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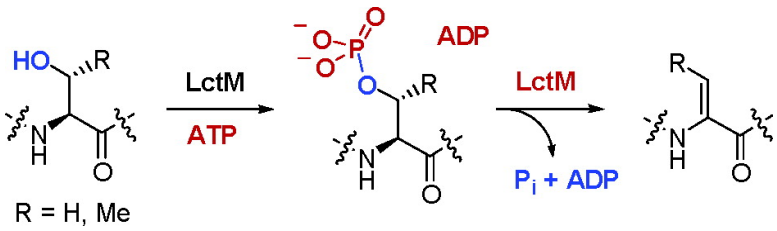

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Lacticin 481 Synthetase Phosphorylates its Substrate during Lantibiotic Production

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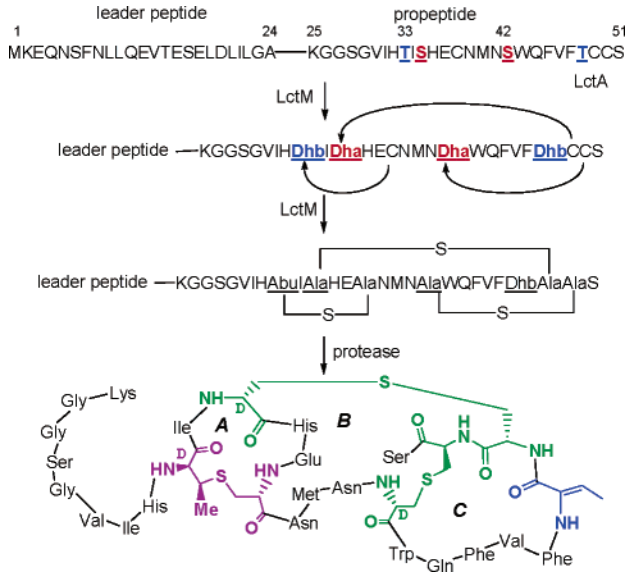
Lantibiotics are genetically encoded, post-translationally modified peptide antibiotics. To date, about 45 family members have been identified.[1] All lantibiotics contain the thioether bisamino acids lanthionine (Lan) and/or methyllanthionine (MeLan) from which they derive their name. These residues are formed by the site-specific dehydration of Ser and Thr residues, respectively, followed by the stereoselective Michael-type addition of a cysteine thiol to the newly formed dehydroalanine (Dha) and dehydrobutyrine (Dhb) (Scheme 1). In the class I lantibiotics, including the most-studied

Scheme 1



member nisin, the (Me)Lan residues are formed by the action of separate dehydratase (LanB) and cyclase (LanC) enzymes. In contrast, a single enzyme (LanM) catalyzes both dehydration and cyclization reactions in class II lantibiotics, including lacticin 481. The activity of the lacticin 481 synthetase LctM was recently reconstituted in vitro and shown to require ATP for the formation of lacticin 481 from the pre-lacticin peptide LctA (Scheme 1).[2] Although LctM bears homology in its C-terminal region to the LanC cyclases, including the presence of a putative zinc binding motif,[3] no homology can be detected between LctM and LanB dehydratases. Moreover, no homology exists with nonlantibiotic producing proteins precluding a prediction of its mode of action based on known mechanisms of dehydratases.

A first clue as to how LctM catalyzes its dehydration reaction came from the use of C-terminally truncated analogues of LctA as substrates. In addition to the expected peaks for products of dehydration, the MALDI-TOF MS spectra displayed peaks that corresponded to +80 Da adducts (Figure 1). Analysis of the isolated M-2 H_2O + 80 ion on a custom-built 8.5 T Quadrupole-FTMS

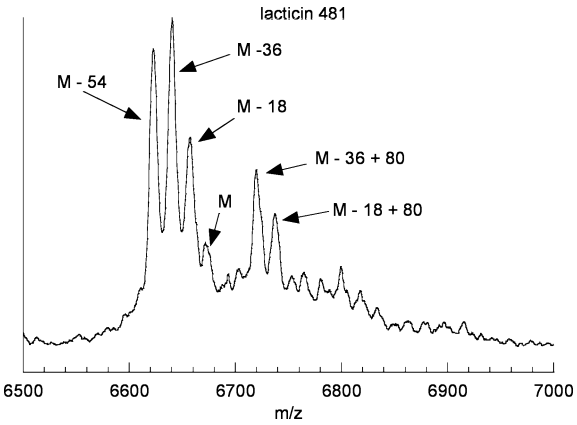


Figure 1. MALDI-TOF spectrum of the assay product formed upon incubation of LctM with His_6-LctA(1−43)W43A in the presence of 10 mM $MgCl_2$ and 5 mM ATP. Masses of observed peaks: 6674 Da (M), 6657.7 Da (M−H_2O), 6640.8 Da (M−2H_2O), 6623.3 (M−3H_2O), 6720.3 (M−2H_2O + 80), 6737.9 (M−H_2O + 80).

instrument[4] using SWIFT for ion isolation and Sustained Off-Resonance Irradiation (SORI)[5] or Electron Capture Dissociation (ECD) for ion fragmentation localized the adduct to the propeptide region of the truncated substrate (Figures S2−S4, Supporting Information). ATP hydrolysis to ADP and phosphate is an absolute requirement for LctM action,[2] suggesting the possibility that it is used to phosphorylate the Ser/Thr residues that are targeted for dehydration, thereby turning the hydroxyl group of these amino acids into a good leaving group. Phosphorylation of the substrate was observed only in the presence of LctM and when non-natural truncated substrates were used, which presumably led to less efficient processing.

To provide further support for this hypothesis, synthetic phosphorylated substrate analogues were prepared[5] and tested for activity with LctM. His_6-LctA(1−27) was overexpressed and purified as the MESNa thioester using intein chemistry.[6] The undecapeptide CGVIHpTISHEA was synthesized on solid phase using Fmoc chemistry and ligated to the HPLC-purified peptide thioester.[7,8] This ligation introduces a cysteine at the position that is occupied by Ser28 in the native substrate, but this Ser is not dehydrated by LctM in the wild-type substrate.[2,9] Furthermore, mutation of Ser28 to Cys in the full length substrate showed that this mutation does not interfere with LctM catalysis as it still leads to full processing of the mutant substrate (not shown). The synthetic peptide also installs an alanine at position 38 normally occupied by Cys (Scheme 1), such that dehydration could be studied without complication by cyclization; cyclized products are highly resistant to fragmentation by tandem mass spectrometry.[2]

Incubation of the HPLC-purified substrate analogue His_6-LctA-(1−38)S28C/T33pT/C38A with LctM resulted in one dehydration as well as phosphate elimination when Mg^{2+} and ATP or ADP

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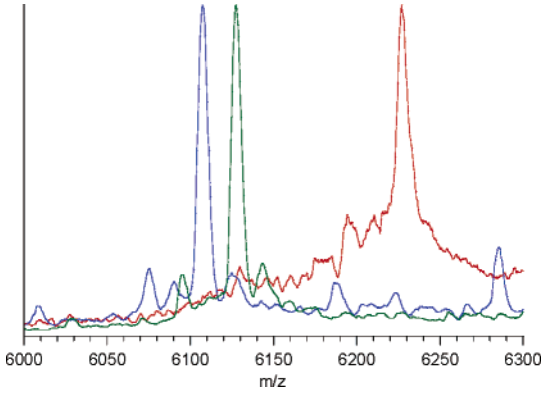

Figure 2. MALDI-TOF mass spectra of assay products of incubation of His_6-LctA(1−38)S28C/T33pT/C38A with LctM in the presence of Mg^{2+}, ATP, and ADP (blue) or just Mg^{2+} and ADP (green). In red is the product of incubating the peptide with LctM in the absence of cofactors. Observed masses: 6108.7 Da ($M-H_3PO_4-H_2O$, blue), 6127.4 Da ($M-H_3PO_4$, green), and 6224.7 Da (M, red).

were present in the assay (Figure 2).[10] No reaction was observed when these cofactors were absent or replaced with the nonhydrolyzable ATP analogues, AMP−PNP or AMP−PCP. Interestingly, elimination of only phosphate ($\Delta m = 98$) was observed when just ADP and Mg^{2+} were added (Figure 2). These data suggest that the phosphorylated peptide by itself is not a competent substrate for elimination, but when both products of the phosphorylation of Thr33 by ATP are present (i.e., the phosphorylated peptide and ADP), LctM eliminates phosphate to generate a Dhb at position 33. These findings suggest a compulsory ordered binding event where the phosphorylated substrate only binds to the enzyme after ADP is bound.[11] Alternatively, it is possible that the presence of ADP is required for either proper positioning of the substrate or lowering the energy barrier for elimination.[12]

The results described so far provide strong support that one of the roles of ATP is phosphorylation of Ser and Thr residues. This model would predict hydrolysis of one molecule of ATP to ADP and phosphate for each dehydration event. Unfortunately, it proved difficult to determine the stoichiometry because ATPase activity was observed even in the absence of the LctA substrate.[10] Hence, an additional role for ATP hydrolysis, such as providing energy for substrate translocation, could not be ruled out. To test this possibility in an alternative fashion, a 17-mer peptide was prepared containing three phosphorylated Ser/Thr residues, CGVIHpTIpSHECNMNpSWA. Ligation of this peptide to His_6-LctA(1−27) resulted in a substrate analogue in which the three Ser/Thr targeted for dehydration were all phosphorylated. Incubation of this peptide with LctM in the presence of ADP resulted in products in which one, two, and three phosphates had been eliminated (Figure S1, Supporting Information). No elimination was detected in the absence of ADP. Hence, ATP is not absolutely required for translocation.[13]

The requirement of ATP for cyclization was investigated with the truncated, phosphorylated peptide His_6-LctA(1−39)S28C/T33pT/S35A/N39A. An analogous peptide in which Thr33 is not phosphorylated undergoes the expected one dehydration (Thr33) and one cyclization upon treatment with LctM, Mg^{2+}, and ATP.[2] As expected, incubation of the phosphopeptide with ADP/Mg^{2+} and LctM resulted in elimination of phosphate. Cyclization activity was investigated by treatment of the starting peptide and the product with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) at pH 3. This reagent cyanylates thiols but not thioethers.[14] Analysis of the products by MALDI-MS showed that, whereas the starting peptide was quantitatively cyanylated twice (Cys28 and Cys38), only a single cyanide adduct was generated with the product of the enzyme catalyzed reaction (Figures S5 and S6, Supporting Information). Hence, LctM catalyzed cyclization of Cys38 onto Dhb33 without the need for ATP.

Dehydratases typically utilize acid−base chemistry,[15,16] pyridoxal 5′-phosphate (PLP),[17] or an iron−sulfur cluster[18] to activate their substrates. Use of ATP such as demonstrated here is unusual. Other examples of phosphorylation of an alcohol prior to elimination are found in PLP-dependent Thr synthase[17] and the mevalonate pathway of isoprenoid synthesis. Mevalonate 5-diphosphate decarboxylase requires ATP for the decarboxylation−dehydration of mevalonate 5-diphosphate to isopentenyl pyrophosphate.[19] Unlike this protein, which is a member of the GHMP kinase family,[20,21] LctM does not display an obvious ATP-binding domain. Structural studies are in progress to provide insights into how LctM binds its cofactor.

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Supporting Information Available: Detailed experimental procedures for preparation of the substrates, assay procedures, and their detailed analysis by MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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