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The Dehydratase Activity of Lacticin 481 Synthetase is Highly Processive

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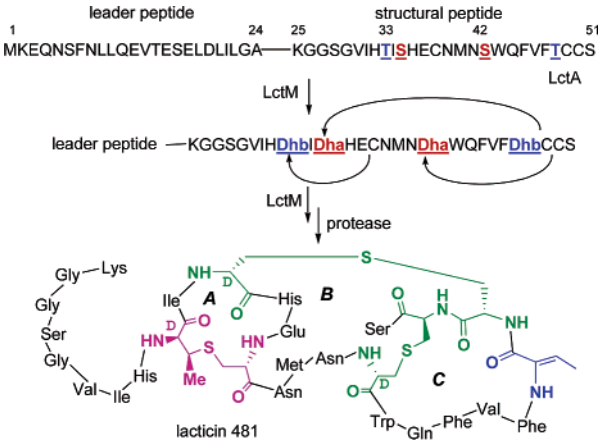
Lantibiotics are gene-encoded peptide antimicrobials that contain the unusual residues lanthionine (Lan) and/or methyllanthionine (MeLan) (e.g., Scheme 1).[1] The formation of these residues involves

Scheme 1



the initial dehydration of specific Ser and Thr residues in the prelantibiotic peptide and the subsequent stereoselective Michael-type addition of cysteine thiols to the newly formed dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively (Figure 1A). A family of bifunctional enzymes (LanM) undertakes both processes for the class II lantibiotics, such as lacticin 481 (Scheme 1).

The activity of the lacticin 481 synthetase, LctM, was recently reconstituted in vitro.[2] This synthetase catalyzes four dehydration and three cyclization reactions in the pre-lacticin peptide, LctA (Scheme 1). Chemical derivatization of the products and intermediates observed in time-dependent assays along with analysis by Fourier transform mass spectrometry (FTMS) analysis has now afforded a first glimpse of the order and timing of both the dehydration and cyclization reactions.

Conventional analysis of the products of the enzymatic modification process by MALDI−MS or ESI−MS is hampered by several experimental obstacles. A single dehydration ($\Delta m = -18$ Da) is difficult to distinguish from oxidation of a doubly dehydrated product ($\Delta m = -36 + 16$), and the potential of disulfide bond formation further clouds the interpretation (Supporting Figure 1). Therefore, it is difficult to distinguish putative dehydrated intermediates from oxidized dead-end products. To overcome this hurdle, high-resolution MS was used and the N-terminally His-tagged substrate, His_6-LctA, was isolated from cells grown in isotopically depleted media (99.9% ^{12}C and 99.99% ^{14}N).[3] This facilitated the precise identification of intermediates versus oxidation artifacts. The time-course of conversion of the precursor peptide to the completely dehydrated product (M − 4 H_2O) was observed by FTMS using a 1:900 enzyme-to-substrate ratio (Figure 1B).

Interestingly, during the course of this assay, very little ion intensity was observed from the build-up of partially dehydrated species. For instance, neither the M − H_2O nor the M − 2 H_2O

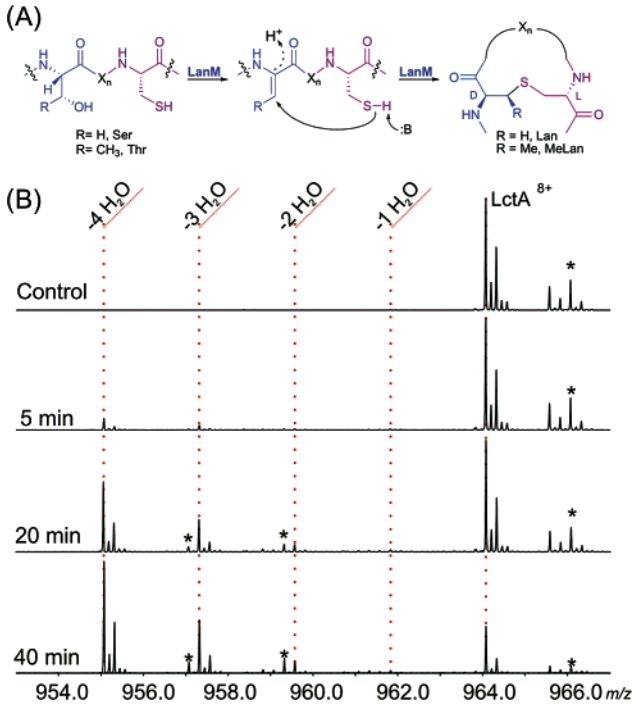


Figure 1. (A) LanM-catalyzed formation of (Me)Lan. (B) Time-dependent ESI/FT mass spectra of the assay of His_6-LctA with LctM; ∗ = oxidized products M+(O).

species was observed at the 5 min time point (Figure 1B), whereas the M − 4 H_2O species was clearly produced in a time-dependent manner. The data in Figure 1 are consistent with a processive mode of action and suggest that the dehydration of all four Ser/Thr residues in His_6-LctA can occur prior to its dissociation from LctM. Processive enzymes, such as nucleotide polymerases, endocellulases, and some protein kinases, remain bound to their polymeric substrate throughout multiple rounds of catalysis.[4−6] Distributive enzymes, however, dissociate from their substrate after each catalytic event, demonstrating a build-up of discrete intermediates, which was not observed for LctM.

To test this hypothesis, rapid-quench assays were conducted with a 1:1 enzyme-to-substrate ratio under single-turnover-like conditions. While no significant reaction was seen within 50 ms, after 3 s the product, substrate, and all partially dehydrated intermediates were observed with similar abundances (Figures 2 and S3). The data indicate that under these conditions the intermediates can be trapped on the enzyme, further supporting a processive model during steady-state turnover.[7]

The timing of cyclization of the dehydrated species was interrogated to determine the relative order of the dehydration and cyclization events. All three Cys residues in His_6-LctA are involved in thioether formation in the mature product (Scheme 1). Ring formation in dehydrated substrates does not lead to a change in mass of the cyclized product, thus precluding direct MS analysis

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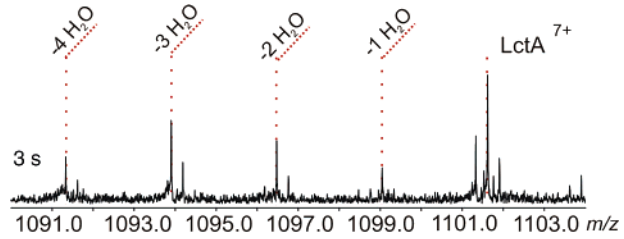

Figure 2. Assay product of LctM with His_6-LctA at 1:1 enzyme-to-substrate ratio. The 3 s time point exhibits the presence of all possible dehydrated forms of LctA (7+ charge state).

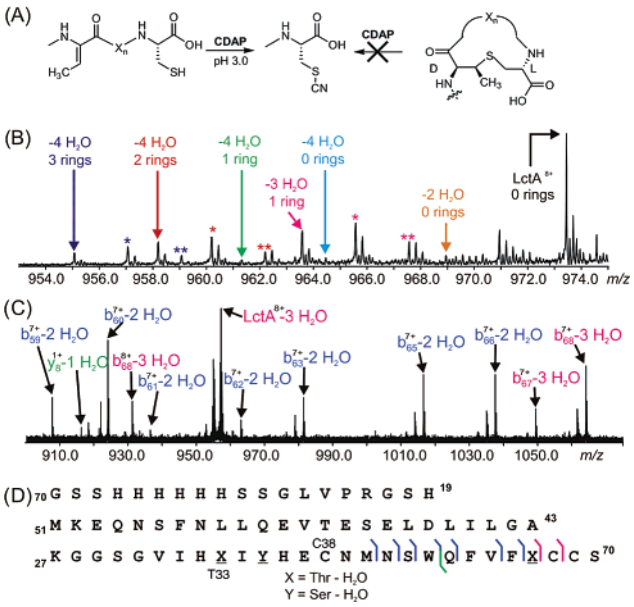

Figure 3. ESI/FT MS/MS interrogation of thioether formation in His_6-LctA during LctM catalysis. (A) Expected cyanylation products. (B) Cyanylation of the assay products after 40 min. Colored asterisks depict oxidation products, M+(O), of the identically colored parent ions. (C) MS/MS analysis of the LctA − 3 H_2O species from Figure 1B (40 min). (D) Fragment ion map of the LctA − 3 H_2O species.

to identify (Me)Lan formation. However, selective chemical derivatization of free thiols in uncyclized products permits assignment of (Me)Lan ring number by high-resolution MS (Figure 3A).[8] To count thioether rings in assay products and intermediates, assays were conducted for 20 min, 40 min, and 2 h. All species present in an assay mixture were co-purified by HPLC under acidic conditions to prevent nonenzymatic cyclization and subjected to thiol cyanylation at pH 3 with excess 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) prior to FTMS analysis.[9]

Upon cyanylation of the 40 min assay product (Figure 1B, final time point) with CDAP, the M − 4 H_2O species is a mixture of all possible ring states (Figure 3B).[10] The presence of a maximum of three uncyclized cysteines in the M − 4 H_2O species clearly indicates that each LctM-catalyzed dehydration is not coupled to a subsequent cyclization. The M − 3 H_2O species contains one ring (cyanylated twice) and the M − 2 H_2O species is cyanylated at all Cys residues (Figure 3B) and, therefore, has no (Me)Lan rings, which supports our conclusion. In contrast, the 2 h time point shows near quantitative conversion to a final product with four dehydrations and three lanthionine rings (Figures S5 and S6).

To localize the residues that underwent modification in the M − 3 H_2O and M − 2 H_2O species, they were interrogated by tandem mass spectrometry (MS/MS) using infrared photodissociation. The M − 3 H_2O ion from the 40 min time point of Figure 1B was isolated by SWIFT[11] and dissociated. The fragment ions observed in Figure 3C localized the three dehydrations in the M − 3 H_2O species to Thr33, Ser35, and Thr48 (Figure 3D), whereas Ser42 remained unmodified. Due to low ion abundance, no single major species could be deduced from the fragmentation ion pattern of the M − 2 H_2O species. The incompletely dehydrated species observed in our assays may be due to intermediates that dissociate from the enzyme,[12] or to the enzyme skipping a residue (Ser42) for dehydration, as seen in the case of the M − 3 H_2O peptide. By virtue of the abundant fragment ions from cleavage of backbone bonds in the B and C ring regions (Figure 3D, bottom right), the one lanthionine ring in the M − 3 H_2O species from the 40 min time point involves residues Thr33 and Cys38 (A-ring).

On the basis of the results presented here, we conclude that the bifunctional enzyme LctM is highly processive in its dehydrative function. Furthermore, the dehydration process precedes the cyclization to form (Me)Lan residues, as seen by the presence of an uncyclized yet completely dehydrated species in our experiments, as well as other partially cyclized products. Finally, the skipping of residues normally targeted for dehydration is observed to some extent in a putatively off-pathway activity of LctM, which accounts for the small amounts of M − 3 H_2O and possibly M − 2 H_2O species observed. Structural interrogation of the "on-pathway" intermediates trapped by rapid quench is underway to assess the directionality and regioselectivity of LctM's dehydration activity.

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Supporting Information Available: Detailed experimental procedures for substrate preparation and FTMS-based assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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