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Deuterium Labeled Peptides Give Insights into the Directionality of Class III Lantibiotic Synthetase LabKC

Bartlomiej Krawczyk,‡ Paul Ensle,‡ Wolfgang M. Müller, and Roderich D. Süssmuth*

Fakultät II-Institut für Chemie, Technische Universität Berlin, Strasse des 17. Juni 124, 10623 Berlin, Germany

Supporting Information

ABSTRACT: The biosynthesis of a considerable number of ribosomally synthesized peptide antibiotics involves the modification of Ser and Thr residues of a precursor peptide. This post-translational processing is performed by one or multiple modifying enzymes encoded in the biosynthetic gene cluster. We present a deuterium-label based enzyme assay, utilizing a series of peptide substrates with α-deuterated Ser, for the determination of the dehydration order during the biosynthesis of class III lantibiotic labyrinthopeptin A2. Remarkably, the data show that, in contrast to other modifying enzymes of class I and II lantibiotics, LabKC has a C- to N-terminal processing mode. This surprising finding, which we consider relevant for the biosyntheses of other class III lantibiotics, underlines significant differences of this class of modifying enzymes compared to other investigated systems.

Lantibiotics are ribosomally synthesized peptides that undergo enzymatic post-translational modifications introducing the characteristic amino acids lanthionine (Lan) and/or methyllanthionine (MeLan).[1] The presence of these cross-linking amino acids is the unifying feature for this growing group of natural products.[2] The mechanism of Lan and MeLan formation has been extensively studied over the last 20 years both *in vitro* and *in vivo*.[2] In an initial step, Ser and/or Thr are dehydrated to yield 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), respectively. This is followed by an enzymatically catalyzed Michael type addition of a Cys thiol side chain to the double bond of Dha or Dhb to establish lanthionine (Lan) and methyllanthionine (MeLan). Because of the lack of crystal structures of lantibiotic synthetases, our knowledge of the precursor peptide processing is still limited. Therefore, studies on the directionality and processivity of lantibiotics biosynthesis contribute to improving the understanding of the modifying enzymes involved.

Previous *in vitro* investigations on microcin B17,[3] and the class II lantibiotics lacticin 481 and haloduracin A2,[4] have revealed a distributive and predominantly directional mode of the precursor peptide processing, with respect to dehydration and/or cyclization reactions, proceeding from the N- to C-terminus. Similar conclusions for the class I lantibiotic nisin A were drawn from *in vivo* investigations.[5]

In this *in vitro* study, we investigated the order of dehydration reactions during biosynthesis of the class III lantibiotic labyrinthopeptin A2. This recently described peptide, which displays remarkable activity in mouse model of neuropatic pain, contains the novel triamino triacid named labionin (Lab), which is structurally related to lanthionine (Figure 1).[6] Lab is

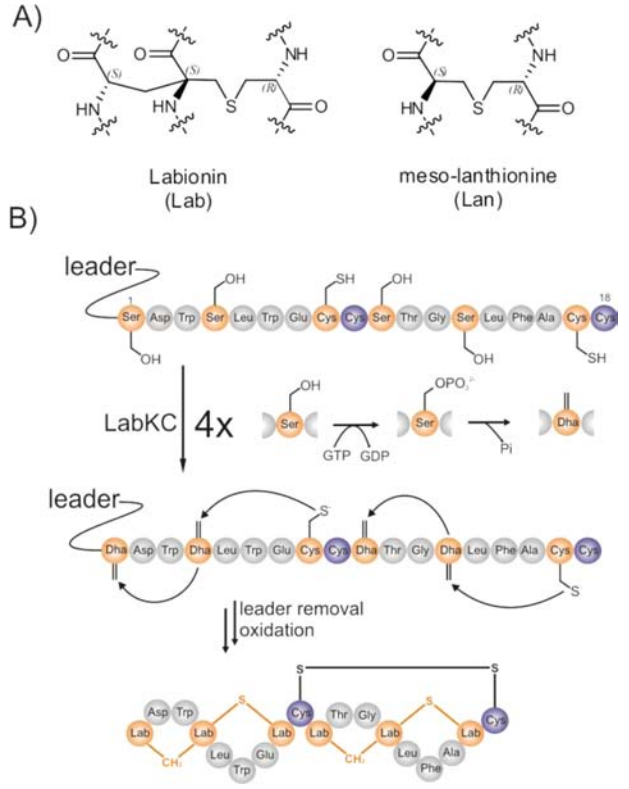

Figure 1. (A) Structures of the amino acids labionin and lanthionine. (B) Proposed model of the labyrinthopeptin A2 biosynthesis performed by the labionin synthetase LabKC.[7]

biosynthetically assembled from a characteristic Ser/Ser/Cys motif in the precursor peptide by the modifying enzyme LabKC (Figure 1).[7] This enzyme is composed of three domains: a lyase, a kinase and a putative cyclase, as identified based on homologies with known proteins from the NCBI database.[7,8]

The unique architecture of LabKC allows every catalytic activity required for Lab formation to be assigned to a specific domain. Our motivation to investigate the processing of the labyrinthopeptin A2 precursor peptide (LabA2) in detail came from previous studies which demonstrated significant differences between biosynthetic enzymes of class II and class III

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lantibiotics.[9] Formally, the modifying enzyme LabKC, which requires GTP instead of the commonly found co-substrate ATP, installs four Dha residues. The corresponding serine precursors are present in positions 1, 4, 9, and 13 of the core peptide (Figure 1).[7] The *in vitro* reconstitution of the LabKC activity with a synthetic precursor peptide revealed the presence of various intermediates during the biosynthesis with different degree of dehydration (Figure S1). The accumulation of these intermediates suggests a distributive mode of processing (i.e., dissociation of intermediates into solution during the biosynthesis) similar to other ribosomally synthesized and post-translationally modified peptides (RiPPs), for example, microcin B17[3] and lacticin 481.[4] In addition, monophosphorylated dehydration intermediates were detected (Figure S1). This observation is in agreement with a general model for Dha formation in which Ser is first phosphorylated and the subsequent phosphate elimination, accompanied by α-proton abstraction, yields Dha.[10]

To further define this mechanism for class III lantibiotics, we synthesized two truncated LabA2 variants with the leader peptide and the N-terminal nine amino acids containing phosphorylated Ser at two different positions (LabA2(1-9)^{pSer1} and LabA2(1-9)^{pSer4} see Supporting Information). Incubation of these peptides with LabKC resulted in a mass shift of −98 Da (Figure 2), corresponding to phosphate elimination and

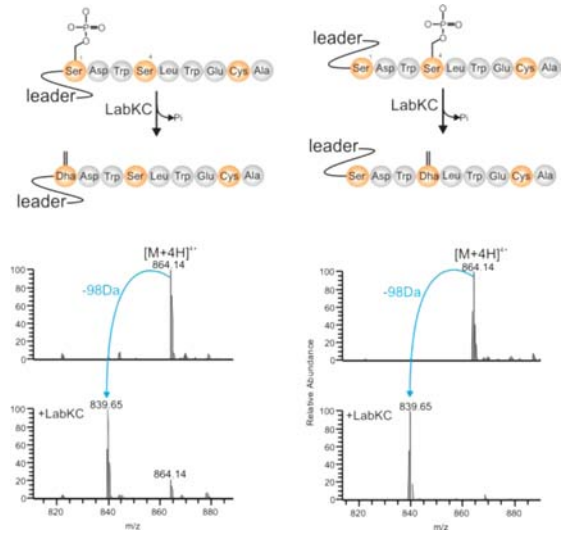

Figure 2. Elimination reaction of LabA2(1-9)^{pSer1} and LabA2(1-9)^{pSer4} peptides, catalyzed by LabKC. The corresponding ESI-MS spectra document the loss of phosphate. Positions of Dha were confirmed by MS/MS.

Dha formation. The positions of Dha residues were further confirmed by MS/MS analysis (Figures S2 and S3). In contrast to the class II enzyme LctM,[10] elimination carried out by LabKC does not require any cofactor. This result is in agreement with a reaction mechanism proposed for protein-lyase-containing enzymes, that is class III and IV lantibiotic synthetases.[8]

To investigate the directionality of the dehydration reactions, we designed four peptides each containing one replacement of Ser with α-deuterated Ser in the core peptide: LabA2^{Ser1}*, LabA2^{Ser4}*, LabA2^{Ser10}*, and LabA2^{Ser13}*. The synthesis of deuterium labeled peptides was performed in analogy to work published previously (see Supporting Information) and labeled positions were confirmed by MS/MS experiments (Figure S4). Dehydration of Ser deuterated in the α-position corresponds to the formal loss of HDO (Δm = −19.02 Da) compared to H_2O elimination (Δm = −18.01 Da) (Figure 3A). Therefore, the ability to distinguish between dehydration of labeled and unlabeled Ser should provide information on the order in which labeled residues are processed. To assess this approach, an enzymatic assay was performed with LabKC and above-mentioned LabA2 precursor peptides, according to protocols described previously (see Supporting Information). A reaction time of ∼5 min was sufficient to detect all the dehydration intermediates (Figure S1). Figure 3C shows the high resolution mass spectra of processed peptides (LabA2^{Ser1}*, LabA2^{Ser4}*, LabA2^{Ser10}*, and LabA2^{Ser13}*) after 6 min of incubation with the enzyme LabKC and GTP. Dehydration intermediates resulting from HDO elimination were identified for all tested peptides based on the most abundant masses in isotopic distributions. The formal elimination of HDO for a singly dehydrated intermediate was observed for the LabA2^{Ser13}* peptide indicating that Ser13 is the first residue dehydrated by LabKC (Figure 3C). In a similar manner, the sequence of the other dehydrations was assigned. Hence, our data indicate a directional processing of the precursor peptide from the C- to N-terminus, with the following order of dehydration: Ser13 → Ser10 → Ser4 → Ser1. The same order was observed when analyzing phosphorylated intermediates (Figure 3C, right-hand side). Detailed inspection of isotopic distributions revealed however some exceptions.

The mass spectra of peptides LabA2^{Ser4}* and LabA2^{Ser10}* also display partial HDO elimination for the singly dehydrated intermediates as judged from the comparison of observed and simulated isotopic distributions (Figures S5−S7). This clearly indicates the presence of intermediates with the first dehydration event taking place at positions 4 or 10. Thus, although the major route for dehydration is Ser13 → Ser10 → Ser4 → Ser1, there is also a minor fraction of peptides which can follow an alternative processing. This observed divergence from the main pathway argues for some flexibility in the enzyme-catalyzed processing. Finally, the directionality of processing was confirmed by assigning fragments from MS/MS analysis of dehydrated intermediates (Figures S8−S10).

Our observation on the directionality is in contrast to previous reports on class I and II lantibiotics, where a predominant N- to C-terminal processing was observed for the dehydration reaction.[4,5] In this context, it is interesting to note that an N- to C-terminus processing was also observed for microcin B17 from another class of ribosomally synthesized peptide antibiotics. Moreover, similar to our data, indicating a fraction of peptides following an alternative processing scheme, Kelleher et al. reported on the presence of parallel processing pathways for the microcin B17 biosynthesis.[3] Thus, although an N- to C-terminal mode of processing seems to be widespread among RiPPs and particularly among lantibiotics, our results clearly show that an alternative C- to N-terminal processing is also occurring. Most recently, a report on the biosynthesis of novel thiazole/oxazole modified microcins from *Bacillus* sp. Al Hakam was published where the authors also observed a C- to N-terminal processing.[11] Together with our results, these are the first reports on this mode of processing for RiPPs.

In summary, we have shown that the dehydration reaction carried out by LabKC, the only so far investigated class III lantibiotic synthetase, follows a mechanism of a repetitive phosphorylation, then elimination. Hence, the processing of the

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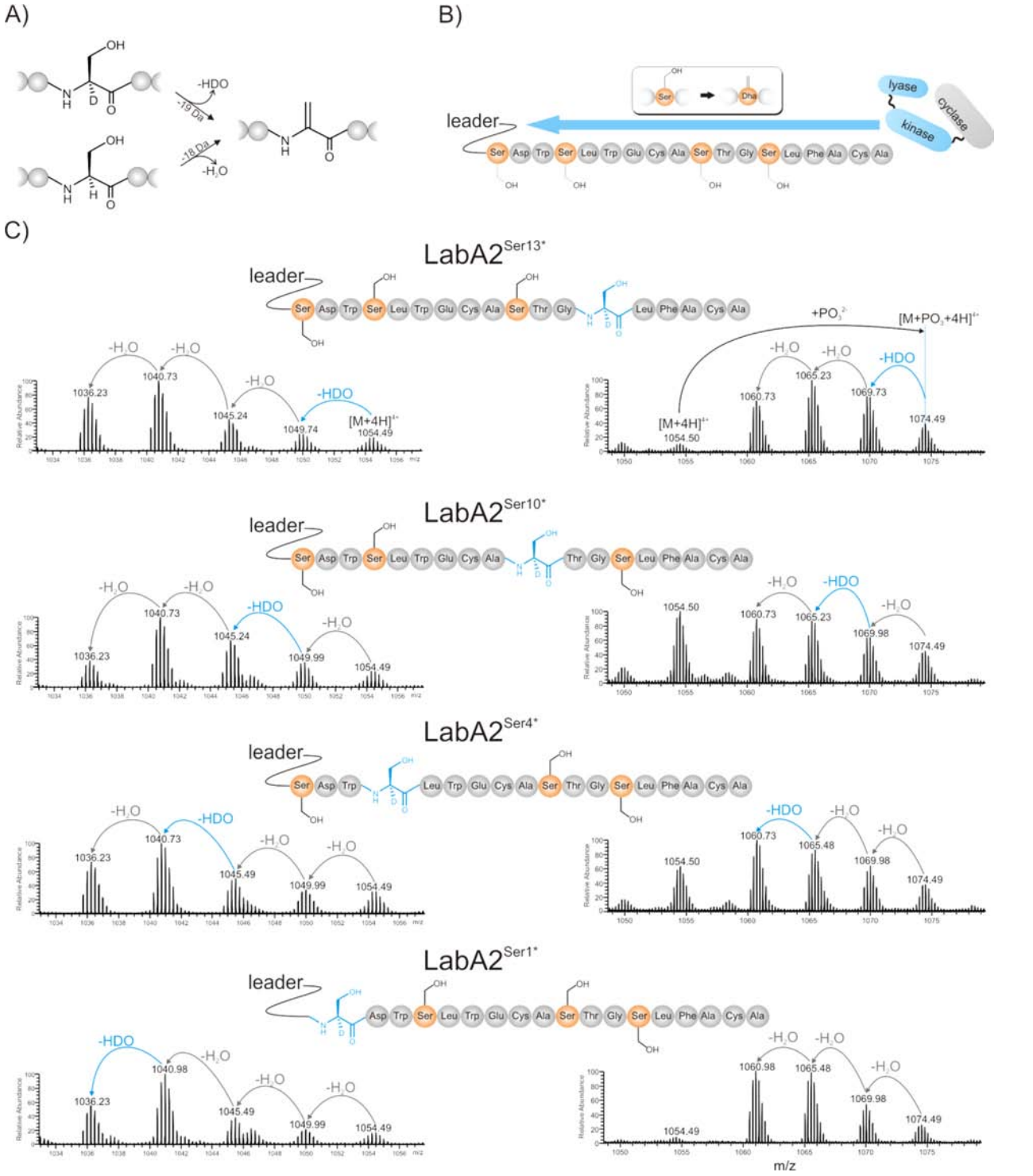

Figure 3. The order of dehydration reactions in labyrinthopeptin A2 biosynthesis. (A) Concept of the method used to assign the order of dehydration reactions: dehydrations of deuterated and nondeuterated Ser are distinguished by the formal elimination of HDO (Δm = −19.02 Da). (B) Proposed order of dehydration reactions catalyzed by the tridomain class III enzyme LabKC. (C) HPLC−ESI−MS spectra of dehydration intermediates (left) and monophosphorylated dehydration intermediates (right) of synthetic $LabA2_{Ser1*}$, $LabA2_{Ser4*}$, $LabA2_{Ser10*}$, and $LabA2_{Ser13*}$.

precursor peptide is directional but, surprisingly, proceeds from the C- to N-terminus. This finding underlines significant differences in the biosynthesis of class III lantibiotics as compared to class I and II. Previously, methods for determination of enzyme directionality have been based on ring protection phenomena of nisin-producing mutants[5] or MS/MS analysis of peptide intermediates (microcin,[3] lacticin,[4] and haloduracin[4]). The introduction of a new method based on α-deuterated Ser allows for a straightforward and clear assignment of the order of dehydration reactions in labyrinthopeptin A2 biosynthesis. Moreover, we believe that the presented labeling method will also prove useful for the biosynthesis of other RiPPs which involve proton abstraction in post-translational modifications.

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■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and mass spectrometric data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

suessmuth@chem.tu-berlin.de

Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

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