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Mechanistic Studies of Ser/Thr Dehydration Catalyzed by a Member of the LanL Lanthionine Synthetase Family[†]

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ABSTRACT: Members of the LanL family of lanthionine synthetases consist of three catalytic domains, an N-terminal pSer/pThr lyase domain, a central Ser/Thr kinase domain, and a C-terminal lanthionine cyclase domain. The N-terminal lyase domain has sequence homology with members of the OspF family of effector proteins. In this study, the residues in the lyase domain of VenL that are conserved in the active site of OspF proteins were mutated to evaluate their importance for catalysis. In addition, residues that are fully conserved in the LanL family but not in the OspF family were mutated. Activity assays with these mutant proteins are consistent with a model in which Lys80 in VenL deprotonates the α-proton of pSer/pThr residues to initiate the elimination reaction. Lys51 is proposed to activate this proton by coordination to the carbonyl of the pSer/pThr, and His53 is believed to protonate the phosphate leaving group. These functions are very similar to the corresponding homologous residues in OspF proteins. On the other hand, recognition of the phosphate group of pSer/pThr appears to be achieved differently in VenL than in the OspF proteins. Arg156 and Lys103 are thought to interact with the phosphate group on the basis of a structural homology model.

Lantipeptides are posttranslationally modified peptide natural products that contain thioether cross-links named lanthionine (Lan)[1] and methyllanthionine (MeLan) (*1*, *2*). Lantipeptides with antimicrobial activities are called lantibiotics (*3*). The characteristic thioethers are incorporated by the site-specific dehydration of Ser and Thr residues in the precursor peptide to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, and subsequent stereoselective intramolecular Michael-type addition of cysteine thiols to the dehydro amino acids. The resulting polycyclic peptides provide protease resistance (*4*) and restrict conformational freedom such that molecular targets can be recognized with higher affinities. Not surprisingly given the highly efficient route to such structures, at least four different biosynthetic routes to lantipeptides have been uncovered to date (Figure 1A) (*1*, *3*, *5*, *6*). On the basis of the type of lanthionine synthetase that forms the lanthionine rings, lantibiotics/lantipeptides have been categorized into four classes. In class I, dedicated dehydratase (LanB) and cyclase (LanC) enzymes are responsible for the dehydration and cyclization (the term Lan is used as a generic descriptor of proteins involved in lantipeptide biosynthesis). In contrast, multifunctional enzymes (LanM, RamC/LabKC, or LanL) catalyze both dehydration and cyclization reactions in the other three classes of lantibiotics/lantipeptides (*1*, *7*, *8*). LanM and LanL enzymes have LanC-like domains in their C-terminal regions (Figure 1B), illustrating that class I, II, and IV lantipeptides are generated using similar cyclization strategies, in which a zinc ion is used to activate the cysteines in the substrate for nucleophilic attack (*9*−*11*). In contrast, the RamC and LabKC proteins of class III lantipeptides lack the zinc ligands but also generate (methyl)lanthionines with Dha and Dhb structures as intermediates (*8*). Whereas the cyclization reaction catalyzed by LanC and LanC-like domains is reasonably well understood (*11*, *12*), the molecular details of dehydration are not known for any of the four classes of lantipeptides. LanM and LanB do not exhibit homology with other known proteins, hampering prediction of their mode of action. In vitro studies on a LanM protein showed that the Ser and Thr residues in the substrate are first phosphorylated to phosphoSer (pSer) and phosphoThr (pThr), respectively, followed by *β*-elimination to form the dehydro amino acids (*13*, *14*), but the detailed molecular mechanism by which these two enzymatic reactions are catalyzed has not been reported.

The recent discovery of the LanL family of enzymes shed the first light on the catalytic mechanism of formation of dehydro amino acids during lantibiotic/lantipeptide biosynthesis. LanL enzymes consist of an N-terminal putative pSer/Thr lyase domain, a central Ser/Thr kinase-like domain, and a C-terminal LanC-like cyclase domain (Figure 1B). This characteristic domain composition and in vitro enzyme assays of deletion mutants of VenL, a LanL enzyme involved in biosynthesis of venezuelin in *Steptomyces venezuelae*, suggested a unique dehydration strategy in which two independent domains install Dha/Dhb via phosphorylation of Ser/Thr by a kinase domain and subsequent

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[1]Abbreviations: Lan, lanthionine; MeLan, methyllanthionine; Dha, dehydroalanine; Dhb, (*Z*)-dehydrobutyrine; pSer, phosphoserine; pThr, phosphothreonine; MAPKs, mitogen-activated protein kinases; MALDI-ToF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; TCEP, tris(2-carboxyethyl)phosphine; DMSO, dimethyl sulfoxide; IPTG, isopropyl *β*-D-1-thiogalactopyranoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TFA, trifluoroacetic acid; His_6, hexahistidine tag.

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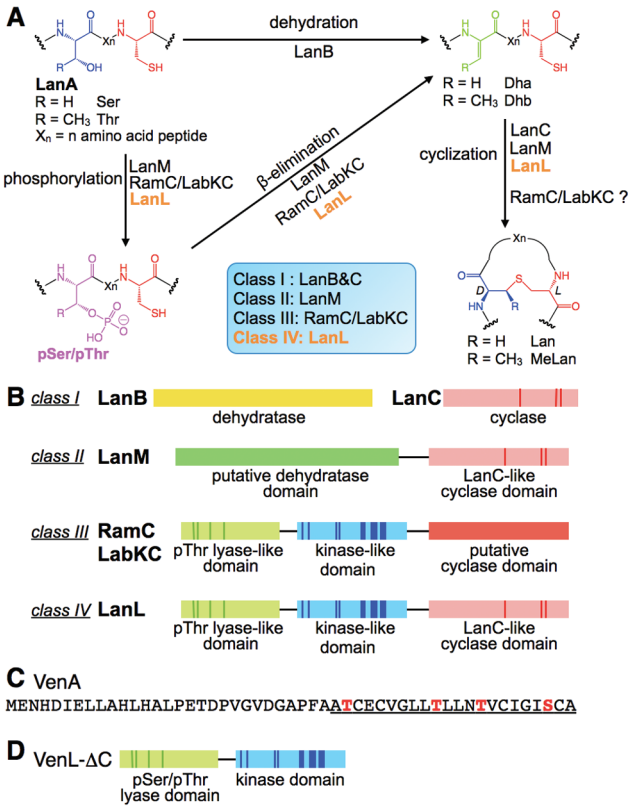

FIGURE 1: Biosynthesis of lantibiotics/lantipeptides by four classes of synthetases. (A) Posttranslational modification of precursor peptides by lantibiotic/lantipeptide synthetases. Following ribosomal synthesis of the precursor peptides (LanA), LanB, LanM, RamC/LabKC, or LanL enzymes catalyze dehydration of Ser/Thr to afford Dha/Dhb. For class II, III, and IV lantipeptides, this modification proceeds through a two-step process, phosphorylation of Ser/Thr and subsequent β-elimination of the phosphate to generate Dha/Dhb. LanC, LanM, or LanL enzymes then catalyze intramolecular addition of Cys thiols onto the dehydro amino acids in a stereo- and regioselective manner to form Lan and MeLan. How the RamC/LabKC proteins catalyze cyclization is currently not known. (B) Illustration of the domain composition of class I−IV lanthionine synthetases. The positions of active site residues (in LanC) or conserved residues in LanM, RamC/LabKC, and LanL corresponding to the active site residues in structurally characterized homologous enzymes are shown in darker colors. (C) Primary sequence of VenA. The dehydration sites are highlighted in red. The putative core peptide is underlined. (D) Domain composition of VenL-ΔC, a truncated analogue of VenL lacking its LanC-like cyclase domain.

elimination of the phosphate from pSer/pThr by a putative pSer/pThr lyase domain (*1*). The lyase domains of the LanL family are also present in the RamC/LabKC proteins (RamC family) of class III lantipeptides. These domains exhibit homology to members of the OspF protein family, effector proteins present in several pathogenic bacteria that inactivate mitogen-activated protein kinases (MAPKs) in the host cells (*15*, *16*). Because OspF enzymes have pThr lyase activity, catalyzing the irreversible β-elimination of a phosphate group from a pThr in the MAPKs to afford a Dhb residue, we hypothesized that LanL and RamC families adopt similar dehydration strategies (*1*).

A sequence alignment of the N-terminal putative pSer/pThr lyase domains of LanL and RamC enzymes with OspF family members is shown in Figure 2. This analysis reveals nine fully conserved residues that include four of the active site residues in the OspF family on the basis of the crystal structure of one member, SpvC (*16*, *17*) (Figure 2, red stars). Other conserved residues in the OspF family are not observed in the lyase domains of class III/IV lantipeptide synthetases and vice versa. To date, direct experimental evidence that supports the hypothesis that LanL and RamC enzymes execute the lyase reaction by a mechanism similar to that of the OspF family is not available. Here, we describe mutagenesis studies aimed at determining the essential residues for lyase activity in VenL.

MATERIALS AND METHODS

General Materials. All oligonucleotides were purchased from Operon Technologies, Integrated DNA Technologies, or Sigma-Aldrich. Restriction endonucleases, DNA polymerases, and T4 DNA ligase were purchased from Invitrogen or New England Biolabs. Media for bacterial culture and chemicals were purchased from Difco laboratories, CalBiochem, Aldrich, or Fisher Scientific unless noted otherwise and used without further purification. *Escherichia coli* DH5α was used as host for cloning and plasmid propagation, and *E. coli* BL21(DE3) was used as a host for protein expression. Cloning vectors (pET15 and pET28) were obtained from Novagen. pET28b vectors containing the *venL*-Δ*C* or *lctM* genes have been described in the literature (*1*, *18*). VenA, LctA, VenL, and LctM were expressed in *E. coli* and purified as previously reported (*1*, *18*).

General Methods. All polymerase chain reaction (PCR) amplifications were conducted with an automated thermocycler (PTC-100 or PTC-150, MJ Research). DNA sequencing was conducted using the appropriate primers by the Biotechnology Center of the University of Illinois. MALDI-ToF MS was conducted on a Voyager-DE-STR instrument (Applied Biosystems).

Construction of venL-ΔC Mutant Genes. Site-directed mutagenesis of *venL*-Δ*C* was performed by multistep PCR. First, the amplification of *venL*-Δ*C* was conducted via 30 cycles of denaturing (94 °C for 20 s), annealing (58 °C for 30 s), and extending (72 °C for 120 s) using the VenL-*Nde*I-FP primer and an appropriate mutant reverse primer (see Table S1 of the Supporting Information for oligonucleotide sequences) to yield a 5′ fragment of the mutated *venL*-Δ*C* gene (FP reaction). The PCR mixtures included 1× FailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), Platinum *Pfx* DNA polymerase (0.025 unit/μL), *Taq* DNA polymerase (0.05 unit/μL), and primers (1 μM each). In parallel, a PCR using an appropriate mutant forward primer and the VenL-ΔC-*Hin*dIII-RP primer was also conducted to produce 3′ fragments of the mutated *venL*-Δ*C* gene using the same PCR conditions that were used for the FP reaction (RP reaction). The overlapping products from the FP reaction and RP reaction were combined in equal amounts and extended by five cycles of denaturing, annealing, and extending in a solution containing 1× FailSafe PreMix G (PICENTRE Biotechnologies), DMSO (4%), and Platinum *Pfx* DNA polymerase (0.025 unit/μL). Following the extension, the VenL-*Nde*I-FP and VenL-ΔC-*Hin*dIII-RP primers were added (final concentration of 2 μM), and the reaction mixture was incubated for 25 additional cycles of denaturing, annealing, and extending. Amplification of the final PCR product was confirmed by 2% agarose gel electrophoresis, and the products were purified using QIAquick PCR purification kits (QIAGEN). The resulting DNA fragment and the pET28 vector were digested in 1× NEBuffer 2 (New England Biolabs) with *Nde*I and *Hin*dIII at 37 °C for 15 h. The digested products were purified by agarose gel electrophoresis followed by use of a QIAquick gel extraction

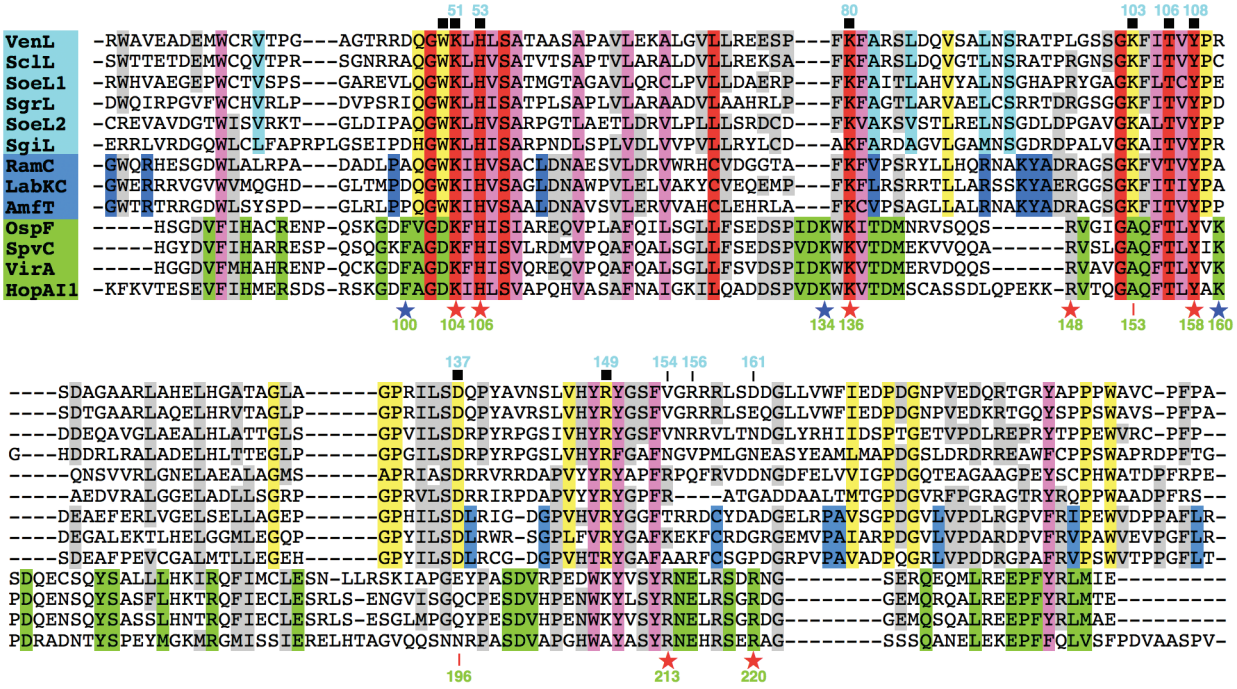

FIGURE 2: Sequence alignment analysis of the lyase-like domains of LanL and RamC proteins and OspF enzymes. Identical, similar, and partially conserved residues among the LanL, RamC, and OspF family members are highlighted in red, pink, and gray, respectively. The residues that are conserved in the LanL family but not in the other families are highlighted in cyan. The residues that are conserved in the RamC family but not in the other families are highlighted in blue. The residues that are conserved in the OspF family but not in other families are highlighted in green. Red stars denote the residues present in the active site in the crystal structure of an OspF family member, SpvC. The residues marked with black squares were mutated in this study. Residue numbering for VenL and SpvC is shown in cyan (top) and green (bottom), respectively. Blue stars indicate the residues responsible for the recognition of pTyr present in the substrate MAPK in SpvC. VenL (GenBank accession number HQ328852), SclL (YP_002193147), SoeL1 (YP_001106221), SgrL (AAP03109), SoeL2 (YP_001106807), SgiL (YP_001821664), RamC (NP_630756), LabKC (CAX48971), AmfT (YP_001823909), OspF (YP_406014), SpvC (YP_002635578), VirA (NP_790745), and HopAI1 (NP_790745).

kit (QIAGEN). The resulting DNA insert was ligated with the digested pET28 vector at 24 °C for 3 h using T4 DNA ligase. The ligation reaction mixture was diluted 10 times with water prior to transformation. *E. coli* DH5α cells were transformed with the ligation product via heat shock, plated on LB-kanamycin agar plates, and grown at 37 °C for 15 h. Three colonies were picked and incubated in 5 mL of LB-kanamycin medium at 37 °C for 15 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing.

Construction of lctM Mutant Genes. The amplification of *lctM*/pET28 was conducted via 30 cycles of denaturing (98 °C for 10 s), annealing (55 °C for 30 s), and extending (72 °C for 135 s) using appropriate mutant primers (see Table S1 of the Supporting Information for oligonucleotide sequences). The PCRs (50 μL) included 1× HF buffer (Finnzymes), DMSO (4%), Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) (0.04 unit/μL), dNTPs (2 mM), *lctM*/pET28 (20 ng), and primers (1 μM each). The PCR product was checked on a 1% agarose gel and purified using a QIAquick PCR purification kit (QIAGEN). The resulting DNA was treated with *Dpn*I at 37 °C for 15 h to digest the methylated template, and *E. coli* DH5α cells were transformed. The desired mutations were confirmed by DNA sequencing.

Overexpression and Purification of VenL-ΔC Mutants and LctM Mutants. BL21(DE3) cells transformed with a pET28 vector carrying each mutant gene were grown in 2 L of LB medium containing 50 mg/L kanamycin at 37 °C until the OD_{600} reached ~0.6. The incubation temperature was then changed to 18 °C, and the culture was induced with 0.2 mM IPTG. The induced cells were shaken continually at 18 °C for an additional 18 h. The cells were harvested by centrifugation (11900*g* for 10 min, Beckman JLA-10.500 rotor). The cell pellet was resuspended in 30 mL of start buffer [50 mM HEPES·Na (pH 7.4), 300 mM NaCl, and 10% glycerol, containing a protease inhibitor cocktail from Roche Applied Science] and stored at −80 °C.

All purification steps were performed at 4 °C. Cell paste in start buffer was sonicated on ice for 20 min. After centrifugation (23700*g* for 30 min, Beckman JA-20 rotor), the supernatant was filtered through a 0.45 μm syringe filter. The sample was then loaded onto a 5 mL HisTrap HP column (GE Healthcare Life Sciences). The column was washed with 20 mL each of start buffer containing 25 and 50 mM imidazole and then 10 mL each of start buffer containing 100, 200, and 500 mM imidazole. The eluent was collected in several fractions, which were analyzed by Tris-SDS−PAGE. The fractions containing the desired protein (200−500 mM imidazole) were combined and concentrated using an Amicon Ultra-15 centrifugal filter unit (30 kDa molecular mass cutoff for His_6-VenL, 10 kDa molecular mass cutoff for other proteins, Millipore) to less than 2 mL. Buffer exchange of the concentrated protein with start buffer was conducted twice using a PD-10 desalting column (GE Healthcare Life Sciences). The resulting protein sample was aliquoted and stored at −80 °C. Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology).

In Vitro Lyase Activity Assay of VenL-ΔC Mutants. The VenA substrate peptide was incubated with a VenL-ΔC mutant (final concentration of 2 μM) in a reaction buffer that contained (final concentrations) 50 mM HEPES·Na buffer (pH 7.5),

10 mM $MgCl_2$, 2.5 mM ATP, 1 mM TCEP, 25 μM VenA, and 5% DMSO. The reaction mixture was incubated at 25 °C for 2 h (standard) or 20 h (see Figures S1 and S2 of the Supporting Information). For mass spectrometric analysis, 10 μL of the reaction mixture was desalted using $ZipTip_{C18}$ (Millipore), eluted with 1 μL of a 75% MeCN/25% water solution containing 0.1% TFA and saturated with sinapinic acid, and spotted onto the target plate for analysis using a Voyager-DE-STR mass spectrometer (Applied Biosystems).

In Vitro Lyase Activity Assay of LctM Mutants. The LctA substrate peptide was incubated with a LctM mutant (final concentration of 2 μM) in a reaction buffer that contained (final concentrations) 50 mM Tris·HCl buffer (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 1 mM TCEP, 25 μg/mL BSA, 25 μM LctA, and 5% DMSO. The reaction mixture was incubated at 25 °C for 2 h. For mass spectrometric analysis, 10 μL of the reaction mixture was desalted using $ZipTip_{C18}$ (Millipore), eluted with 1 μL of a 75% MeCN/25% water solution containing 0.1% TFA and saturated with sinapinic acid, and spotted onto the target plate for analysis using a Voyager-DE-STR mass spectrometer (Applied Biosystems).

RESULTS

pSer/pThr Lyase Activity of VenL-ΔC Proteins Mutated at Residues Strictly Conserved among Members of the LanL and OspF Families. VenL is a member of the LanL family, and its activity has been reconstituted in vitro (*1*). The enzyme catalyzes four dehydrations followed by four cyclizations of its substrate peptide VenA (Figure 1C). The deletion mutant VenL-ΔC lacks its C-terminal cyclase domain; it retains the native dehydratase activity but has no cyclase activity (Figure 1D). We used this deletion mutant to further investigate the dehydration reaction. The residues that are conserved among the OspF, LanL, and RamC families and that are located in the active site in OspF family member SpvC (K104, H106, K136, and Y158) were first targeted for mutation. Thus, the genes encoding His_6-VenL-ΔC variants with K51M, H53N, H53F, K80M, and Y108F mutations were generated via overlap extension PCR. The proteins were overexpressed in *E. coli* and purified by immobilized metal affinity chromatography.

To evaluate the lyase activity of each VenL-ΔC mutant, we incubated the VenA peptide with each mutant and then subjected each to MALDI-ToF MS. Incubation of VenA with wild-type VenL-ΔC at 25 °C for 2 h in the presence of ATP, $MgCl_2$, and TCEP resulted in 4-fold dehydrated VenA (Figure 3A,B). In contrast, VenL-ΔLC, a VenL variant lacking both lyase and cyclase domains, catalyzed conversion of VenA into a mixture of peptides carrying multiple (up to four) phosphate groups (Figure 3C). Under these reaction conditions, His_6-VenL-ΔC-K80M yielded only the 4-fold phosphorylated intermediate, indicating that this mutant lost pSer/pThr lyase activity (Figure 3D). In the mass spectrum of VenA after treatment with VenL-ΔC-K51M, peaks corresponding to the fully phosphorylated substrate as well as 3-fold phosphorylated peptide that also had undergone elimination of a phosphate group were detected (Figure 3E). After incubation for 20 h, the 2-fold dehydrated peptide was detected but no further dehydrated VenA peptides were observed (Figure S1B of the Supporting Information). This result shows that VenL-ΔC-K51M does not catalyze efficient β-elimination. Similarly, replacement of His53 with an Asn resulted in a mutant VenL-ΔC that was able to partially but not completely

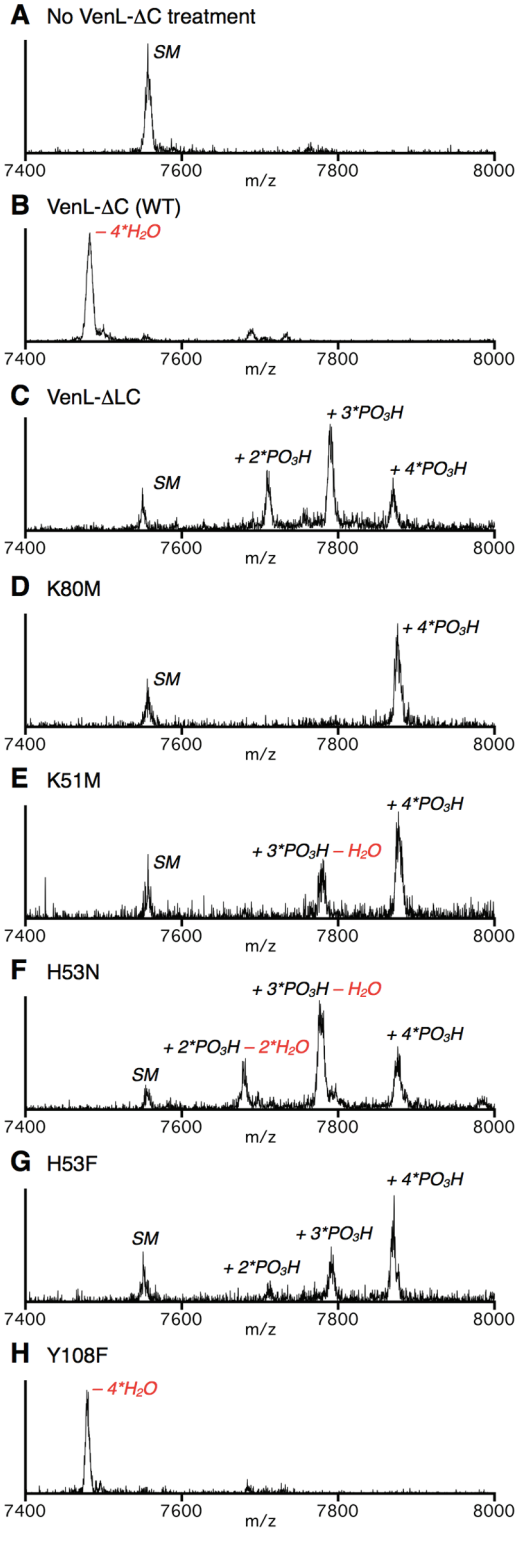

FIGURE 3: MALDI-ToF MS analysis of VenA processed by VenL-ΔC derivatives having a mutation at the predicted catalytic site residues. Mass spectra of (A) VenA and of VenA after incubation with (B) wild-type VenL-ΔC, (C) VenL-ΔLC, (D) VenL-ΔC-K80M, (E) VenL-ΔC-K51M, (F) VenL-ΔC-H53N, (G) VenL-ΔC-H53F, and (H) VenL-ΔC-Y108F. The assignments for the observed peaks are shown in the spectra, in which SM indicates the starting material (VenA).

dehydrate the substrate, and VenL-ΔC-H53F was incapable of eliminating phosphate from pSer/pThr on VenA (Figure 3F,G). In contrast, mutation of Tyr108, which corresponds to Tyr158 in SpvC that forms a hydrogen bond with the carbonyl oxygen of

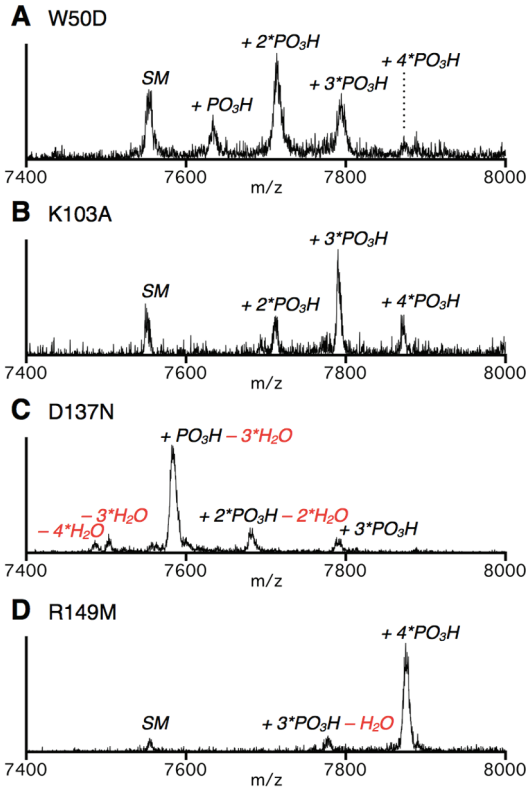

FIGURE 4: MALDI-ToF MS analysis of VenA processed by VenL-ΔC derivatives having a mutation of the residues conserved only in the LanL and RamC families but not in the OspF family. Mass spectra of VenA after incubation with (A) VenL-ΔC-W50D, (B) VenL-ΔC-K103A, (C) VenL-ΔC-D137N, and (D) VenL-ΔC-R149M. The assignments for the observed peaks are shown in the spectra, in which SM indicates the starting material (VenA).

pThr in the substrate and assists deprotonation of the α-proton (*16*), was not critical in the lyase reaction catalyzed by VenL as the completely dehydrated peptide was detected in the mass spectrum of VenA incubated with VenL-ΔC-Y108F (Figure 3H). Mutation of Thr106 to Ala also resulted in a protein that was able to complete the 4-fold dehydration of VenA in 2 h (Figure S2A of the Supporting Information). A double mutant, VenL-ΔC-T106A/Y108F, also dehydrated the substrate (Figure S2B,C of the Supporting Information).

pSer/Thr Lyase Activity of VenL-ΔC Proteins Mutated at Residues Conserved Only in the LanL Family. In addition to investigation of the residues that are conserved in both the LanL and OspF families, the importance of residues that are conserved only in the LanL and RamC families was assessed. Mutagenesis of Trp50 (W50D and W50A) resulted in enzymes that still phosphorylated VenA but did not display detectable dehydration activity (Figure 4A and Figures S1E and S2D,E of the Supporting Information). Furthermore, the His_6-VenL-ΔC-K103A mutant predominantly phosphorylated the substrate peptide, with some dehydrated peptides observed after incubation for 20 h (Figure 4B and Figure S1F of the Supporting Information), demonstrating that Lys103 is important but not essential for the lyase activity of VenL. In the case of VenL-ΔC-D137N, although 4-fold dehydrated VenA was not the major product, varying amounts of peptides with three or two dehydrations were observed after incubation for 2 and 20 h (Figure 4C and Figure S1G of the Supporting Information), suggesting decreased but not completely abolished lyase activity of the mutant. However, His_6-VenL-ΔC-R149M yielded 4-fold phosphorylated VenA as the major product along with small amounts of mono- and didehydrated peptides, demonstrating the weak lyase activity of this mutant (Figure 4D and Figure S1H of the Supporting Information). Conversely, mutation of Arg156 to Met did not have a deleterious effect as the mutant enzyme catalyzed the 4-fold dehydration of VenA (Figure S2F of the Supporting Information).

Sequence Homology among All Four Lantibiotic/Lantipeptide Synthetase Families and Mutagenesis of the Conserved Residues. Although the lyase domain of members of the LanL and RamC families does not display obvious sequence similarity with that of members of the two other lantipeptide dehydratase families (LanB and LanM), close examination of multiple-sequence alignments exhibits two weakly homologous regions (Figure 5A,B). The first is a "G-(K/R)-(F/A)" motif that includes Lys103 of VenL that was shown in the studies discussed above to be important for elimination. The second motif was found in only LanL, RamC, and LanM; it is located around the invariant Arg149 of VenL and contains a conserved basic residue flanked by multiple aromatic or hydrophobic residues. Because both homologous areas include a conserved residue that plays an important role in the lyase activity of VenL, we hypothesized that the detected conserved residues might also be important for the dehydration reaction by LanM and LanB. To address this hypothesis, the putative conserved residues were mutated in LctM, a representative LanM enzyme whose in vitro activity has been studied (*7*, *12*−*14*, *19*−*21*). Unfortunately, we could not test the activity of mutants of LanB family members, as in vitro reconstitution of dehydratase activity of the LanB family has not been achieved thus far (*22*−*24*).

To evaluate the reliability of the first homologous region, we constructed His_6-LctM-K144A. When LctA, the canonical substrate peptide of LctM, was incubated with His_6-LctM-K144A in the presence of ATP and Mg^{2+}, completely (4-fold) dehydrated peptide was generated, suggesting that Lys144 of LctM is not essential for lyase activity (Figure 5C). Three additional LctM mutants, LctM-Y225A, LctM-K227M, and LctM-R226M/K227M, were prepared to evaluate the importance of the second homologous region. All mutants were able to dehydrate LctA four times (Figure 5D−F). Taken together, the mutagenesis analysis of LctM suggests that the observed sequence homologies among the lantibiotic/lantipeptide synthetase families shown in Figure 5 do not have functional significance.

DISCUSSION

The mutagenesis studies of VenL-ΔC performed in this work provide further experimental support for the idea that LanL enzymes utilize their pSer/pThr lyase domain to complete dehydration of Ser/Thr residues in their substrates. Some of the amino acids that have been reported to be important for this activity in members of the OspF family are shown here to be important for VenL also. In the proposed catalytic mechanism of SpvC based on its X-ray structure (*16*, *17*) (Figure 6A), the carbonyl oxygen of pThr of the substrate makes two hydrogen bonding contacts with the ε-amine of Lys104 and the hydroxyl group of Tyr158, resulting in a decrease in the pK_a of the α-proton of the pThr. Lys136 is believed to be the catalytic base that abstracts this α-proton to form an enolate and trigger the β-elimination reaction, with His106 protonating the bridging oxygen of the phosphate ester (*16*). In addition, several other residues could be involved in the recognition of the phosphate group of pThr as several

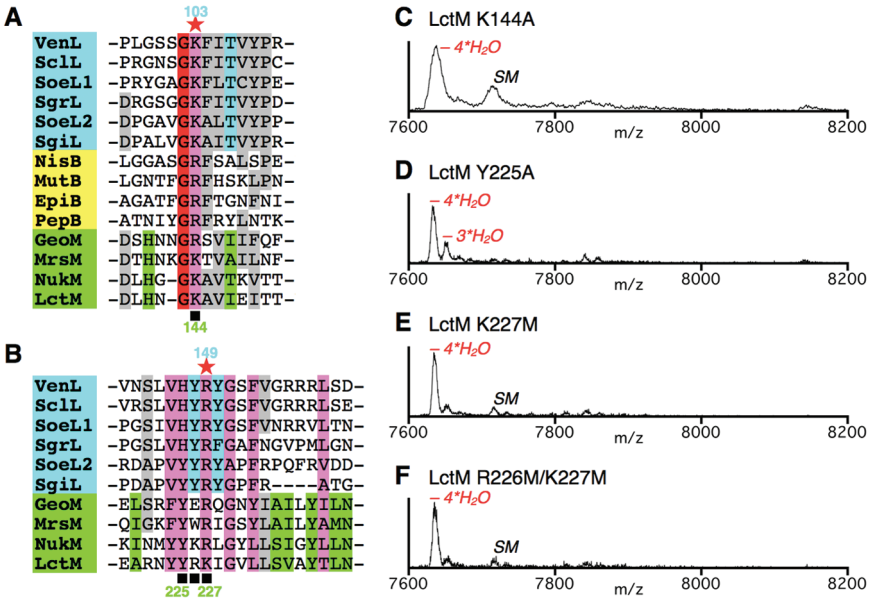

FIGURE 5: Site-directed mutagenesis of LctM. (A) Partial sequence alignment of LanL, LanB, and LanM proteins. The residue numbering at the top is from VenL, whereas the residue numbering at the bottom is from LctM. (B) Partial sequence alignment of LanL and LanM proteins. Identical, similar, and partially conserved residues are highlighted in red, pink, and gray, respectively. The conserved residues within only one protein family are highlighted in cyan (LanL) and green (LanM). Red stars indicate the residues that are important for the lyase activity of VenL. The residues in LctM marked with black squares were mutated in this study. Residue numbering for VenL and LctM is shown in cyan (top) and green (bottom), respectively. NisB (GenBank accession number CAA48381), MutB (AAG48566), EpiB (CAA44253), PepB (CAA90025), GeoM (YP_001126159), MrsM (CAB60261), NukM (NP_940773), and LctM (AAC72258). (C−F) MALDI-ToF MS analysis of LctA processed by (C) LctM-K144A, (D) LctM-Y225A, (E) LctM-K227M, and (F) LctM-R226M/K227M. The assignments for observed peaks are shown in the spectra, in which SM indicates the starting material (LctA).

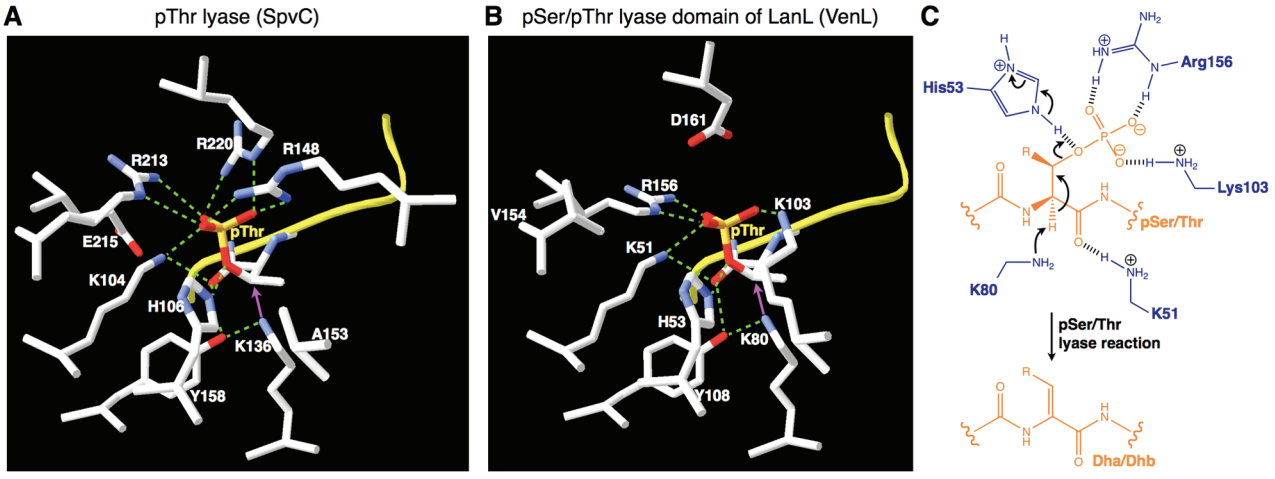

FIGURE 6: Proposed mechanism of the lyase reaction catalyzed by VenL. (A) Modeled structure of the active site of SpvC. The structure is based on the crystal structure of the SpvC-K136A mutant−substrate complex (Protein Data Bank entry 2Q8Y) and was generated by replacement of Ala136 with a lysine. (B) Modeled structure of the lyase domain of VenL. The model was generated by the SWISS-MODEL homology modeling server on the basis of the crystal structure of a SpvC mutant complexed with its substrate peptide (Protein Data Bank entry 2Q8Y) and the alignment shown in Figure 2. Substrate peptides containing a pThr residue are shown as yellow ribbons with the pThr shown as sticks. Predicted hydrogen bonds are represented by green dashed lines. Interactions between the proposed catalytic base residue and the α-proton of pThr in the substrate are denoted with magenta arrows. (C) Proposed mechanism for β-elimination of the phosphate of pSer/Thr catalyzed by the lyase domain of VenL. The substrate peptide and active site residues are colored orange and navy, respectively.

possible hydrogen bonds with Arg148, Arg213, and Arg220 were suggested by the crystal structure (*16*, *17*).

The proposed catalytic base residue in the OspF family (Lys136) is well-conserved in LanL enzymes, and mutation of the corresponding Lys80 in VenL-ΔC shows it is essential for catalysis of the β-elimination reaction. The observation that the VenL-ΔC-H53N and VenL-ΔC-H53F mutants also lost the lyase activity is consistent with the hypothesis that this invariant His (corresponding to His106 in SpvC) acts as a catalytic acid protonating the bridging oxygen of the phosphate leaving group. Lys51 of VenL, corresponding to Lys104 in SpvC, also plays an important role in VenL on the basis of the mutagenesis results, suggesting that this residue activates the carbonyl oxygen of pSer/pThr and assists deprotonation of its α-proton.

On the other hand, mutagenesis of Tyr108, which is completely conserved among members of the LanL, RamC, and OspF families, did not affect the lyase activity of VenL-ΔC. This observation is in sharp contrast to replacement of Tyr158 in

SpvC with a Phe, which resulted in a drastic decrease in catalytic activity (*16*). Thr106, located near Tyr108 and also conserved among members of the three families, was also not critical for the β-elimination reaction by the lyase domain of VenL. Thus, it is not clear which residue in LanL may play a role similar to that of Tyr158 of SpvC. Perhaps activation of the carbonyl group of pSer/pThr by Lys104 in the lyase domain of LanL is sufficient.

The sequence alignment and mutagenesis results also demonstrate some differences between the LanL/RamC and OspF families. For instance, a (V/I)-D-K sequence conserved in OspF (Val132−Lys134 in SpvC) near the catalytic base is not present in the LanL and RamC proteins. Lys134 in SpvC recognizes a phosphate of a phosphotyrosine (pTyr) present in its substrate MAPK in a pTyr-X-pThr motif. Presumably because such a pTyr residue is not present in the LanA substrate peptides, Lys134 is not conserved in the LanL and RamC families.

Mutation of residues that are conserved in members of the LanL and RamC families but not in members of the OspF family showed that several are important for lyase activity. VenL-ΔC-W50D did not catalyze β-elimination of pSer/pThr in the VenA peptide; members of the OspF family have a conserved Asp residue at the corresponding position that has not been investigated by mutagenesis. Likewise, when Lys103 was replaced with Ala (the residue that is conserved at this position in OspF), the resulting VenL-ΔC mutant was devoid of measurable lyase activity. The function of these two residues is not immediately clear from the SpvC structure. Asp103 and Ala153 in SpvC, the residues corresponding to Trp50 and Lys103 in VenL, respectively, are located near the active site, but they do not make contacts with the substrate peptide in the crystal structure (Figure 6A). VenL-ΔC-D137N also exhibited a significant decrease in lyase activity; Gln and Asn are found at the corresponding positions in SpvC and HopAI1, respectively. Determination of the exact role of these residues (Trp50, Lys103, and Asp137) in the VenL-catalyzed lyase reaction would be aided by structural information.

To date, we have been unable to obtain a crystal structure of VenL or its individual domains. To still provide insights into the potential role of these residues, we built a structural model of the VenL lyase domain with the SWISS-MODEL server using the crystal structure of SpvC (Protein Data Bank entry 2Q8Y) and the sequence alignment between SpvC and VenL shown in Figure 2 (Figure 6B). The active site model provides further support for the idea that Lys51, His53, and Lys80 in VenL likely play roles similar to those of Lys104, His106, and Lys136 in SpvC. On the other hand, it appears that recognition of the phosphate leaving group is achieved quite differently. In the crystal structure of SpvC, in addition to His53, three arginines (Arg148, -213, and -220) interact with the phosphate, but none of these arginines is conserved in VenL. Instead, a different arginine and a lysine (Arg156 and Lys103 corresponding to Glu215 and Ala153 in SpvC, respectively), which are not conserved in members of the OspF family, are located near the phosphate on the substrate peptide. The observation that mutation at Lys103 resulted in a loss of the catalytic activity is consistent with an important role of this residue. In contrast, Arg156 does not appear to be important because mutation in VenL-ΔC to Met did not inactivate its lyase activity. The homology model did not provide insights into why the Trp50, Asp137, and Arg149 mutants are impaired with respect to lyase activity. On the basis of the collective results obtained in this study, we propose the mechanism shown in Figure 6C for the anti elimination reaction catalyzed by LanL.

Although weak sequence homologies among members of the LanL, LanB, and LanC families in the regions containing the residues important for the lyase activity in VenL [Lys103 and Arg149 (Figure 5A,B)] suggested the possibility that all three enzyme families share a common strategy for catalyzing dehydration of Ser/Thr, mutagenesis analysis of the corresponding residues in LctM showed that they do not play essential roles in dehydration. Thus, the question of whether the elimination reaction in LanM proteins takes place in the same active site as phosphorylation or whether this class of enzymes also has a separate lyase domain remains unresolved.

In summary, the mutagenesis study of VenL-ΔC in this paper provides the first mechanistic insights into the dehydration reaction catalyzed by members of the LanL enzyme family. The data described here indicate that LanL enzymes and the OspF family share a similar catalytic strategy for β-elimination of phosphate groups of pSer/pThr to afford dehydro amino acids, but that their substrate recognition mechanism differs. These findings support our previous hypothesis that LanL and OspF proteins have evolved from a common primitive pSer/pThr lyase. The OspF proteins evolved high substrate specificity in part by the recognition of the pTyr present in their substrates. On the other hand, the LanL family may have evolved via gene fusion events among stand-alone Ser/Thr kinases, pSer/pThr lyases, and LanC-like cyclases, which retained their primordial substrate promiscuity that allows these enzymes to process multiple Ser/Thr residues in a substrate peptide.

SUPPORTING INFORMATION AVAILABLE

Mass spectrometric data of assays with mutant enzymes and sequences of oligonucleotide primers used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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