Communication

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The Leader Peptide Is Not Required for Post-Translational Modification by Lacticin 481 Synthetase

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Lantibiotics are post-translationally modified antimicrobial peptides containing multiple cyclic thioethers that constrain their conformational freedom and that are required for their potent antimicrobial activities.[1] These cross-links are installed in a two-step process that involves dehydration of Ser and Thr residues to the corresponding dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, and the subsequent conjugate addition by cysteine thiols onto the dehydro amino acids (e.g., Figure 1A for lacticin 481). The ribosomally synthesized prepeptides contain an N-terminal leader peptide that is not modified during maturation and a C-terminal structural region that is transformed into the lantibiotic. The role of the leader peptide has been the subject of much speculation. Previous studies have shown that its removal is required for biological activity.[2,3] These findings suggest that it might play a protective role for producing organisms as the leader peptide is proteolytically removed in the final step of biosynthesis. Alternatively, the leader peptide may provide a recognition sequence for either the protease or the transporter that secretes the product. Finally, the leader peptide might function as a scaffold for the biosynthetic enzymes that generate the cross-links because several recent studies have shown that lantibiotic dehydratases can process non-lantibiotic substrates attached at the C-terminus of a leader peptide.[4−6] The recent in vitro reconstitution of the activities of the biosynthetic enzymes for nisin, haloduracin, and lacticin 481[3] allows detailed investigation of the role of the leader peptide in the post-translational modifications. In this study we show that, surprisingly, the leader peptide is important but not required for dehydration by lacticin 481 synthetase (LctM).

LctM tolerates a wide range of nonproteinogenic amino acids incorporated into the substrate peptide by expressed protein ligation.[6,7] While this approach allowed investigation of the substrate specificity of LctM, scale-up to produce the amounts required for quantitative SAR-studies of lacticin 481 analogues proved difficult. We therefore investigated an alternative route to LctA analogues containing non-proteinogenic amino acids. A recent report showed that a substrate with three Ala residues inserted between the leader peptide and structural region of LctA was fully processed by LctM.[6] This observation prompted the evaluation of the non-peptidic linkers **1** and **2** between the C- and N-terminal regions of LctA. In an initial test experiment, peptide **3** was obtained via the copper(I)-catalyzed [3+2] cycloaddition[8] of peptides **4** and **5**.[9] Incubation of peptide **3** with LctM, ATP, and Mg^{2+} resulted in 4-fold dehydration (Figure 2A). Surprisingly, when peptides **4** and **5** were incubated with LctM to test enzymatic templation of the cycloaddition reaction, cycloaddition did not take place, but partially dehydrated products were observed. A similar result was obtained when a heterologously expressed, His_6-tagged LctA analogue was cleaved between Lys25 and Gly26 with endoproteinase Lys-C followed by incubation of the proteolytic fragment peptides with LctM.[9]

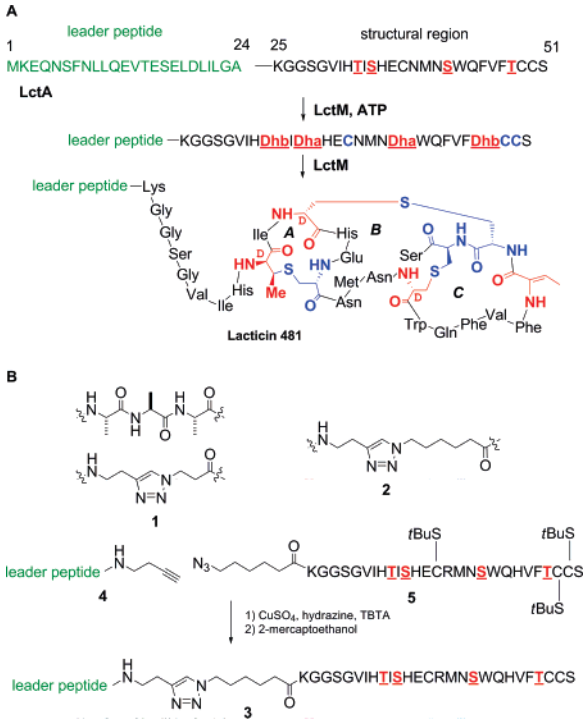

Figure 1. (A) Dehydration and cyclization of the LctA prepeptide catalyzed by lacticin 481 synthetase. (B) Preparation of a substrate analogue containing a non-peptidic linker between the leader and structural peptides.

These unexpected observations prompted the synthesis of the authentic N-terminal leader peptide and the C-terminal structural region by solid-phase peptide synthesis. Incubation of these peptides with LctM in the presence of ATP and Mg^{2+} resulted in three observed dehydrations in the structural peptide (Figure 2B) out of the four dehydrations that take place when the two peptides are linked via an amide bond in wild-type LctA. This activity of the two peptides *in trans* was significantly lower than the activity observed with full-length LctA with the major product consisting of a 3-fold dehydrated peptide after 2.5 h compared to complete, 4-fold dehydration of wild-type LctA in less than 15 min.

Previous experiments have shown that LctM dehydrates LctA in a highly processive manner,[10] but this processivity appears to be lost in the *in trans* reaction, with intermediates corresponding to one and two dehydrations present at early time points in the mass spectra (Figures 2B and S1, Supporting Information). To obtain insights into the localization of the dehydrations, the mixture of processed structural peptides was treated with cyanogen bromide, resulting in cleavage between Met40 and Asn41 (Figure S2).[11] Analysis of the resulting two fragments by MALDI-MS revealed that Thr33 and Ser35 were both dehydrated, whereas Ser42 and/or Thr48 were only partially processed (Figures S3 and S5). This

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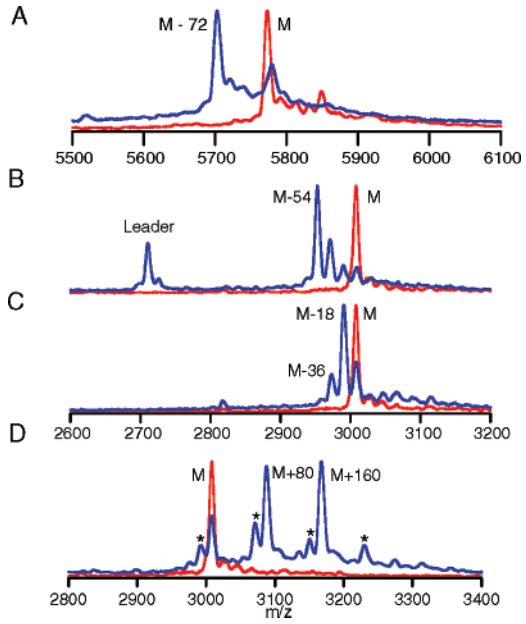

Figure 2. MALDI-MS spectra of products of incubation of LctM with ATP, Mg^{2+}, and (A) peptide **3**, (B) LctA(1−24) and LctA(25−51)N39R/F45H,[9] and (C) LctA(25−51)N39R/F45H. (D) Incubation of LctA(1−24) and LctA(25−51)N39R/F45H with LctM-R399M; only the structural peptide is shown. Asterisks represent phosphorylated peptides that have undergone one elimination.

observation suggests that the dehydration is directional in the *in trans* experiment and proceeds from N- to C-terminus. We also analyzed the cyclization activity of LctM using a recently developed protocol that allows distinction between enzymatic and nonenzymatic cyclization.[11] The results showed that LctM also catalyzes cyclization when presented with leader and structural peptide *in trans* (Figures S6−S8).

Incubation of the structural peptide with LctM in the *absence* of the leader peptide still resulted in partial processing of the structural peptide, showing that the leader peptide is *not* required for catalysis. However, the major product corresponded to just one dehydration (Figure 2C).[12] Localization of the dehydration by cyanogen bromide treatment followed by MALDI-MS analysis showed that in this case, both fragments contain about the same level of product with one dehydration (Figure S4), suggesting that the directionality is lost when the leader peptide is absent. The net dehydration reaction proceeds via initial phosphorylation of the Ser/Thr residues targeted for dehydration and a subsequent *anti* elimination step[13] with Arg399 fulfilling a critical role in the second step.[14] When R399M-LctM was incubated with the leader peptide and structural region, phosphorylated structural peptides were produced (Figure 2D).

These findings provide interesting new insights into the role of the leader peptide in the post-translational modifications of lacticin 481 biosynthesis. Clearly, the leader peptide is important to increase the dehydration activity of LctM, and it also appears to be the main reason for processivity and, possibly, directionality.

Thus far, investigations of the directionality of catalysis have not been possible because reaction intermediates with wild-type LctM have only been observed in rapid-quench studies of single-turnover experiments, which never provided the required quantities of intermediates for MS−MS analysis.[11] The generation of such intermediates in the *in trans* experiments with wild-type LctM may now allow investigation of the directionality of dehydration.

The data presented here are consistent with a model in which the leader peptide provides important binding affinity that allows the peptide to remain associated with the enzyme between successive dehydration events, resulting in directional processivity. Because dehydration is still observed in the absence of the leader peptide, a model in which binding of the leader peptide is required to activate LctM, perhaps through a conformational change, does not accommodate the observations. On the other hand, it is feasible that the active form of LctM is present in a very small equilibrium population in the absence of the leader peptide and that binding of the leader peptide results in an increase in the concentration of active LctM. Ongoing crystallographic analysis in the presence and absence of substrate may test this hypothesis.

In summary, this work shows that the leader peptide, while promoting the efficiency of dehydration, is not required for dehydration activity of lacticin 481 synthetase.

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Supporting Information Available: Figures showing mass spectra of CNBr cleavage of LctM products. This material is available free of charge via the Internet at http://pubs.acs.org.

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