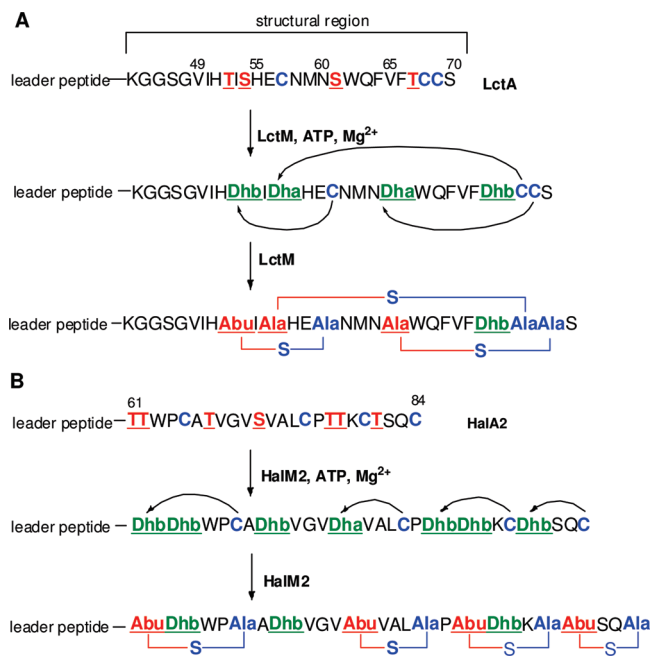


Figure 1. (A) Lactacin 481 synthetase, LctM, catalyzes the dehydration of four Ser/Thr residues in the structural region of its substrate peptide LctA. Subsequently, it catalyzes the Michael-type addition of three cysteine thiols to the dehydroamino acids to generate lanthionine (from Ser) and methylanthionine (from Thr) cross-links. Leader peptide of LctA: MKEQNSFNLLQEVTESELDLILGA. Abu-S-Ala = methylanthionine; Ala-S-Ala = lanthionine. (B) Haloduracin β synthetase (HalM2) catalyzes similar dehydrations and cyclizations of its substrate HalA2. Leader peptide of HalA2: MVNSKDLRNPFRKAQGLQFVDEVNEKELSSLAGSGDVHAQ.

suggest that this observation is not due to a processive mechanism of the enzyme but rather to a slow deaggregation of the peptide substrate followed by faster enzymatic processing.⁷ At much lower substrate concentrations, partially dehydrated intermediates are observed, but the amounts of materials accessible under these conditions have greatly limited investigation of the positions of dehydration. A possible solution to obtaining sufficient amounts of intermediates for investigation by tandem mass spectrometry is provided by the Arg399Met mutant of LctM that still carries out the phosphorylation step but has strongly impaired elimination activity, resulting in build-up of phosphorylated intermediates as observed by MALDI-TOF MS⁶ and ESI-FTMS (*vide infra*). The presence of three cysteines in these phosphorylated peptides proved a hurdle to investigate the location of the phosphorylations and therefore all three cysteines were replaced with alanines by site-directed mutagenesis. The resulting mutant peptide will be referred to in this work as Cys-free LctA. This peptide proved a good substrate for LctM, resulting in the expected four dehydrations and it was therefore utilized with the LctM-Arg399Met enzyme.

A time course of the phosphorylation activity for LctM-Arg399Met is shown in Figure 2A. After 1 min of incubation, an intermediate product containing a single phosphorylation was detected, and after 5 min, a mixture was observed corresponding to one and two phosphorylations. By 10 min, the predominant ions are associated with a peptide with two phosphorylations, and after 40 min some 3-fold phosphorylated product was generated. As the number of phosphorylated species increased, it was observed that the charge state distribution had a slight

tendency to shift toward lower charge (higher m/z) (Supplementary Figure 1, Supporting Information).

Localization of Phosphorylations by Tandem Mass Spectrometry. Use of octupole collisionally activated dissociation (OCAD) for ion fragmentation did not result in the number of backbone cleavages required for precise localization of phosphorylations on intermediates. Additionally, due to OCAD being a “slow heating” fragmentation method⁸ and the intact mass of the lactacin precursor being somewhat low relative to intact phosphoproteins,⁹ the MS/MS data resulted in partial dephosphorylation (unpublished results, Supplementary Figure 2, Supporting Information) and the resulting fragments that incorporate the corresponding dehydroamino acids did not allow localization of the former phosphorylated intermediates. Use of an orthogonal fragmentation method, electron capture dissociation (ECD),¹⁰ resulted in better fragment coverage in the structural region of the LctA substrate, with the Cys-free LctA mutant providing the best data for retention and localization of each phosphorylation. The observation of an unmodified c50 fragment ion in all phosphorylated peptides indicates that all modified serine and threonines are localized to the propeptide/structural region of Cys-free LctA. We note that the fragment ion designation takes into account an N-terminal 19-amino acid His-tag and linker peptide and does not correspond to the amino acid numbering of the LctA substrate. The numbering used herein is depicted in Figure 1. Fragmentation of the early intermediate(s) containing a single phosphorylation resulted in detection of both (c53 + 80)⁴⁺ ions (major, Figure 2B) and unmodified c53⁵⁺ ions (minor), indicating the presence of positional isomers for this monophosphorylated peptide. Analysis of the intermediate containing two phosphorylations unambiguously showed that these are located at Thr52 and Ser54, as shown by the observation of (c53 + 80)⁴⁺ and (c60 + 160)⁵⁺ ions. All c-ions resulting from fragmentation C-terminal to Asn60 also had mass shifts of +160 Da, consistent with complete phosphorylation at Thr52 and Ser54. A second key fragment ion that was observed was (z20 + 160)²⁺, further corroborating the assignment of the first two phosphorylations to Thr52 and Ser54. Collectively, these results indicate that LctM-Arg399Met slightly favors Thr52 with respect to the first phosphorylation but that the enzyme can phosphorylate Ser54 first. In addition, the mutant LctM always phosphorylates these two residues prior to any Ser/Thr located more C-terminal. Indeed, the third phosphorylation was localized to Ser61 by the observation of an additional (c64 + 240)⁷⁺ fragment ion in the peptide that had been phosphorylated three times. A schematic representation in Figure 2C summarizes the key c- and z-fragment ions that localize each phosphorylation and demonstrate that Arg399Met displays a distributive and not strictly directional type of catalysis in moving from the leader peptide toward the C-terminus.

Directionality of Dehydration of HalA2 by HalM2. To probe the generality of the conclusion from the phosphorylation studies that LctM acts directionally in a largely N-to-C fashion, a second lantibiotic synthetase was investigated. HalM2, one of the two synthetases involved in the biosynthesis of the two-component

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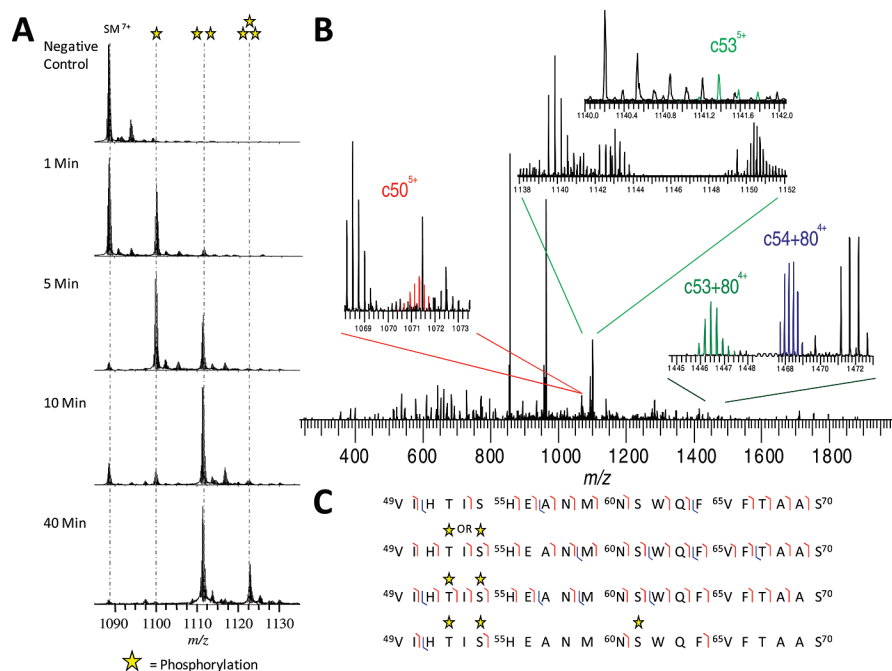


Figure 2. (A) Time dependence of phosphorylation of Cys-free His₆-LctA (12 μM) by LctM-R399 M (1.2 μM) monitored by ESI-FT-ICR MS operating at 12 T; SM, Starting Material. Stars indicate phosphorylated Ser/Thr residues and in the negative control, the substrate peptide was not treated with LctM-R399M. (B) ECD fragmentation of the intermediate after one phosphorylation event. The c50 ions (red) localize all modifications to the structural region. The c53 (light green, minor) and c53 + 80 (forest green, major) ions along with a c54 + 80 ion (blue) demonstrate that there are two isomers, one with the phosphorylation on Thr52 and one with the phosphorylation on Ser54. (C) Schematic representation of all observed fragment ions used to localize the phosphorylations in the structural region. The top entry shows the fragments in the starting peptide. Depicted below are in order the fragments observed in peptide phosphorylated once, twice, and thrice.

lantibiotic haloduracin,^{3,11} was chosen due to the good solubility of its substrate HalA2. As a result, unlike LctM, HalM2 does generate partially dehydrated intermediates in the presence of relatively high concentrations of substrate, as shown in the time course in Figure 3A. After three minutes, ions of approximately equal intensity corresponding to intermediates that had undergone one to seven dehydrations were detected. Over time, the substrate was converted to intermediates with an increasingly higher number of dehydrations, until all substrate was converted to a 7-fold dehydrated product (Figure 3A, bottom). Thus, like LctM at low substrate concentrations,⁷ HalM2-catalyzed dehydration takes place in a distributive manner.

The distributive nature of HalM2 afforded the opportunity to investigate all intermediates resulting from dehydration with respect to directionality. The directionality of HalM2 dehydration was determined by isolation and fragmentation of each peak corresponding to an increasing number of dehydrations in a linear ion trap using collisionally activated dissociation (CAD), with detection of fragment ions at high resolution in the FT-ICR cell. Analysis of the resulting fragments for each intermediate demonstrated that the first four dehydrations were localized to Thr61, Thr62, Thr67, and Ser71, through the observations of b61, b62, b63, b67, and b68 ions that directly encompass each dehydrated residue and the presence of b75 containing a dehydrated Ser71 (Supplementary Figure 3A–C, Supporting Information). Fragment ions b81 and b82 ions also confirmed recent conclusions based on site-directed mutagenesis experiments that Ser82 escapes dehydration in HalA2.¹¹ The presence of b77 and b78, both containing five dehydrations, localize the fifth dehydration unambiguously to T77 (Supplementary

Figure 3D, Supporting Information). The sixth and seventh dehydrations were localized to Thr78 and Thr81, respectively, through the observation of b78 through b82 with exact mass values matching with <10 ppm mass accuracy (Supplementary Figures 3E and F, Supporting Information). It should be noted that, based on MS/MS data, the dehydrations can be attributed to enzymatic dehydrations, and are not a result of MS² neutral loss. In Supplementary Figure 3A (Supporting Information), no isotopic distributions were observed at *m/z* 951.896 or *m/z* 963.759 in Supplementary Figure 3B (Supporting Information) that would indicate dehydration due to CAD fragmentation. Similarly, in Supplementary Figure 3C (Supporting Information), the absence of peaks at *m/z* 843.415 and *m/z* 866.674 further points to enzymatic dehydration. Similar observations were made for subsequent dehydrations as shown in Supplementary Figure 3D–F (Supporting Information).

Directionality of Cyclization of HalA2 by HalM2. In addition to providing sufficient amounts of intermediates to follow the order of the dehydrations, the build-up of intermediates during processing of HalA2 by HalM2 also provided the opportunity to probe the order and possible directionality of thioether ring formation which has hitherto not been achieved. Because products of dehydration and cyclization are not distinguishable by mass, alkylation of all free cysteines present in intermediates was utilized to differentiate cyclized and noncyclized cysteines. Alkylation with iodoacetamide (IAA) adds a +57.021 Da shift in mass to each free (i.e., uncyclized) cysteine. A time course for processing of HalA2 by HalM2 is shown in Figure 3B, producing intermediates of nearly all possible combinations of dehydrations and cyclizations. Each observed ion could be annotated with respect to the number of dehydrations (indicated by solid circles) and cyclizations (indicated by stars) based on

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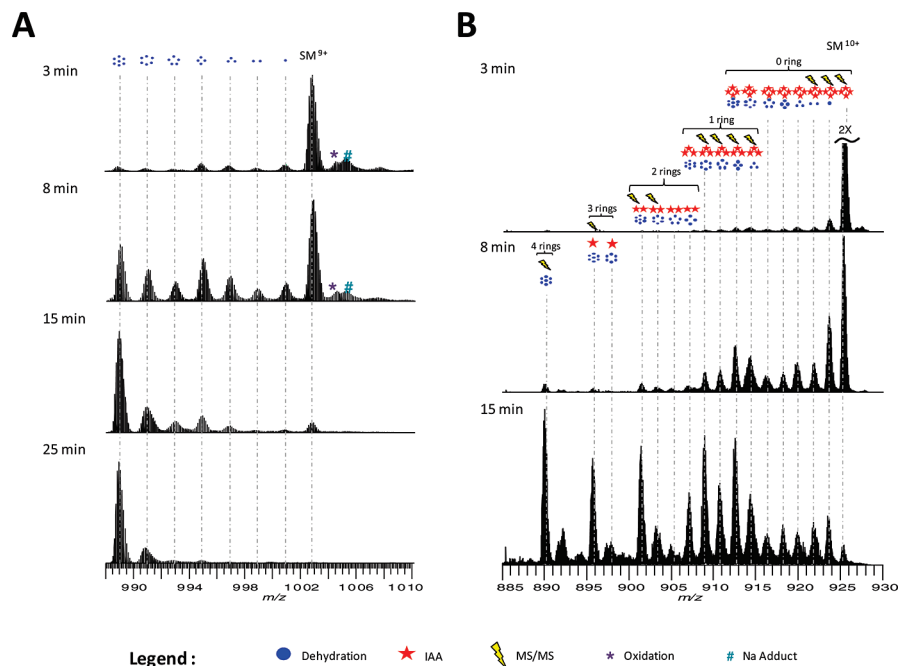


Figure 3. (A) Time dependence of dehydration of His₆-HalA2 (111 μM) by His₆-HalM2 (0.7 μM) monitored by ESI-FT-ICR MS. The number of dehydrations associated with each group of ions is indicated with blue circles. Oxidation products (M+16) are marked with an asterisk and sodium adducts (M+23) are marked with a #. (B) Time dependence of dehydration and cyclization of His₆-HalA2 by His₆-HalM2. Assay samples were quenched at the indicated time points and treated with IAA to alkylate non-cyclized cysteines. The number of alkylations and dehydrations are annotated by red stars and blue dots, respectively. The yellow lightning bolts signify peaks that were targeted for MS².

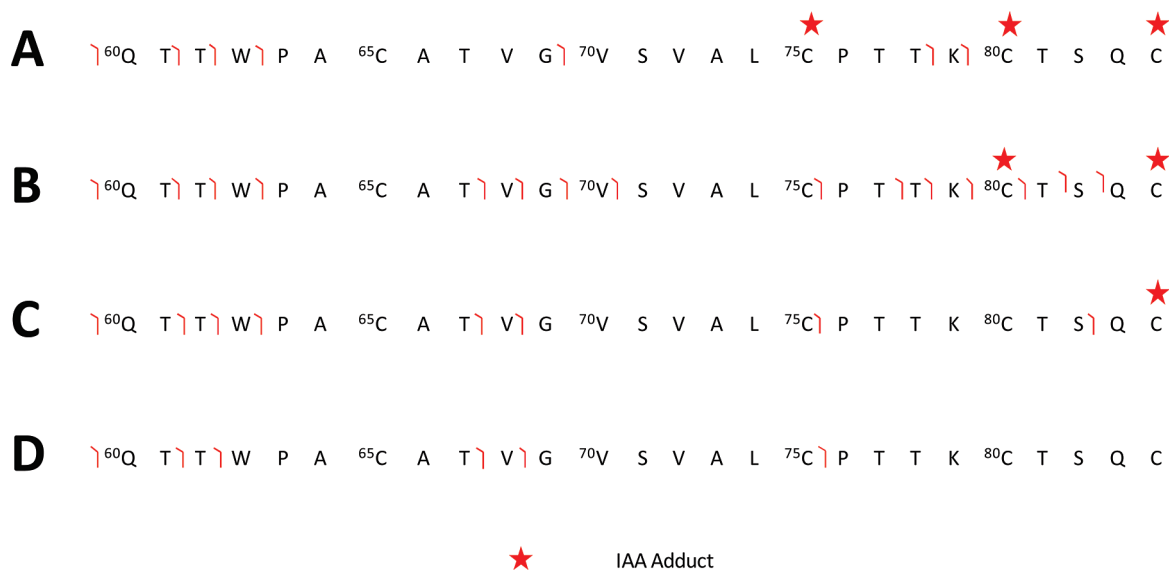


Figure 4. Localization by tandem MS of the IAA adducts in products dehydrated 6-fold (A) or 7-fold (B–D). The ions targeted for MS² to create this schematic representation are indicated in Figure 3B with yellow lightning bolts.

the number of observed alkylations. Unfortunately, even with this derivatization, significant mixing of fragmentation spectra was observed due to partially overlapping isotopic distributions in such a mixture. To ease such overlap and increase signal intensity, HalA2 was heterologously expressed in *Escherichia coli* cells grown on minimal medium containing ¹³C-depleted glucose and ¹⁵N-depleted (NH₄)₂SO₄ (Supplementary Figure 4, Supporting Information). The combination of an isotopically depleted substrate and IAA alkylation allowed the use of tandem Fourier transform mass spectrometry to assign the position of each alkylated cysteine (Cys) in the fragment ions containing different degrees of cyclization. The ions containing six and seven dehydrations and between zero and four IAA adducts were

analyzed in particular depth. Observation of an unmodified b59 ion localizes all dehydrations and IAA adducts to the structural region, as anticipated. Fragmentation of the peptide product of seven dehydrations and one IAA alkylation resulted in b82, b75, b68, and b67 ions without alkylated cysteines, demonstrating that the single alkylation was present C-terminal to Ser82 (which localizes it to Cys84; Figure 4 and Supplementary Figure 5, Supporting Information). These data suggest that all other cysteines underwent cyclization and, therefore, that the D-ring of Halβ is formed last. Fragmentation of the peptide product of seven dehydrations and two IAA alkylations resulted in b79, b78, b77, b75, b70, b69, b68, and b67 ions without any alkylated cysteines, and b82, b81, and b80 ions with one alkylated

cysteine. Therefore, these ions show that one alkylation again occurred on Cys84 and the second alkylation must be on Cys80. Hence, the C-ring is formed penultimately. Finally, fragmentation of the peptide product of six dehydrations and three IAA alkylations resulted in a b69 ion without alkylated cysteines, and b79 and b78 ions with one alkylated cysteine, which must be Cys75 (Figure 4). Thus, collectively these data show that cyclization is largely distributive and that the first ring formed is the A-ring, followed by the B-ring, the C-ring, and finally the D-ring. Hence, the cyclization activity of HalM2 also shows a basic propensity for directional processing, from N- to C-terminus. Given the complexity of the MS and MS/MS data, minor processing pathways that do not follow this strict directionality cannot be ruled out based on cleanliness of intact ion isolation and the signal-to-noise of the key fragment ions (Supplementary Figure 5, Supporting Information).

Discussion

Lantibiotic synthetases are remarkable enzymes that carry out multiple post-translational modifications with precise control over regio- and stereoselectivity. For instance, HalM2 first breaks fourteen chemical bonds during seven dehydrations of Ser/Thr residues in the structural region of the HalA2 peptide, and then forms eight new chemical bonds in four cyclization reactions (Figure 1B). Recent studies have shed light on the residues of these bifunctional enzymes that are important for both dehydration and cyclization activity,^{6,12} but how the enzyme exerts control over especially the regioselectivity of catalysis is still poorly understood. The current study shows that both the dehydration and cyclization processes take place in a distributive fashion. That is, the products of each successive dehydration/cyclization are released into solution, an important observation with implications for understanding the mechanism of catalysis.

The leader peptide is generally believed to be important for substrate recognition,^{13–15} but it was recently shown to not be required for dehydration activity by LctM.¹⁶ In the absence of the leader peptide, a low level of background dehydration activity was observed, suggesting that leader peptide binding does not induce a requisite conformational change to activate the enzyme. Instead, the observations are more in line with an equilibrium between an active and inactive form of the enzyme favoring the latter, with leader peptide binding shifting this equilibrium toward the active form.⁷ Indeed, when the leader peptide was provided *in trans* with the structural peptide to LctM, dehydration catalysis was significantly enhanced, albeit still slower than when the leader peptide was attached covalently to the structural region.¹⁶ Interestingly, when the leader peptide was provided *in trans* the dehydrations were non-directional,¹⁶ in contrast to the data presented herein that provide strong support for an N-to-C-terminal direction of dehydration by both LctM and HalM2 when the leader peptide is covalently attached

to the structural region of LctA and HalA2, respectively. The greatly enhanced dehydration activity and the observed directionality when the leader peptide is covalently attached are consistent with a model in which leader peptide binding brings the Ser and Thr in the structural region in closer proximity to the dehydration active site.⁷ Ser/Thr residues in the structural region nearer to the leader peptide would have a greater probability of being dehydrated than residues that are located more C-terminal. This static binding model for the leader peptide can account for the observation that directionality is not strict because residues that are close to each other, such as Thr52 and Ser54 of LctA, are not strongly differentiated.

Directional activity has been reported previously for the post-translational modifications during microcin B17 biosynthesis, a peptide antibiotic produced by certain *E. coli* strains. In this system, Cys and Ser residues are converted by a three-subunit synthetase to thiazoles and oxazoles in a precursor peptide that also contains an obligatory leader sequence.¹⁷ Analysis of intermediates in the formation of these heterocycles demonstrated that the rings are formed distributively, with the regioselectivity resulting from a basic N-to-C directionality occasionally overridden by an increased chemoselectivity of Cys-to-thiazole versus Ser-to-oxazole transformations.⁴ A difference between the lantibiotic synthetases and microcin B17 synthetase is that the latter shows no activity when the leader and structural peptides are presented *in trans*.¹⁸ Multistep processing of other peptide substrates, such as the domains of factor IX containing gamma-carboxyglutamic acids have also been interrogated by mass spectrometry-based kinetic assays. Here, the vitamin K-dependent gamma-glutamyl carboxylase active site induces an allosteric effect that may achieve processive carboxylation and regulate the release of carboxylated product.¹⁹

Lantibiotic synthetases are typically depicted as first carrying out all dehydrations prior to the cyclizations (*e.g.*, Figure 1), but inspection of the data in Figure 3B shows that cyclization takes place on a similar time scale as dehydration; some intermediates that have not been completely dehydrated already contain rings within their N-terminal structural regions. More definitive conclusions regarding the molecular logic used by these multifunctional enzymes to control regioselectivity and directionality will require enzyme structural information, which at present is not available for the LanM-type synthetases capable of both dehydration and lantionine ring formation. When this information becomes available, mechanistic models will have to account for the distributive, directional catalysis revealed here.

Experimental Section

Materials. HPLC-grade acetonitrile was purchased from Sigma-Aldrich. His₆-LctA, His₆-LctM, His₆-HalA2, and His₆-HalM2 were obtained as previously described.^{2,3}

Preparation of Overexpression Plasmid for Cys-Free LctA. The *lctA* gene was amplified using the primers LctAFPNdeI: 5'-GGGAATTCCATATGAAAGAACAACCTTTTAA-3' and LctAC49AC50ABamHIRP: 5'-CGCGGATCCCTAAGATGCAG-CAGTA-3' with the plasmid pET15b-LctA as the template. The PCR product was digested with *NdeI* and *BamHI* restriction enzymes (Invitrogen and NEB) and ligated into the pET15b vector

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digested with the same enzymes generating pET15b-LctA-C49A/C50A. Using this plasmid as template, the partial *lctA* gene was amplified using the primers LctAC38A/C49A/C50A FP: 5'-ACAATTTCTCATGAAGCTAATATGAATAGCTGG-3' and T7 terminator: 5'-GCTAGTTATTGCTCAGCGG-3'. The full length triply mutated *lctA* gene was generated in a second round of PCR using the mutant 3' fragment generated by the first round of amplification as the reverse primer and the long T7 promoter (5'-CGCGAAATTAATACGACTCACTATAGGGGAATTGTGAG-3') as the forward primer. The resulting PCR products were digested with *Bam*HI and *Nde*I in buffers supplied by the manufacturer at 37 °C for 12 h. Typical reaction volumes were 40 or 50 μ L and contained approximately 1 μ g of DNA per unit enzyme. Products were purified *via* Qiagen's QIAquick PCR Purification Kit. The resulting product was ligated into pET15b vector from Novagen (previously digested with *Bam*HI and *Nde*I and gel purified) for 2 h at 25 °C utilizing T4 DNA ligase (Invitrogen). Ligation reactions were conducted with a 9:1 ratio of insert:vector in T4 DNA ligase buffer and the reaction volume was 15 μ L. After 2 h, the ligase was denatured at 65 °C for 10 min. Chemically competent *E. coli* DH5 α cells were transformed with 9 μ L of ligation product at 42 °C for 1 min. Cells were rescued with 150 μ L of Luria-Bertani (LB) media and incubated at 37 °C with vigorous shaking (200 rpm). Cells were plated on LB agar plates containing 100 μ g/mL ampicillin and grown for 16 h at 37 °C. DNA sequencing revealed a nucleotide change (A33G) leading to a silent mutation (Q11Q) in addition to the desired mutations. Overexpression and purification of His₆-LctA-C38A/C49A/C50A was performed as described for His₆-LctA.²

Time Dependent Phosphorylation Assay. The Cys-free version of LctA was assayed with LctM-R399M⁶ using a 1:10 enzyme:substrate ratio, unless noted otherwise, in 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, 25 μ g/mL BSA at pH 7.5 at 25 °C. Assays were quenched at various time points with a solution of 5% TFA (added to 10% of the total reaction volume). Each sample was purified using a Phenomenex C4 analytical column on an HP1100 HPLC (solvent A: water, 0.1% TFA; B: acetonitrile, 0.1% TFA). Once purified, samples were frozen and lyophilized. Dried samples were resuspended in 100 μ L of water, 20 μ L of which were diluted with 10 μ L of ESI spray solution (49% CH₃CN, 49% water, 2% acetic acid) for 30 μ L total.

Overexpression of the His₆-Hala2 Substrate Using ¹³C, ¹⁵N-Double-Depletion.²⁰ The *halA2* gene was overexpressed in *E. coli* BL21 (DE3) cells. The cells were grown in 545 mL of modified M9 minimal media consisting of 54.5 mL of 10 \times stock minimal media (15 g KH₂PO₄, 30 g Na₂HPO₄, 2.5 g NaCl in 500 mL H₂O, pH 7.4), 1.45 mL of a 40% solution of (¹⁴NH₄)₂SO₄ (99.99% ¹⁴N), 1 g ¹³C-depleted glucose (99% ¹³C), 18.7 μ L of a FeSO₄ and thiamine stock (2 g FeSO₄ and 200 mg thiamine in 10 mL water), 727 μ L of 1 M MgSO₄, 36.3 μ L of 1 M CaCl₂, and 272.5 μ L of trace element solution (0.55 g CaCl₂, 0.17 g ZnCl₂, 0.044 g CuCl₂·H₂O, 0.06 g CoCl₂·6H₂O, 0.06 g Na₂MoO₄·2H₂O in 1 L of H₂O at pH 7.4). Ampicillin was added to a final concentration of 100 μ g/mL and sterile Millipore water was added to achieve the 545 mL final volume. The cells were grown to an OD₆₀₀ = 0.6 to 0.7, induced with 0.7 mM IPTG, grown for an additional 6 h at 37 °C, harvested by centrifugation (~2.2 g cell pellet) at 4 °C and stored at -80 °C.

Purification of WT and ¹³C, ¹⁵N-Depleted His₆-Hala2. The cell pellet was resuspended in start buffer (20 mM Na₂HPO₄, 500 mM NaCl, 0.5 mM imidazole, pH 7.5). The cells were lysed *via* sonication for 15 min and the lysate cleared by centrifugation at 11,900g for 30 min. The supernatant was discarded and the pellet was resuspended a second time in start buffer. The sonication and centrifugation steps were repeated and the supernatant discarded. The resulting cell pellet was dissolved and sonicated in denaturing

buffer 1 (20 mM NaH₂PO₄, 500 mM NaCl, 0.5 mM imidazole, 6 M guanidine HCl, pH 7.5) to solubilize the peptide substrate. The remaining insoluble material was pelleted by centrifugation. The supernatant was subsequently filtered through a 0.45 μ m syringe-tip filter (Millipore) and loaded onto a 5 mL HiTrap chelating column (GE Healthcare Life Sciences) charged with 0.1 M NiSO₄ that was equilibrated with buffer 1. The column was washed with five column volumes of buffer 1 followed by five column volumes of buffer 2 (20 mM NaH₂PO₄, 300 mM NaCl, 30 mM imidazole, 4 M guanidine HCl, pH 7.5). The substrate was eluted off the column with three column volumes of elution buffer (20 mM Na₂HPO₄, 1 M NaCl, 1 M imidazole, pH 7.5). The fraction containing Hala2 was further purified using a Waters preparatory C4 HPLC column with monitoring at 220 nm. Hala2 eluted between ~16 to 26 min with a gradient of 2 to 100% B over 80 min (A = 0.1% TFA in H₂O B = 0.086% TFA in 80% CH₃CN). The fractions were lyophilized and stored at -80 °C until use for assays with HalM2.

Hala2 and HalM2 Kinetic Assays with Iodoacetamide Alkylation of Free Cysteines. Hala2 and HalM2 assays were conducted at 25 °C in 50 mM MOPS pH 7.6, 100 mM MgCl₂, 5 mM ATP, 3 mM TCEP, 111 μ M substrate, 0.7 μ M enzyme, with a final substrate:enzyme ratio of ~100:1 after removal of a small amount of precipitate. The assay was allowed to progress until a desired time point, at which time an aliquot was taken and quenched with 0.5% TFA. To alkylate any free cysteines, the aliquots (400 μ L) were treated with fresh iodoacetamide (IAA, 5 mM final concentration), and 40 μ L of 1 M NaOH (final pH 8.5) at room temperature in the dark for 2 h. After 2 h, the assays were stored at -80 °C prior to HPLC purification for analysis by mass spectrometry. Each assay sample was injected onto an analytical Phenomenex Jupiter C4 column (5 μ m bead size, 300 Å pore size, 4.6 mm \times 150 mm) using an HP 1100 diode array detector. The processed peptides with IAA modification eluted between 17 and 26 min with a linear gradient from 2–98% B over 60 min. (solvent A: water, 0.1% TFA; b: acetonitrile, 0.086% TFA). The fractions were concentrated on a speedvac and resuspended in ESI solution (49:49:2 of CH₃CN:H₂O:glacial acetic acid).

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS) Analysis. Two systems were used in this study. The first was a custom-built 8.5 T quadrupole-FT-ICR MS hybrid.²¹ Ions from ESI were directed through a heated metal capillary, skimmer, and multiple ion guides into the ICR cell (~10⁻⁹ Torr) of the FT-ICR MS instrument. Each intermediate species was isolated and enhanced using selective ion accumulation to give approximately a 10-fold increase in signal. Tandem mass spectrometry (MS/MS) experiments were performed by selective enhancement of precursor ions and stored waveform inverse fourier transform (SWIFT) isolation^{22,23} down to a 1 *m/z* window prior to fragmentation *via* electron capture dissociation (ECD)¹⁰ to produce *c* and *z*[•] fragment ions or collisionally activated dissociation (CAD)²⁴ to produce *b* and *y* ions. The second instrument was a LTQ FT 12 T Ultra constructed in collaboration with Thermo Fisher Scientific (San Jose, CA) using direct infusion with an Advion TriVersa NanoMate (Ithaca, NY). All data were collected at a resolution setting of 171,000 at 400 *m/z*. Each lantibiotic intermediate was isolated in the LTQ using a set of two or three nested isolation windows (each 3 *m/z* wide) to achieve high quality precursor ion selection (1–1.5 *m/z*) before MS/MS fragmentation. Isolated

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isotopic distributions were subsequently fragmented with ECD, CAD, infrared multiphoton dissociation (IRMPD)²⁵ and octupole collisionally activated dissociation (OCAD).²⁶

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Supporting Information Available: Supplementary Figures 1–5. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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