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Characterization of Leader Peptide Binding During Catalysis by the Nisin Dehydratase NisB

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Abstract

The dehydratase NisB performs stepwise tRNA^{Glu}-dependent glutamylation of Ser/Thr residues and subsequent glutamate elimination to effect eight dehydrations in the biosynthesis of the antibacterial peptide nisin. Its substrate, NisA, bears a C-terminal core peptide that is modified and an N-terminal leader peptide (LP) that is not modified but that is required for efficient dehydration. To elucidate the mechanism of LP-NisB interactions during dehydration, we engineered a disulfide that covalently links the NisA LP to NisB. The enzyme fully dehydrated tethered NisA, confirming the functional LP binding site and supporting a mechanism where NisB uses a single LP binding site for glutamylation and elimination. We also show an order of NisA and tRNA^{Glu} binding to NisB that enables dehydration.

Natural products that can be readily diversified are attractive frameworks for engineering new therapeutics. Ribosomally synthesized and post-translationally modified peptides (RiPPs) encompass more than 25 compound families.¹ For most RiPPs, the precursor peptides contain a C-terminal core peptide (CP) that is post-translationally modified by the biosynthetic enzymes, and an N-terminal leader peptide (LP) that is required for efficient enzymatic modification of the CP but that does not itself undergo post-translational modification (PTM).^{2,3} The LP is ultimately proteolytically removed. For many RiPPs, the PTM machinery will function on an altered or even completely distinct CP as long as the appropriate LP is present.^{4–19} Because changes to the CP are readily introduced at the genetic level, RiPPs and their enzymatic machinery are finding increasing use for engineering applications.

As RiPP biosynthesis relies on enzyme recognition of the LP, an understanding of how this recognition occurs is beneficial to take full advantage of the engineering opportunities. Recent crystallographic^{20,21} and bioinformatic analyses²² have demonstrated a conserved

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Supporting Information

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Notes

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LP-binding motif in many biosynthetic enzymes, but mechanistic insights of how LPs interact with the biosynthetic machinery during the PTM process are largely missing. In this study, we describe our investigation into LP binding for NisB, the dehydratase in the biosynthetic pathway of nisin, a commercially used food preservative belonging to the lanthipeptide family of RiPPs.^{24,25} The PTMs in nisin include NisB-catalyzed conversion of eight Ser and Thr residues in the CP of NisA to dehydroalanine (Dha) and 2,3-*Z*-dehydrobutyrine (Dhb) residues, respectively (Figure 1A). This process occurs via glutamyl-tRNA^{Glu} dependent glutamylation of Ser/Thr followed by elimination (Figure 1B).²⁰ NisC catalyzes the 1,4-conjugate addition of five Cys residues in NisA to select Dha and Dhb to give the thioether cross-links that are characteristic of lanthipeptides.

Two main mechanistic models for LP binding during the PTM process have been proposed. One model suggests that the LP translocates to enable distal modification, and is supported by NisB-catalyzed dehydration of Ser/Thr in NisA that can be located as far as 47 residues from the LP (Scheme 1, Model 1).^{23,26} A second model suggests that the LP ensures the core peptide is appropriately positioned in the active site, but that it does not translocate during catalysis (Scheme 1, Model 2).

We set out to engineer a system that could distinguish between these two models. We envisioned covalent cross-linking of NisB to unmodified NisA through its LP. Efficient modification (or not) of this covalent complex would test the LP translocation mechanism (Model 1). We selected NisB for these studies because NisB has been reconstituted *in vitro* and can fully dehydrate NisA in the absence of NisC;²⁰ this independent activity is not general to lanthipeptide enzymes.²⁷ Additionally, a cocrystal structure of NisA and NisB provides critical information on where to generate a covalent cross-link.²⁰ NisB is a homodimer and contains separate glutamylation and elimination domains. In the cocrystal structure the LP binds to the glutamylation domain. However, previous studies could not determine whether the LP occupies different binding sites to facilitate glutamylation and elimination (Scheme 1, Model 3) or remains fixed while the core peptide moves between the glutamylation and elimination sites (Model 2). Our intended covalently locked complex could distinguish between these models and test if the cocrystal structure depicts a functionally important engagement of NisA and NisB.

On the basis of the NisA-NisB complex structure, we engineered an intermolecular disulfide by mutating Ser-12 in the LP of NisA and Val169 of NisB to Cys (Figure 2A). We generated the NisA mutant bearing a Twin-Strep-tag (TST)²⁸ and the NisB mutant with a hexahistidine tag (His₆), such that the desired disulfide complex could be purified from free TST-NisA using immobilized nickel resin. All naturally occurring Cys residues in the core peptide of NisA were mutated to Ala to avoid competing disulfide formation that could interfere with substrate binding and dehydration, giving our desired substrate termed TST-NisA-S-12C_{noCysCP}. This peptide was processed efficiently by wild-type NisB and wild-type NisA was processed efficiently by His₆-NisB-V169C (Figure S1). Thus, removing all native Cys from NisA and installing a Cys at proximal sites on the NisA and NisB is well tolerated. Incubation of TST-NisA-S-12C_{noCysCP} with His₆-NisB-V169C *in vitro* under ambient air afforded the desired intermolecular disulfide as detected by SDS-PAGE (Figure 2B) or Western blotting using a Strep-tag antibody (Figure S2A). Importantly, disulfide formation

was not detected in control experiments with wild-type NisB (Figure 2B), demonstrating that the disulfide forms regioselectively via the engineered Cys residues. Nickel affinity chromatography successfully removed non-cross-linked NisA as determined by MS and SDS-PAGE (Figure S3).

Based on the intensity of the SDS-PAGE bands, it appears that approximately 1.2 NisA is tethered per NisB dimer (Figure S2B). Importantly, dithiothreitol (DTT) was able to reduce the disulfide (Figure 2C).

We next tested whether NisB would modify the tethered NisA. The covalent TST-NisA-S-12C_{noCysCP}-His₆-NisB-V169C complex was incubated in a 1:2 ratio with *in situ* generated glutamyl-tRNA^{Glu}. After 1.5 h, the reaction was quenched with RNase A, followed by reduction of the disulfide with DTT (Figure S4) and analysis of the released NisA by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Eight- and nine-fold dehydrated TST-NisA-S-12C_{noCysCP} containing one equivalent of DTT were detected as the major species with each dehydration evidenced by a decrease in mass of 18 Da and with a +154 Da mass shift for DTT adduct formation (Figure 3B). The DTT adducts presumably form by addition to a dehydro amino acid. The presence of the DTT adduct on each peptide was confirmed by treatment of the modified NisA with endoproteinase Arg-C to remove the LP and obtain higher resolution MS data (Figure 3B, inset). Attempts to prevent adduct formation by using alternative reductants were not successful (Supporting Information). When un-cross-linked TST-NisA-S-12C_{noCysCP} was dehydrated *in vitro* by wild-type NisB and then treated with DTT, again a single adduct was observed, demonstrating that this reactivity is independent of the disulfide-linked complex. Tandem MS showed that the adduct formed at Dha5 (Figure S5).

The complete dehydration observed for the covalent NisA-NisB complex shows that the peptide does not need to translocate for full activity. These observations argue against Model 1 and support Model 2 where the LP binds at a single site. Our results also demonstrate that the LP binding site in the cocrystal structure is functional for catalysis. Furthermore, the data show that association of tRNA^{Glu} to NisB does not need to occur prior to binding of NisA for modification to proceed and can occur once NisA is bound to NisB.

We next decreased the extent of modification by adding less tRNA^{Glu} (Figures 3 and S6–S8). Thus, early intermediates ranging from 1- to 4-fold dehydrated NisA were detected (Figure 3C,D). Importantly, we did not observe glutamylated NisA in these intermediates, which demonstrates that the LP requires only a single binding site on NisB to enable both glutamylation and Glu elimination; in other words, the LP does not require dissociation and reassociation with a different binding mode for elimination. Thus, Model 3 is not supported.

To compare the tethered system with dehydration of noncovalently bound NisA, we investigated the directionality of dehydration, since previous studies have shown that manipulating lanthipeptide biosynthetic systems can drastically alter directionality.²⁹ *In vitro*, wild-type NisB first dehydrates Thr2 in NisA and the second and third dehydrations occur at various N-terminal residues (Ser3, Ser5, Thr8) and Thr23, respectively.³⁰ In agreement with the wild-type system, we found through MS/MS analysis of partially

dehydrated tethered NisA that the one-fold dehydrated intermediate exists as a single species containing Dhb2 (Figures 4 and S9–S11).

Analysis of the 3-fold dehydrated intermediates formed in tethered NisA showed additional dehydration of Thr8, Thr13, and Thr23 (Figures S12–S14), consistent with a preference for dehydration of Thr over Ser residues.³¹ Thus, both covalent and noncovalent binding of NisA results in a mostly N-to-C directional dehydration process, but small differences do exist. Such changes are not surprising given that our core peptide, which lacks all native cysteines, likely does not exactly recapitulate the native energy landscape for dehydration.

In conclusion, by covalently tethering NisA to its dehydratase NisB this study demonstrates that the LP does not need to translocate during dehydration, that the crystallographically observed binding site can support both glutamylation and glutamate elimination, and that NisA likely binds deeper into the glutamylation pocket than glutamyl-tRNA. Because our strategy required removing core peptide cysteines from NisA, it is not amenable to studying lanthipeptide cyclases such as NisC. Future work will be focused on devising and implementing strategies for understanding leader peptide recognition by such cyclases and other RiPP biosynthetic enzymes to determine the generality of the findings with NisB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Biosynthesis of the lanthipeptide nisin. The figure shows complete dehydration by NisB and then cyclization by NisC for clarity, but it is more likely that the two enzymes alternate.

²³ (B) Mechanism of NisB. Abu:α-aminobutyric acid.

Figure 2.

(A) Modeling of the engineered disulfide complex based on the cocrystal structure.²⁰ (B,C) SDS-PAGE analysis; (B) disulfide formation of TST-NisA-S-12C_{noCysCP} with His₆-NisB-V169C (lane 3) but not wild-type His₆-NisB (lane 4); (C) disulfide complex purified using nickel resin and treated with or without DTT.

Figure 3.

MALDI-TOF MS analysis of the TST-NisA-S-12C_{noCysCP}-His₆-NisB-V169C disulfide complex treated with varying ratios of *in situ* prepared glutamyl-tRNA^{Glu}, quenched at different time points with RNase A, and then treated with DTT. Assays performed (A) in the absence of tRNA; (B) with 2:1 tRNA^{Glu}:disulfide complex, after 1.5 h; (C) with 1:2 tRNA^{Glu}:disulfide complex, after 15 min; (D) with 1:2 tRNA^{Glu}:disulfide complex, after 30 min. Inset in panel B: (top) ArgC treatment of the assay from (B) and (bottom) ArgC treatment of unmodified TST-NisA-S-12C_{noCysCP}. M: unmodified NisA. The numbers 1–9 represent 1- to 9-fold dehydrated NisA.

Figure 4. Tandem MS analysis after reduction of the singly dehydrated TST-NisA-S-12C_{noCys}CP-His₆-NisB-V169C disulfide-linked complex. Key b and y ions are labeled.

Scheme 1.

Mechanistic Possibilities for NisA–NisB Interactions During Catalysis^a

^aOnly one monomer of the NisB dimer²⁰ is shown. NisB is in blue and the LP and CP are black and pink, respectively.