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Mechanistic Investigations of the Dehydration Reaction of Lacticin 481 Synthetase Using Site-Directed Mutagenesis[†]

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ABSTRACT: Lantibiotic synthetases catalyze the dehydration of Ser and Thr residues in their peptide substrates to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, followed by the conjugate addition of Cys residues to the Dha and Dhb residues to generate the thioether cross-links lanthionine and methyllanthionine, respectively. In this study ten conserved residues were mutated in the dehydratase domain of the best characterized family member, lacticin 481 synthetase (LctM). Mutation of His244 and Tyr408 did not affect dehydration activity with the LctA substrate whereas mutation of Asn247, Glu261, and Glu446 considerably slowed down dehydration and resulted in incomplete conversion. Mutation of Lys159 slowed down both steps of the net dehydration: phosphorylation of Ser/Thr residues and the subsequent phosphate elimination step to form the dehydro amino acids. Mutation of Arg399 to Met or Leu resulted in mutants that had phosphorylation activity but displayed greatly decreased phosphate elimination activity. The Arg399Lys mutant retained both activities, however. Similarly, the Thr405Ala mutant phosphorylated the LctA substrate but had compromised elimination activity. Finally, mutation of Asp242 or Asp259 to Asn led to mutant enzymes that lacked detectable dehydration activity. Whereas the Asp242Asn mutant retained phosphate elimination activity, the Asp259Asn mutant was not able to eliminate phosphate from a phosphorylated substrate peptide. A model is presented that accounts for the observed phenotypes of these mutant enzymes.

Lantibiotics are ribosomally synthesized and posttranslationally modified peptide antibiotics (*1*, *2*). The modification reactions common to all lantibiotics are dehydration of Ser and Thr residues to dehydroalanine (Dha)[1] and dehydrobutyrine (Dhb), respectively, and a subsequent cyclization reaction during which Cys residues carry out a conjugate addition to Dha and Dhb to generate the thioether cross-links lanthionine (Lan) and methyllanthionine (MeLan), respectively (Figure 1A). These latter structures have given lantibiotics their family name (*3*). The recent in vitro reconstitution of the biosynthesis of the lantibiotics lacticin 481 (*4*), haloduracin (*5*), and nisin (*6*) has provided the opportunity to investigate the mechanistic details of these reactions. Two separate enzymes carry out the dehydration (NisB) and cyclization (NisC) reactions during nisin biosynthesis, the prototype for class I lantibiotics (*7*). In the case of the class II lantibiotic lacticin 481, the dehydration and cyclization reactions are catalyzed by one bifunctional enzyme LctM that dehydrates four Ser and Thr residues and generates three (Me)Lan cross-links (Figure 1B) (*4*, *8*−*10*). The enzyme demonstrates remarkably relaxed substrate specificity, thereby showing great promise for lantibiotic engineering (*11*).

Mechanistic investigations of the dehydration reaction have demonstrated that ATP and Mg^{2+} are used by LctM to phosphorylate the Ser and Thr residues that are targeted for dehydration (*14*). Subsequent elimination then results in the net dehydration and the formation of the Dha and Dhb structures. The enzyme performs these reactions in a processive manner such that all four dehydrations are completed before release of the substrate (*15*). At present, no information is available regarding which amino acid residues of LctM are involved in catalysis. LctM has sequence homology with other enzymes involved in lantibiotic biosynthesis (LanM and LanC proteins) but not with other proteins in the databases. The LanM proteins are large polypeptides of about 120 kDa. At their C-termini, they show homology with the LanC cyclases involved in class I lantibiotic biosynthesis including three residues that coordinate to a zinc (*6*, *16*), but they have no homology with the corresponding LanB dehydratases (*17*). It is assumed that the N-terminal domain of LctM is responsible for the dehydration reaction. A sequence alignment of the N-terminal domains of a subset of LanM enzymes is shown in Figure 2, revealing ten fully conserved amino acids with side chain functionalities that could be directly involved in catalysis. In this study, we performed site-directed mutagenesis experiments on all of

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[1] Abbreviations: Dha, dehydroalanine; Dhb, dehydrobutyrine; FPLC, fast protein liquid chromatography; LanB, class I lantibiotic dehydratase enzymes; LanC, class I lantibiotic cyclase enzymes; LanM, class II lantibiotic synthetase enzymes; MALDI-TOF MS, matrix-assisted laser desorption ionization−time of flight mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

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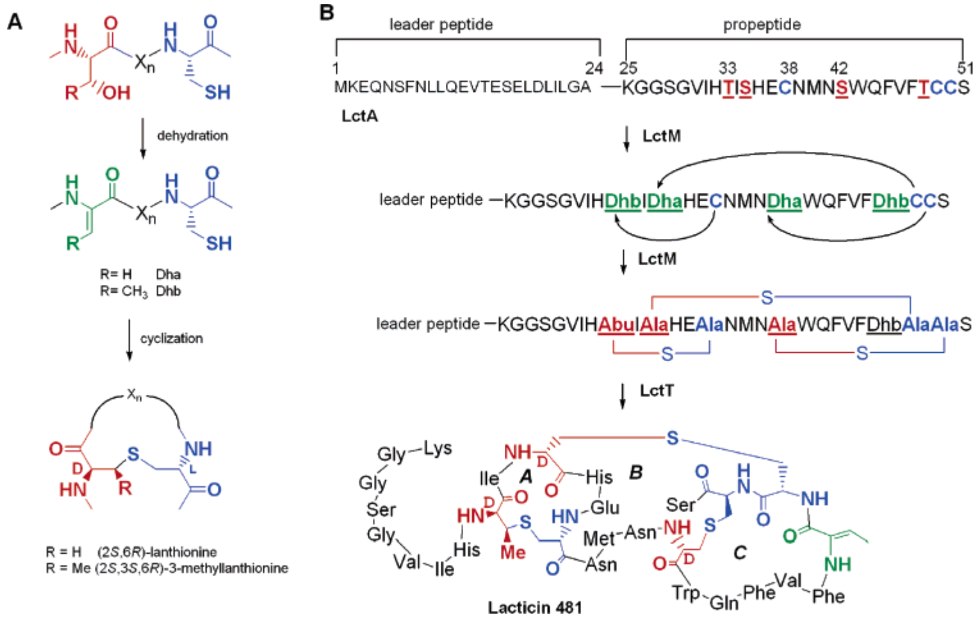

FIGURE 1: (A) Dehydration of serine and threonine residues results in the formation of dehydroalanine (Dha) or dehydrobutyrine (Dhb), respectively. Subsequent Michael-type addition of cysteines onto the unsaturated amino acids results in (methyl)lanthionine formation. (B) Biosynthesis of lacticin 481 (*12*). Following ribosomal synthesis, lacticin 481 synthetase (LctM) dehydrates serine and threonine residues in the propeptide region of the substrate peptide LctA and catalyzes (methyl)lanthionine formation. LctT then removes the unmodified leader peptide and secretes the final product (*13*).

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             159         242             259           399    408           446
              *           * *  *           * *           *      *  *          *
NukM   QLLYKPRSL....NISDLHFENILC....ILIDLETIF....VEVRILPRSTIEYSVL....NSEI
LctM   KLIYKPKSL....NLTDLHFENVIS....CIIDLETMF....VTCRILFRNTMEYSVL....KSEI
MutM   SLFYKP-HL....NMTDLHFENIIS....VILDMETIC....LTCRVIFRQTAHYSLM....ESEK
ScnM   KVVFKKKSS....NISDLHFENLIS....KLVDVETVF....IKTRILFRNTRDYSLV....LSEK
RumM   KIVYKPTRG....GMNDIHYENLIA....VITDVETIF....VESRIILNMTKGYSKI....EQEV
MrsM   KIVYKPRSL....NAVDFHMQNLIA....ILVDLESLF....VTVRQILRGTSRYANL....NSEK
CylM   KIVYKPK-I....NVTDLHYENIIA....VIIDNETFF....LIVRNVIRPTQRYADM....HYEF
CinM   EFFYKPRSV....GGSDLHFENVIV....FVCDAETVL....VTARFINWGTQIYAQL....GREV
LasM   ELLYKPRSN....GTTDIHSENIIT....IVIDIETLS....YVSRQLLRDTQVYADF....KNEV
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FIGURE 2: Partial sequence alignment of the N-terminal region of selected LanM enzymes. Fully conserved residues are highlighted in yellow. For a complete sequence comparison, see ref *9*.

these conserved residues to provide the first insights into the mechanism of catalysis.

MATERIALS AND METHODS

General Materials. Restriction endonucleases were purchased from NEB or Invitrogen. IPTG was obtained from Calbiochem. Taq DNA polymerase and Pfx DNA polymerase were purchased from Invitrogen. T4 DNA ligase was purchased from NEB or Invitrogen. dNTPs and DNA polymerases were purchased from Invitrogen. Chelating Sepharose Fast Flow resin and SP-Sepharose Fast Flow resin were purchased from Amersham Pharmacia Biotech. Chitin resin was obtained from NEB.

General Methods. Escherichia coli strains DH5α and BL21(DE3) were used for plasmid DNA preparation and protein expression, respectively. pET-28b containing the *lctM* gene and pET-15b containing the *lctA* gene were reported previously (*4*). His-tagged LctM mutants were purified from *E. coli* BL21(DE3) by Ni^{2+} affinity chromatography followed by SP cation-exchange chromatography as previously described for wild-type LctM (*4*). Truncated substrates His_6-LctA(1−37) or His_6-LctA(1−37)S35A were obtained using intein chemistry and were purified with chitin resin by previously described methods (*14*). A Waters system was used for preparative HPLC with a Waters C4 PrepLC (25 mm module) column. The flow rate for preparative HPLC was 8 mL/min with detection at 220 nm. Solvents for RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile/20% water). For analytical HPLC, a Beckman Gold system and Vydac analytical C4 column were used. The flow rate was 1 mL/min. MALDI-TOF mass spectrometry was carried out on an Applied Biosystems Voyager-DE-STR in the Mass Spectrometry Laboratory at the School of Chemical Sciences, UIUC.

Samples for MALDI-TOF MS were prepared using a C_{18} ziptip (Millipore), eluting with a solution of 0.1% TFA and 50% ACN as previously described (*14*). DNA fragments amplified by PCR and/or treated with restriction endonucleases were purified with a QIAquick PCR purification kit or gel extraction kit (QIAGEN). A QIAGEN plasmid midi kit or QIAprep spin miniprep kit was used for plasmid isolation. DNA sequencing was carried out at the Biotechnology Center of UIUC.

Site-Directed Mutagenesis and Overexpression Constructs. Construction of the pET-28b plasmid containing the *lctM* gene was reported previously (*4*). Plasmids containing *lctM* mutant genes were generated by PCR using the wild-type *lctM* gene as template, the mutagenesis primers provided in the Supporting Information (Table S1), Taq DNA polymerase, and Pfx DNA polymerase. The PCR products were treated with endonuclease *Dpn*I followed by DH5α transformation. Alternatively, overlap-extension PCR methodology was used. For example, PCR fragments were generated by primers NheI-38 and K159MFP (Table S1) or K159MRP and MfeIRP using Taq DNA polymerase and Pfx DNA polymerase. After purification, the two resulting PCR fragments were used to generate an extended DNA fragment with primers NheI-38 and MfeIRP. The final PCR products were treated with *Nhe*I and *Mfe*I followed by ligation into a pET-28b vector treated with the same restriction enzymes. The ligation products were used for DH5α transformation using agar plates containing 50 μg/mL kanamycin. Colonies were selected and grown in 5 mL of LB containing 50 μg/mL kanamycin and used for plasmid preparation. The presence of the mutations was confirmed by sequence analysis.

His$_6$-LctA N39R/F45H. This mutant was generated by PCR using a previously reported plasmid carrying wild-type *lctA* (*4*) as the template. PCR cycles using the primers LctAN39R/F45H FP (5′-ATGAATGTCGTATGAATAGCTGGCAACATGTATTTA-3′) and T7 terminator (5′-GCTAGTTATTGCTCAGCGG-3′) generated a mutant of the partial *lctA* gene. In a second PCR, this DNA fragment was used as the reverse primer with the T7 promoter primer (5′-CGCGAAATTAATACGACTCACTATAGGGGAATTGTGAG-3′) to yield the full *lctA* gene. The PCR product was digested with *Nde*I and *Bam*HI restriction enzymes and ligated into the pET-15b vector. DNA sequencing confirmed the desired mutations.

Overexpression and Purification of Wild-Type His$_6$-LctA and His$_6$-LctA N39R/F45H. BL21(DE3) cells hosting a pET-15b plasmid containing genes encoding wild-type or His$_6$-LctA N39R/F45H were induced by adding 1 mM IPTG (final concentration) at 37 °C at OD_{600nm} = 0.6−0.7, followed by 3 h additional growth. The cells were harvested by centrifugation at 4 °C for 15 min at 11900*g*. The cell pellet (∼3 g/L) was resuspended in start buffer (∼3 mL/g of cell pellet, 20 mM NaH_2PO_4, pH 7.5, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and stored at −80 °C. When needed, the cell pellet was thawed and lysed by sonication for 15 min followed by centrifugation for 30 min at 23700*g*. After centrifugation, the supernatant was discarded, and the pellets were resuspended in start buffer. The mixture was sonicated for 5 min and centrifuged, and the pellet was resuspended in denaturing LctA buffer 1 (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5, 0.5 mM imidazole, 500 mM NaCl). The resuspended mixture was sonicated and centrifuged. The supernatant from the last centrifugation was filtered through a 0.45 μm filter and loaded onto a precharged Ni^{2+}-NTA column. Then the resin was washed with 50 mL of LctA buffer 1 and 50 mL of LctA buffer 2 (4 M guanidine hydrochloride, 20 mM PO_4^{3-}, pH 7.5, 30 mM imidazole, 300 mM NaCl). His$_6$-LctA was eluted with elution buffer (20 mM Tris•HCl, 100 mM NaCl, 1 M imidazole, 4 M guanidine hydrochloride, pH 7.5). The elution fraction containing His$_6$-LctA peptides was further purified by preparative HPLC (see General Methods). The HPLC peaks were collected and lyophilized. After analysis by MALDI-TOF mass spectrometry, the lyophilized powder containing the desired His$_6$-LctA peptides was collected and stored at −20 °C.

Assay for Dehydration Activity of LctM Mutants. His$_6$-LctA or the more soluble mutant His$_6$-LctA N39R/F45H was dissolved in buffer (50 mM Tris•HCl, pH 7.5, 1 mM ATP, 10 mM Mg^{2+}, 10 mM DTT) to a final concentration of 2−12 μM and incubated with about 0.1 mg/mL (final concentration) LctM or LctM mutant at 25 °C for 15 min to 6 h depending on the time required for complete consumption of the substrate. In the case of active mutants such as H244N and Y408F, the dehydration reactions were completed within 30 min. LctM mutants with partial activity, like K159M and E446M, required more than 2 h to complete substrate consumption, whereas Asn247Leu did not completely process the LctA substrate even after 6 h. In the case of inactive LctM mutants, D242N and D259N, the substrate was incubated with enzyme for 6 h, and no dehydration/phosphorylation was observed. Each reaction was stopped by adding 5% TFA to give a final concentration of 0.5% and analyzed by MALDI-TOF mass spectrometry.

Assay for Phosphate Elimination Activity of LctM-D242N, -D259N, and -R399M. A semisynthetic peptide corresponding to a mutant of truncated LctA spanning residues 1−39 [His$_6$-LctA(1−39)S28C/T33pT/S35A/N39A] was prepared by Dr. Chatterjee as previously described (*14*). The reaction conditions for the elimination reaction were also as previously described (*14*).

Assay for Phosphorylation Activity of LctM-R399M. His$_6$-LctA N39R/F45H, His$_6$-LctA(1−37), and His$_6$-LctA(1−37)-S25A (final concentration of 10 μM) were incubated with 0.1 mg/mL LctM-R399M, 1 mM ATP, 10 mM Mg^{2+}, and 10 mM DTT at pH 7.5 at 25 °C. The reactions were stopped by adding 5% TFA to a final concentration of 0.5% at 1, 5, 10, 20, 30, and 60 min time points. Each reaction product was checked by MALDI-TOF MS.

Purification of the Phosphorylated His$_6$-LctA(1−37)S35A Produced by LctM-R399M. A sample containing a 30 min time point of the assay of His$_6$-LctA(1−37)S35A with LctM-R399M was purified by analytical HPLC using a gradient of 2% solvent B (0.086% TFA in 80% acetonitrile/20% water) to 100% solvent B over 50 min. Two peaks with retention times of 28 and 28.5 min were collected separately. MALDI-TOF MS showed that the peak at 28 min contained a peptide corresponding to M + 80 whereas the 28.5 min fraction consisted of starting material. The phosphorylated peptide was used for phosphate elimination experiments with wild-type LctM.

RESULTS

Generation of LctM Mutants and Assay Conditions. The residues selected for mutagenesis in this study were Lys159,

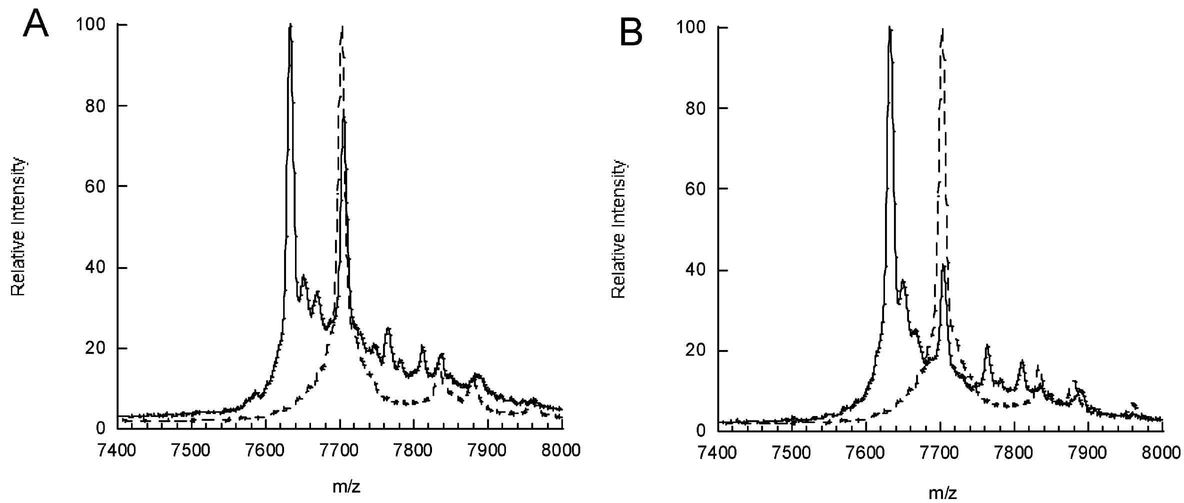


FIGURE 3: MALDI-TOF mass spectra of substrates (dashed line) and dehydration assays after 15 min (solid line) containing His_6-LctA and (A) LctM-H244N or (B) LctM-Y408F.

Asp242, His244, Asn247, Asp259, Glu261, Arg399, Thr405, Tyr408, and Glu446 (Figure 2). All LctM mutants were constructed as hexahistidine fusion proteins (His_6-LctM; see Materials and Methods), expressed in *E. coli*, and purified by Ni-based immobilized metal affinity chromatography. The mutant proteins were then incubated for 15 min to 6 h with 1 mM ATP, 10 mM Mg^{2+}, 10 mM DTT, and 12 μM His_6-LctA or an LctA mutant with better solubility than the wild-type sequence (His_6-LctA N39R/F45H). Note that the solubility of both peptides is poor, with the substrate often only partially soluble under the reaction conditions preventing detailed kinetic experiments (see Supporting Information and Figure S1). Attempts to use quantitation of ADP produced in the phosphorylation step as a measure of catalytic activity was hampered by various degrees of ATPase activity displayed by these mutants, which prevented correlation of ADP concentrations with dehydrations observed by mass spectrometry (MS). Despite these technical difficulties, incubation of the LctA substrates with the LctM mutant proteins provided new insights into the importance of the conserved residues.

Mutagenesis of His244 and Tyr408. LctM-H244N and -Y408F were incubated with substrate, ATP, and Mg^{2+}, and the assay products were analyzed at several time points by matrix-assisted laser desorption ionization (MALDI) mass spectrometry to assess dehydration activity in comparison to identical reactions with His_6 wild-type LctM. Under the reaction conditions used, wild-type (wt) LctM completed the 4-fold dehydration of both of these substrates in less than 5 min. As shown in Figure 3, His244Asn and Tyr408Phe were both able to dehydrate His-tagged wild-type LctA with a peptide corresponding to four dehydrations as the major product. The Tyr408Phe mutant achieved complete conversion slightly slower than wild-type LctM under identical conditions, and the His244Asn mutant showed a relative activity that was decreased about 6-fold as complete consumption required about 30 min (data not shown).

Mutagenesis of Glu261, Glu446, Asn247, and Lys259. Unlike the results with His244Asn and Tyr408Phe, LctM-Glu261Gln and LctM-Glu446Met were significantly less active than wild-type LctM, requiring 1 and 6 h, respectively, for complete consumption of the substrate. In addition, the 4-fold dehydrated product was no longer the major product as varying amounts of peptides corresponding to two or three dehydrations were observed that could not be forced to a 4-fold dehydrated product (Figure 4). Increasing the Mg^{2+} and ATP concentrations from 10 to 20 mM and from 1 to 2 mM, respectively, did improve the activity of the Glu261Gln mutant such that the peptide with four dehydrations was the major product (Figure S2). The Lys159Met mutant was also much less active, requiring more than 6 h for complete consumption of the starting peptide. This mutant also produced significant quantities of phosphorylated intermediates (M + 80 Da, Figure 4C,D). LctM-Asn247Leu was the least active in this group of mutants, but it did produce a small amount of products corresponding to one to four dehydrations after 6 h (Figure S3).

Mutagenesis of Asp242 and Asp259. In contrast to the conserved residues discussed in the previous section, mutation of Asp242 and Asp259 to Asn resulted in proteins with no discernible dehydration activity (Figure 5A). They were then investigated for the ability to eliminate phosphate from a phosphorylated substrate peptide prepared using methodology developed previously (*14*). In short, a truncated LctA substrate (residues 1−27) was expressed in *E. coli* as an intein fusion peptide, which upon purification and treatment with the sodium salt of 2-mercaptoethylsulfonic acid (MESNa) resulted in the MES thioester of LctA(1−27). This thioester was then ligated (*18*) with a synthetic nonapeptide containing the phosphorylated Thr. The resulting ligation product corresponds to LctA(1−37) with a phosphorylated Thr at position 33 and a Cys instead of a Ser at position 28. Previous experiments have shown that the replacement of Ser28 with a Cys has no consequences with respect to the posttranslational modification carried out by LctM (*14*). The semisynthetic phosphorylated peptide represents an intermediate in the dehydration of Thr33 and can be converted into the dehydroamino acid containing peptide by incubation with wild-type LctM as long as ADP and Mg^{2+} are added to the assay mixture (*14*). When LctM-Asp242Asn and LctM-Asp259Asn were analyzed by this method, the former was able to eliminate the phosphate (Figure 5B) whereas the latter was not (Figure 5C). Thus, Asp242 is critical for phosphorylation but not elimination, and Asp259 is important for both steps.

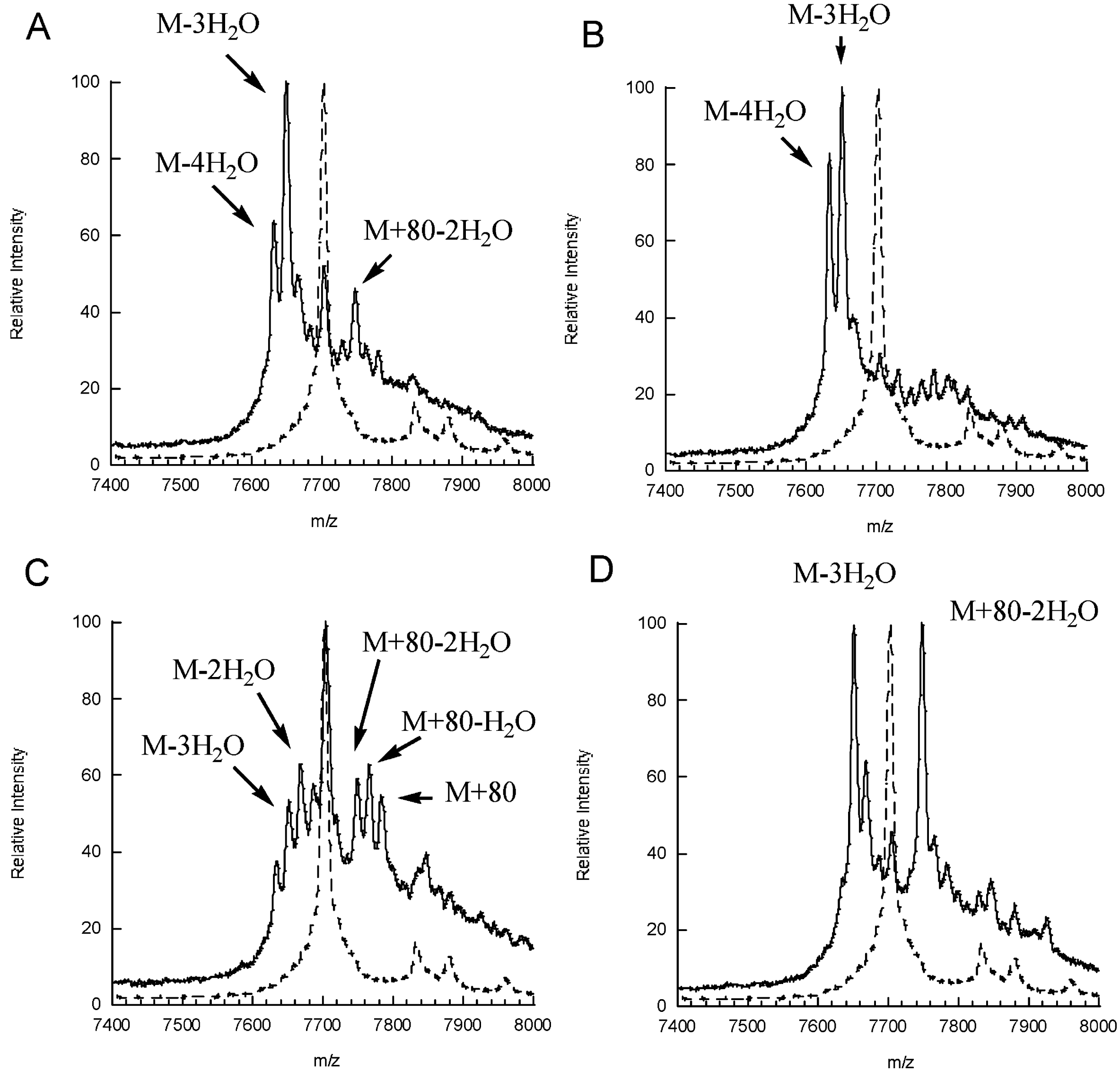


FIGURE 4: MALDI-TOF mass spectra of substrates (dashed line) and dehydration assays (solid line) after incubation of His_6-LctA with (A) LctM-E261Q for 1 h, (B) LctM-E446M for 6 h, (C) LctM-K159M for 1 h, or (D) LctM-K159M for 6 h.

Mutagenesis of Arg399 and Thr405. Replacement of Arg399 with a Met resulted in a mutant LctM that predominantly phosphorylated substrate peptides (Figure 6). The protein catalyzed conversion of His_6-wt LctA or the more soluble mutant His_6-LctA N39R/F45H into a mixture of peptides carrying one, two, or three phosphate groups as well as small peaks corresponding to a phosphorylated peptide that also had undergone elimination of a phosphate group (asterisks, Figure 6A). Treatment of the mixture of phosphorylated peptides with the protease LysC, which cleaves after Lys25 (see Figure 1B), and subsequent MS analysis showed that the phosphorylations took place in the propeptide region. Similar results were obtained with an Arg399Leu mutant (not shown). The Arg399Met mutant could also phosphorylate a truncated LctA substrate (residues 1−37) (Figure 6B). On the other hand, when the Arg was replaced with a Lys residue, the mutant protein had only about 2-fold reduced dehydration activity compared to wild-type LctM with no buildup of phosphorylated intermediates (Figure 6C). As expected, LctM-Arg399Met and LctM-Arg399Leu were incapable of eliminating phosphate from Thr33 in the semisynthetic phosphorylated peptide. The behavior of LctM-Thr405Ala resembled that of the Arg399Met mutant. Incubation with wild-type LctA also resulted in a mixture of peptides carrying one, two, or three phosphate groups (data not shown).

Investigation of the Phosphate Elimination Reaction. The behavior of the R399M mutant provides a much-improved route to rapidly prepare phosphorylated peptides compared to our previously reported method that required solid-phase peptide synthesis and subsequent ligation to expressed peptides (*14*). The capability to access these phosphorylated substrates more conveniently allowed investigation of the phosphate elimination step in more detail than previously reported. Hence, a truncated LctA substrate corresponding to residues 1−37 was constructed in which Ser35 was mutated to Ala. This peptide [His_6-LctA(1−37)S35A] contained one residue (Thr33) that is dehydrated in wild-type LctA, and it was envisioned that treatment of this peptide with the LctM-R399M would result in a product with a single phosphorylation. Indeed, this assay resulted in the generation of a M + 80 peak as the major product (Figure 7A). This phosphorylated peptide was then purified by HPLC and used to investigate the phosphate elimination reaction by wild-

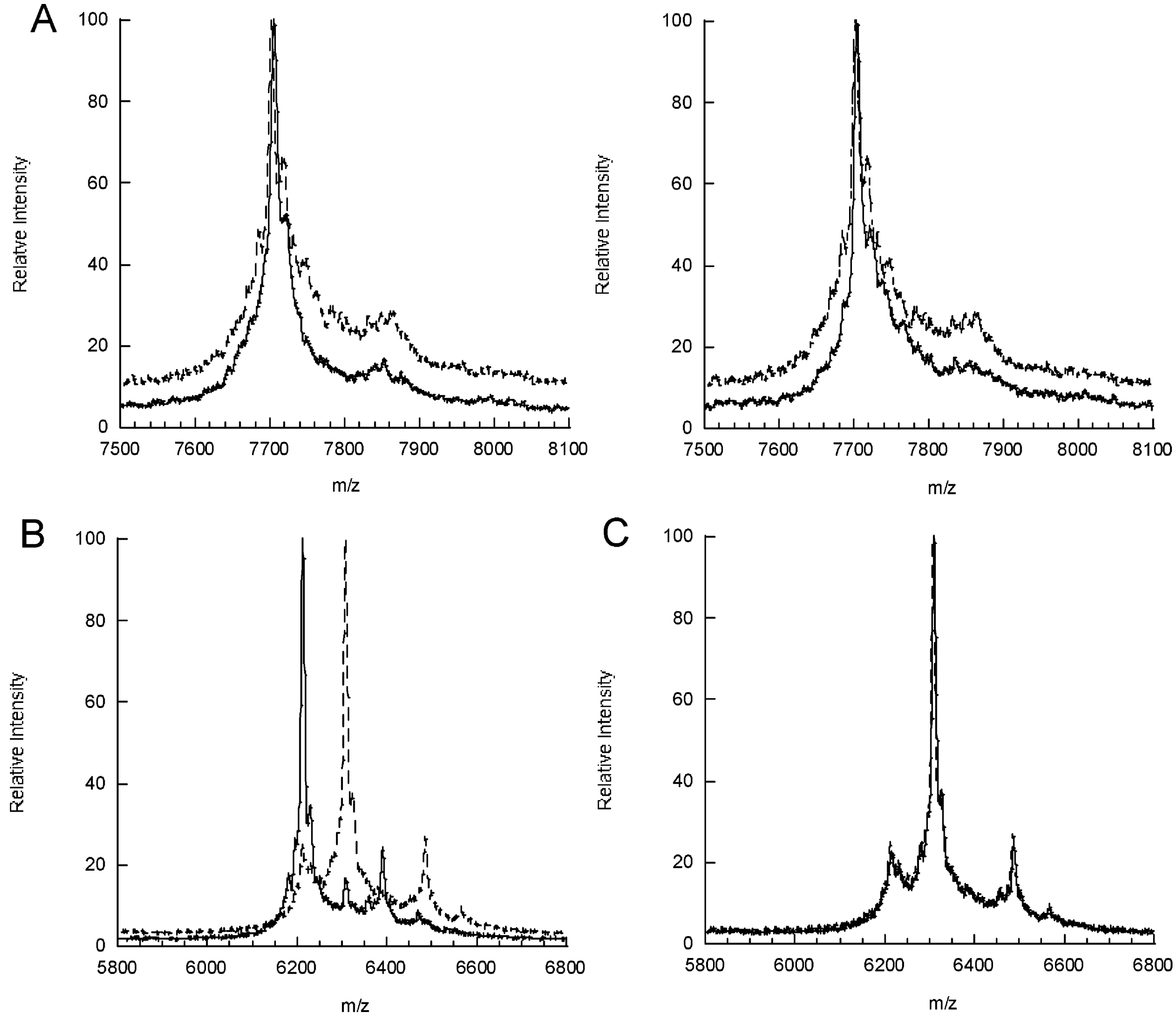


FIGURE 5: MALDI-TOF mass spectra of assays of His$_6$-LctA with LctM-D242N and LctM-D259N. Substrates are shown in dashed line and assay product in solid line. (A, left) Dehydration assay with D242N and (A, right) dehydration assay with LctM-D259N. (B) Phosphate elimination assay of a truncated, phosphorylated LctA analogue reacted with LctM-D242N in the presence of ADP and Mg^{2+} resulting in a M − 98 peak indicative of phosphate elimination. (C) Phosphate elimination assay of LctM-D259N.

type LctM. ADP is required for phosphate elimination (Figure 7B) since in the absence of ADP or in the presence of ATP and Mg^{2+} no elimination took place. In addition, no elimination was observed when Mg^{2+} was omitted but ADP was present in the assay (Figure 7C).

DISCUSSION

The dehydration reactions catalyzed by LctM during lacticin 481 biosynthesis and by HalM during haloduracin biosynthesis require the presence of ATP and Mg^{2+} (*4*, *5*). In this work we provide additional evidence for our proposed mechanism (*14*) that the cofactor is utilized to phosphorylate Ser and Thr residues that are converted to Dha and Dhb residues, respectively. The LanM proteins have no sequence homology with known enzymes other than lantibiotic synthetases, and no ATP binding motif is found in conserved domain searches (*19*). These proteins lack the Walker motif (*20*) characteristic of the phosphate binding loop (P-loop) (*21*) in kinases that phosphorylate small molecules and in some instances proteins (*22*). They also do not contain the typical ATP binding motifs found in eukaryotic protein kinases (*23*) that more recently have also been characterized in bacteria (*24*, *25*), nor do they have the ATP binding motif found in the PurM superfamily of proteins (*26*). Nevertheless, the lessons learned from studies of these classes of kinases provide a good starting point for the formulation of a mechanism for the LanM-catalyzed dehydration reaction. A minimal putative mechanism for the overall dehydration reaction is shown in Scheme 1. A general base could deprotonate the Ser/Thr targeted for dehydration, which in turn would attack the γ-phosphate of ATP producing the phosphorylated peptide and ADP. Mg^{2+} is absolutely required for the LctM reaction, but its binding motif cannot be predicted as different binding modes have been observed in kinases (*27*). In most Ser/Thr kinases Mg^{2+} is coordinated by the nonbridging oxygens of the β- and γ-phosphates of ATP (as drawn) with sometimes additional contacts made with the α-phosphate (*28*, *29*). Alternatively, a select group of kinases utilizes two Mg^{2+} ions for catalysis (*27*, *29*). The octahedral coordination geometry of Mg^{2+} in protein kinases is typically completed by water molecules, backbone amides, and/or side chains of conserved Ser, Thr, Asx, and/or Glx residues in the ATP binding site (*27*, *29*, *30*). In almost all protein kinases an additional conserved Lys residue provides a further contact to the γ-phosphate that is critical for the phosphoryl transfer reaction (*31*, *32*).

After the phosphoryl transfer step, the LanM reaction deviates from the typical reactions in biological phosphoryl

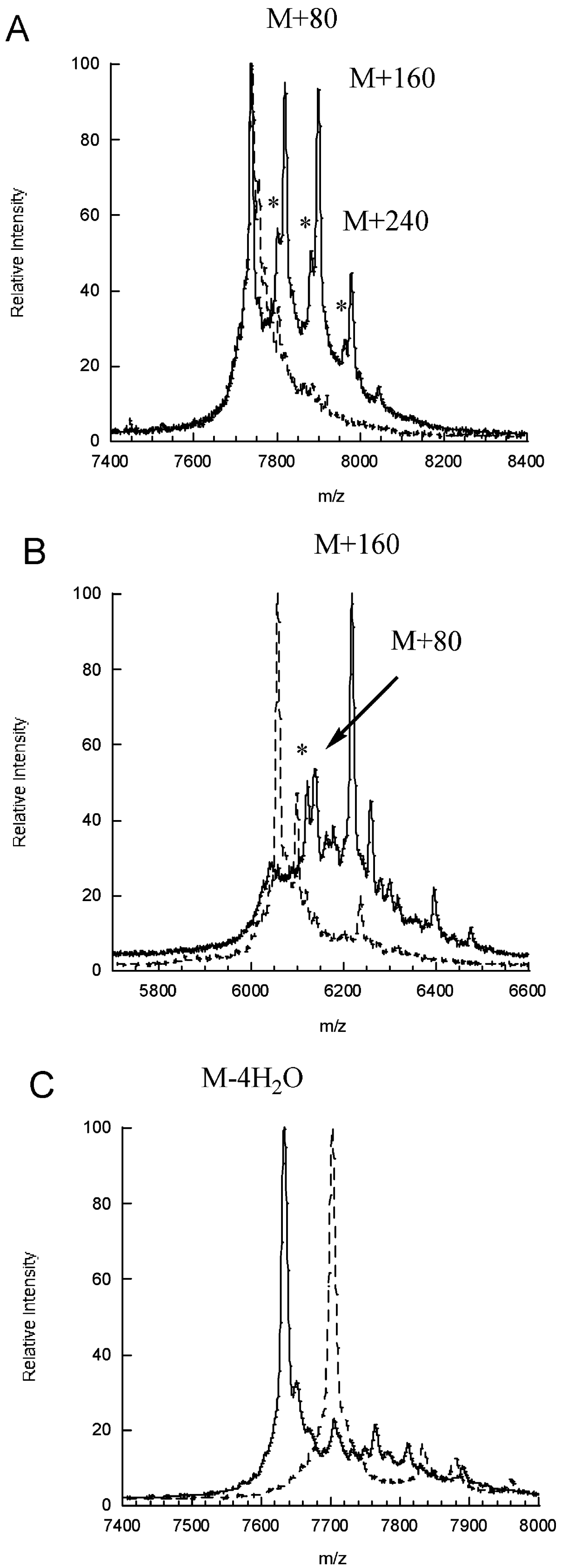


FIGURE 6: MALDI-TOF mass spectra of assays of various peptides with LctM-R399M. Substrates are depicted in dashed line and assay products in solid line. R399M was incubated with His$_6$-LctA N39R/F45H (A) and His$_6$-LctA(1−37) (B) for 30 min. LctM-R399K was also incubated with His$_6$-LctA for 15 min (C). The peaks labeled with an asterisk (*) are showing single eliminations of phosphates from phosphorylated peptides.

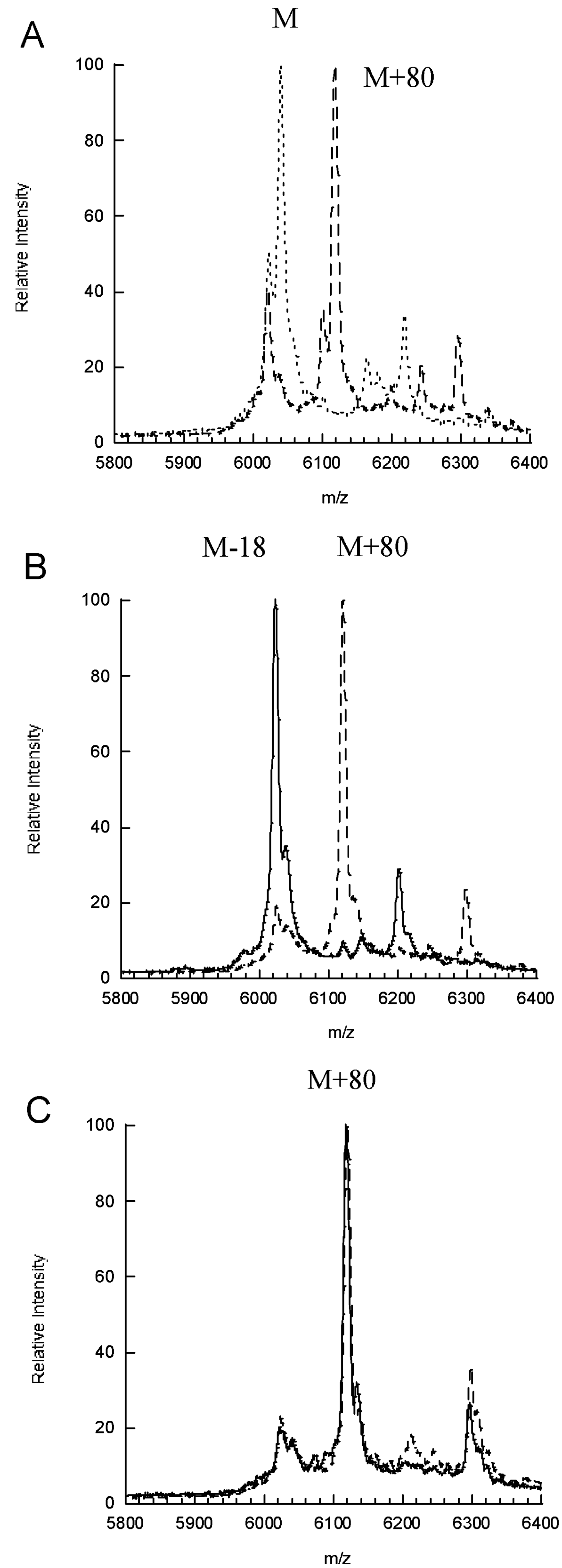
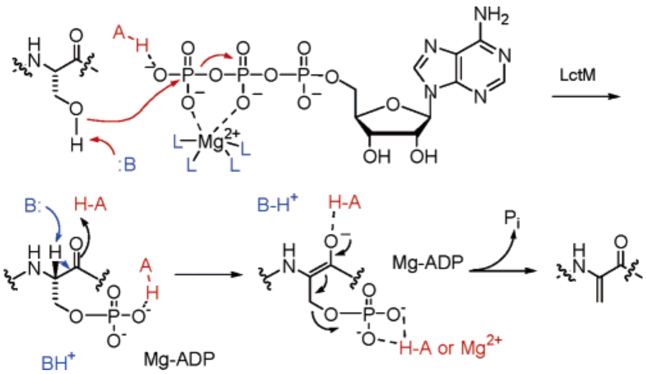


FIGURE 7: MALDI-TOF mass spectra of phosphate elimination reactions. (A) His$_6$-LctA(1−37)S35A substrate is depicted in dotted line and phosphorylated substrate after 30 min incubation with R399M in dashed line. (B) Incubation of wild-type LctM with phosphorylated LctA(1−37)S35A in the presence of 500 μM ADP and 10 mM Mg^{2+}. (C) Incubation of wild-type LctM with phosphorylated LctA(1−37)S35A in the presence of 500 μM ADP but in the absence of Mg^{2+}.

Scheme 1: Proposed General Mechanism of Dehydration of Ser by LctM



transfer processes involving protein substrates. Rather than removal of the phosphate by hydrolysis or phosphorolysis, LanM proteins eliminate the phosphate group. In this reaction, a general base is expected to deprotonate the α-carbon to generate an enolate, which subsequently eliminates the phosphate group, possibly assisted by a general acid and/or Mg^{2+}. At present, it cannot be ruled out that the deprotonation and elimination occur in concerted fashion. Given the stereochemistry of the (*Z*)-dehydrobutyrine formed from Thr, the elimination proceeds with *anti* stereoselectivity. Our studies with synthetic phosphorylated peptides suggest that ADP and Mg^{2+} must be bound in the active site during the elimination step.

To date, attempts to crystallographically characterize LctM or other LanM proteins that have been reconstituted in vitro (*5*) have not been successful. We therefore turned to site-directed mutagenesis studies to provide insights into the possible identity of the active site residues in the dehydratase domain of LctM. Mutation of Asp242 resulted in a protein that still retained the ability to eliminate phosphate but not to dehydrate the substrate. We interpret these results to indicate that the phosphorylation step is impaired. In addition, the Asp259Asn mutant lacks both dehydration and phosphorylation activity. Previously characterized Ser/Thr and Tyr protein kinases contain a conserved Asp that makes a hydrogen-bonding contact to the hydroxyl group of the substrate, as seen for instance in the crystal structure of a transition state mimic of cAMP-dependent protein kinase (*29*). Mutation of the Asp to Glu in the C-terminal Src kinase (Csk) greatly decreased the rate of phosphorylation (~10^4-fold), indicating the importance of this residue (*33*). A similarly large decrease in phosphorylation activity for the LctM-D242N and D259N mutants is not detectable in our current assay due to the insolubility of the substrate, the relative instability of the LctM protein, and the sensitivity and dynamic range of the mass spectrometric readout. Thus, Asp242 or Asp259 may be the base that accepts a proton from Ser/Thr of the substrate. We note that the aforementioned studies on Csk as well as other mechanistic studies on Tyr and Ser protein kinases indicate that the relevant Asp, while very important for catalysis, does not function as a general base that deprotonates the nucleophile prior to or concomitant with attack onto ATP (*34*−*38*). Instead, the residue is proposed to position the nucleophile and to accept the proton late in the reaction process.

Unlike Asp242 and Asp259, other potential active site general acids or bases such as Glu261, His244, Asn247, Tyr408, and Glu446 did not abolish dehydration activity when mutated, suggesting that they are not critical for catalysis. Of these, the His244 and Tyr408 mutants behaved much like wild type but the Asn247, Glu261, and Glu446 mutants required longer times to consume the substrate and produced a significantly larger fraction of partially dehydrated peptides (Figures 3 and 4), which are not typically seen for wild-type LctM because of its processive mechanism of dehydration (*15*). We tentatively assign roles of Mg^{2+} coordination to these three residues.

Perhaps the most interesting phenotypes are displayed by mutants involving Arg399 and Thr405. Both Arg399Met and Arg399Leu phosphorylated the LctA substrate in the propeptide region of the peptide. The heterogeneity of the product has not yet allowed precise localization of the phosphates, but time-dependent studies in combination with Fourier transform mass spectrometry (*15*) are in progress to investigate whether the phosphorylation events have order and/or directionality or are random. Thus far, such investigations have not been possible because reaction intermediates with wild-type LctM have only been observed in rapid quench studies of single turnover experiments (*15*), which never provided the required quantities of product. With respect to the role of Arg399 in the dehydration mechanism, it might represent the active site base that deprotonates the α-carbon of a phosphorylated Ser/Thr (Scheme 1). Because of their relatively high pK_a of 12.5 in solution, arginines have not usually been associated with roles of general base or general acid in enzyme catalysis. However, several examples of such roles have been documented recently in the literature (*39*−*46*), including enzymes that catalyze elimination reactions in which an Arg general base deprotonates a carbon acid adjacent to a carbonyl group. On the other hand, the phenotype of the Thr405Ala mutant is much the same as the Arg mutants, and it is unlikely to be the active site base. Therefore, it is possible that Arg399 and/or Thr405 activate(s) another residue that is the actual general base for deprotonation. If so, only Asp259 appears to be a possible candidate for the true general base since all other mutants of conserved residues are not impaired in the phosphate elimination step. Interestingly, the LctM-Arg399Lys mutant retained nearly full dehydration activity, which appears to argue against a role of general base for Arg399. However, the Arg418Lys mutant in IMP dehydrogenase also displayed a rate similar to that of the wild-type enzyme for the step that involves Arg418 as general base (*40*). Thus, the possibility of Arg399 serving as the general base that initiates phosphate elimination cannot be ruled out.

Finally, mutation of Lys159 resulted in a protein with much reduced dehydration activity. The mutant also produced phosphorylated intermediates, but these never accumulated to the same extent as what was observed with Arg399Met. Thus, it appears that the mutation predominantly slows down the phosphorylation step but also affects the elimination step. As mentioned above, Ser/Thr kinases contain a conserved Lys that contacts the γ-phosphate of ATP or ATP analogues in structures of substrate-like complexes as well as the phosphate of phosphorylated Ser/Thr in complexes of the product. This Lys is believed to be critical for the phosphoryl transfer reaction. We hypothesize that Lys159 in LctM fulfills a similar role and that it also may aid in the phosphate elimination step as a general acid.

In summary, this study provides the first investigation of the residues responsible for catalysis in the LanM lantibiotic synthetase family. We propose a model in which Asp242 or Asp259 accepts the proton from the Ser/Thr substrate, Asn247, Glu261, and Glu446 serve as ligands to Mg^{2+}, Arg399 or Asp259 deprotonates the α-carbon in the elimination step, and Lys159 guides the phosphate group that is transferred and later eliminated.

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SUPPORTING INFORMATION AVAILABLE

Sequences of oligonucleotide primers used for site-directed mutagenesis, a discussion of the solubility of the LctA substrate, and mass spectrometric data on assays with some mutant enzymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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