Identification of the Catalytic Residues in the Cyclase Domain of the Class IV Lanthipeptide Synthetase SgbL

Julian D. Hegemann*[a] and Roderich D. Süssmuth*[a]

Lanthipeptides belong to the family of ribosomally synthesized and post-translationally modified peptides (RiPPs) and are subdivided into different classes based on their processing enzymes. The three-domain class IV lanthipeptide synthetases (LanL enzymes) consist of N-terminal lyase, central kinase, and C-terminal cyclase domains. While the catalytic residues of the kinase domains (mediating ATP-dependent Ser/Thr phosphorylations) and the lyase domains (carrying out subsequent phosphoserine/phosphothreonine (pSer/pThr) eliminations to yield dehydroalanine/dehydrobutyrine (Dha/Dhb) residues) have been characterized previously, such studies are missing for LanL cyclase domains. To close this gap of knowledge, this study reports on the identification and validation of the catalytic residues in the cyclase domain of the class IV lanthipeptide synthetase SgbL, which facilitate the nucleophilic attacks by Cys thiols on Dha/Dhb residues for the formation of β-thioether crosslinks.

Representatives of the RiPP natural product family are defined through a shared biosynthetic logic, where genetically encoded precursor peptides are matured into the final natural products by the activity of processing enzymes.[1]

Lanthipeptides are members of the RiPP family that contain characteristic β-thioether crosslinks; so-called (methyl) lanthionine ((Me)Lan) residues.[1] These crosslinks are installed by: 1) activation/elimination of Ser/Thr hydroxy groups to yield Dha/Dhb residues, and 2) nucleophilic attacks on these unsaturated double bonds by Cys thiolates to yield, after an additional protonation step, the (Me)Lan moieties.

The lanthipeptide precursors contain N-terminal leader and C-terminal core peptide regions.[1] The leader regions feature conserved motifs needed for substrate recognition by the processing enzymes.[1–2] The core peptide regions are those where the dehydroalanine/dehydrobutyrine (Dha/Dhb) residues are intermediately formed and (sometimes only partially) converted into the (Me)Lan crosslinks (Figure 1). After full modification of the core peptide, the mature lanthipeptide is released via proteolytic removal of the leader and subsequently often exported into the extracellular space.[1–3]

Lanthipeptides can be further subclassified on the basis of their corresponding biosynthetic enzymes and there are currently five different classes known.[1,4] Class IV lanthipeptide synthetases, so-called LanL enzymes, feature a three-domain architecture consisting of a lyase, a kinase, and a cyclase domain (Figure 1A).[1,3a,4e,5] LanLs accomplish the generation of the Dha/Dhb residues through the concerted action of the lyase and kinase domains. First, the central kinase domain binds the precursor and facilitates the ATP-dependent Ser/Thr phosphorylation.[1,2,6] Then, the N-terminal lyase domain catalyzes the phosphate elimination from the pSer/pThr residues, yielding the Dha/Dhb residues[1,7] (Figure 1B). Finally, the C-terminal cyclase domain mediates the nucleophilic attack of the Dha/Dhb residues by Cys thiolates and facilitates the subse-

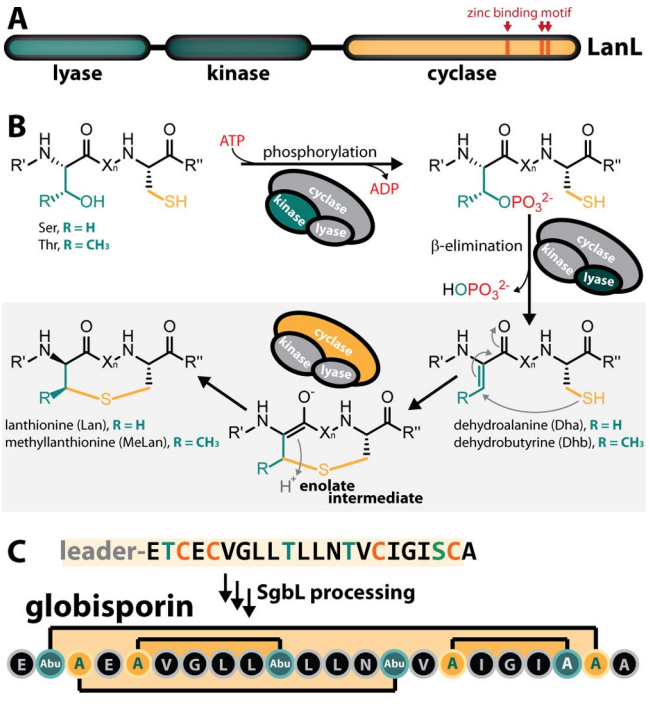

Figure 1. (A) Schematic representation of the domain organization in class IV lanthipeptide synthetases. (B) (Me)Lan formation by class IV lanthipeptide synthetases. The domain active during a specific step is shown in color, while the domains not participating in this step of catalysis are shown in grey. (C) Schematic representation of the class IV lanthipeptide globisporin; Abu = aminobutyric acid.

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202100391

quent protonation of the resulting enolates to yield the (Me)Lan crosslinks (Figure 1B).[1,2b,4e,6]

In our recent review[1b] on class III and IV lanthipeptide synthetases, we noted that whereas the catalytic residues of the lyase[7] and kinase[6] domains of class IV lanthipeptide synthetases have been reported, the catalytic residues of the LanL cyclase domains still remained uncharacterized. Due to the homology between the cyclases of class I, II, and IV lanthipeptide synthetases, we further hypothesized that class IV cyclase domains would feature the same conserved catalytic residues that were previously reported[1b,c,8] for class I and II cyclases.

Indeed, an alignment of well-studied class I[1c,8a–d] and II cyclases[1c,8b,e] with the cyclase domain of the previously characterized class IV lanthipeptide synthetase SgbL[2b,6] as well as with the cyclase domains of a selection of other reported class IV enzymes[2b,3a,4e,5] (Figure 2A) shows the full conservation of the putative catalytic residues. To experimentally validate the role of these residues in (Me)Lan formation, we performed a mutational analysis of the SgbL enzyme that catalyzes the core peptide modification during the biosynthesis of the class IV lanthipeptide globisporin (Figure 1C).[2b,6]

By mutation of the *his$_6$-sgbL* gene in an expression vector, heterologous expression in *E. coli*, and *in vitro* reconstitution, the five predicted catalytic residues were exchanged to Ala. According to bioinformatic analysis, three of these residues (Cys769, Cys814, His815) would be coordinating a zinc ion that would act as a Lewis acid to increase the nucleophilicity of the Cys thiols in the SgbA core region (Figure 2B).[1b,c,8a–d] In addition, His710 would fulfill the role of the catalytic acid that protonates the enolate intermediates and that is further activated by interaction with Asp642 (Figure 2B).[1b,c,8a–d]

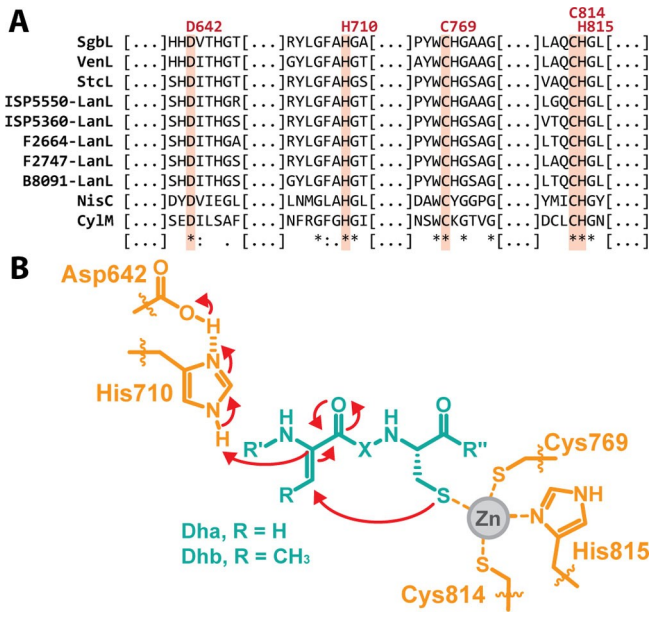

Figure 2. (A) Excerpt of the alignment of a selection of known functional LanL enzymes[2b,4e,5] with the representative cyclases of class I (NisC[8a]) and class II (CylM[8e]) lanthipeptide synthetases. The red labels above the alignment refer to the corresponding predicted catalytic residues in SgbL. (B) Putative catalytic mechanism of the SgbL cyclase-mediated (Me)Lan formation.

To assess if the generated SgbL variants (SgbL(D642A), SgbL(H710A), SgbL(C769A), SgbL(C814A), SgbL(H815A)) are indeed unable to catalyze the (Me)Lan formation, a series of *in vitro* assays were performed using the SgbL WT enzyme as a positive control. However, whereas Ser/Thr dehydrations are easily observable by mass spectrometry (MS) due to the loss of a water molecule (−18 Da), the (Me)Lan formation is mass neutral. Hence, the lack of (Me)Lan formation can only be detected indirectly by labeling of the free Cys residues present. This labeling can be accomplished by addition of N-ethylmaleimide (NEM), a thiol-selective electrophile.[2b,6,9] Whenever a free thiol adds to an NEM molecule, the mass of the compound increases by 125 Da, which can be readily tracked by MS.

Another important aspect to consider for these assays is that the steric hindrance imposed by the methyl groups in Thr-derived Dhb side chains efficiently suppresses non-enzymatic cyclizations to occur, while the less sterically hindered unsaturated double bonds in Dha residues can be more readily attacked by Cys thiol nucleophiles in the absence of an enzyme.[2b] Thus, a variant of the precursor peptide SgbA was heterologously produced, where the only ring-forming Ser in the core region was replaced with a Thr (His$_6$-SgbA(S20T), Figure 3A) to suppress the background of non-enzymatic Lan formation in our assays.

Accordingly, the His$_6$-SgbA(S20T) precursor variant was incubated overnight under assay conditions either by itself (negative control), with SgbL (positive control), or with one of the five SbgL variants (Figure 3B).

On the next day, the reaction mixtures were digested with trypsin to facilitate the MS analysis of the modified SgbA(S20T) core peptides.[2b] The samples were then split to enable the comparison of the mass spectra before and after NEM treatment. Hence, one part of each sample was directly desalted and applied to MS, while the other part was first treated with NEM before desalting and MS analysis (Figure 3B).

Indeed, all SgbL variants introduced up to four dehydrations into the His$_6$-SgbA(S20T) precursor peptide as did the WT enzyme (Figure 4), which was expected as it was previously[2b,4e,6] shown that the LanL lyase/kinase domains can incorporate the dehydrations independently from the cyclase domain.

However, when comparing the results of the NEM assays, it became apparent how the Ala exchanges affected the ability of SgbL to introduce the MeLan crosslinks into His$_6$-SgbA(S20T). The assay with WT SgbL yielded a significant amount of fully-cyclized core peptide that did not add any NEM. In contrast, none of the SgbL variants were able to install all four MeLan crosslinks into the precursor peptide and the major products of all the SgbL variant assays always had four NEM molecules added. Thus, these experiments clearly demonstrate that the Ala exchange of any of the five catalytic residues predicted through homology analysis (almost) completely abrogates the ability of SgbL to introduce β-thioether crosslinks into His$_6$-SgbA(S20T).

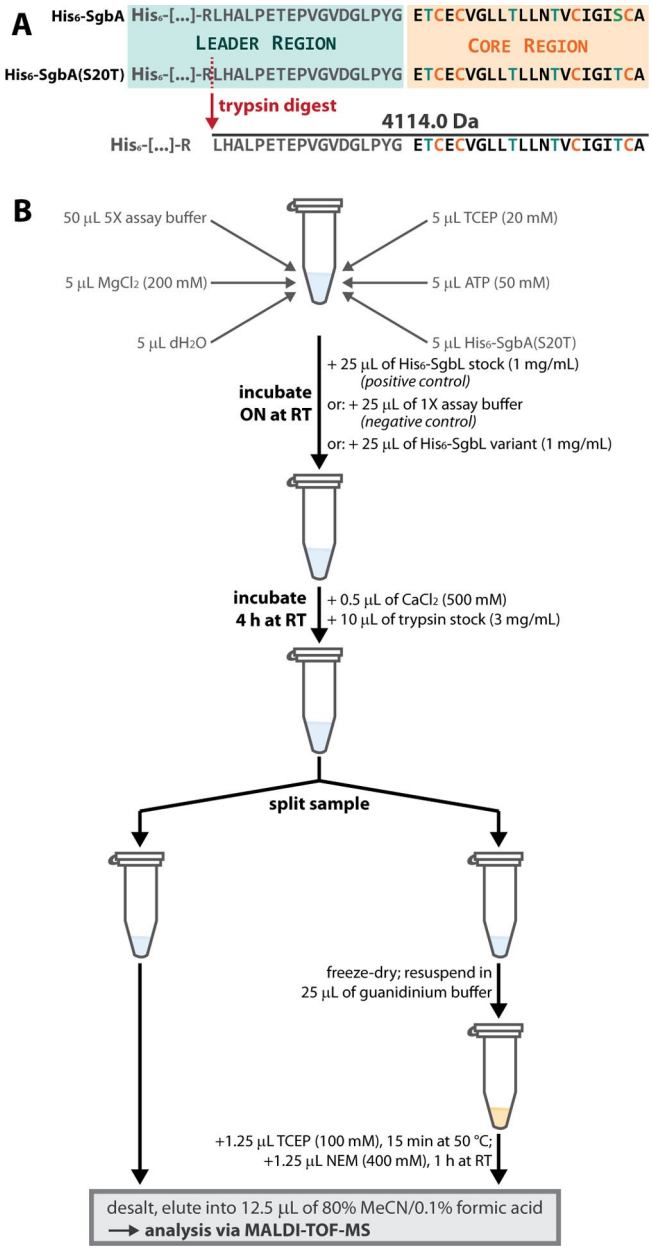

Figure 3. (A) An S20T exchange in the SgbA precursor peptide yields a core peptide that can only form MeLan crosslinks. (B) Schematic representation of the general workflow of the *in vitro* modification assays.

In conclusion, this study provides the first experimental validation of the function of the predicted[1b] conserved catalytic residues in the cyclase domain of a class IV lanthipeptide synthetase. Adding to previous studies that were focused on identifying and characterizing the catalytic residues situated in the kinase[6] and lyase[7] domains of LanL enzymes, these experiments therefore lead to a more complete understanding of the underlying principles of class IV lanthipeptide biosynthesis.

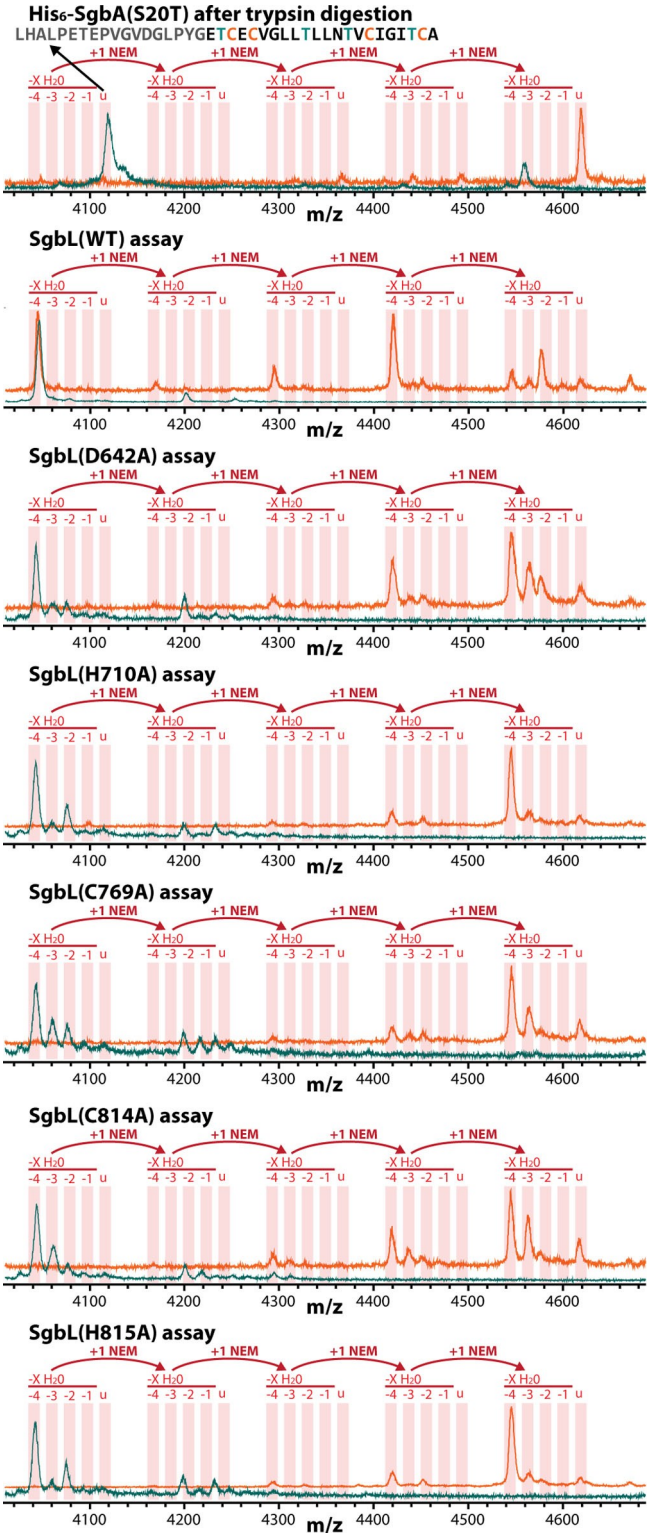

Figure 4. Results of the *in vitro* modification assays of His_6-SgbA(S20T) with SgbL and variants thereof. Traces in teal and orange show the MS data before and after NEM labeling, respectively. For the negative control shown on top, the precursor was incubated overnight under assay conditions in the absence of any modification enzyme. The MS signals shown were obtained by MALDI-TOF-MS analysis of the samples after desalting and using sinapic acid as MALDI matrix; u = unmodified peptide.

Acknowledgements

We would like to thank Dr. Chris Weise (Freie Universität Berlin) for assistance with the MALDI-TOF-MS measurements. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: biocatalysis · biosynthesis · lanthipeptides · natural products · RiPPs

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Manuscript received: August 3, 2021
Revised manuscript received: September 6, 2021
Accepted manuscript online: September 7, 2021
Version of record online: September 12, 2021