When the Leader Gets Loose: In Vivo Biosynthesis of a Leaderless Prenisin Is Stimulated by a trans-Acting Leader Peptide

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The nisin leader is believed to be crucial for nisin biosynthesis. Here, by using a construct completely lacking the leader peptide, we show that an up to fivefold-dehydrated leaderless prenisin can be obtained, as judged by MALDI-TOF MS, and that some of these species are biologically active, thus suggesting that at least three lanthionine rings are present. Notably, by expressing the leader peptide in trans together with the leaderless prenisin, we were able to increase the dehydration/cyclization efficiency of both NisB and NisC, but still with limited efficiency until the fifth dehydratable residue (Thr13) was processed, thereby enabling three rings to form. This, for the first time, demonstrates that 1) the leader is not absolutely necessary for the dehydration reaction of class I lantibiotics to occur in vivo; 2) the leader acts in trans in vivo; 3) the leader increases the efficiency of modification. Based on previous work and our current study, a model for the interactions of NisB and NisC with prenisin is proposed, in which the leader induces a more conformational and/or productive complex formation of the biosynthetic machinery, and, when covalently bound, is involved in increasing the efficiency of dehydration to the C-terminal end of the prenisin substrate molecule.

Introduction

One of the major challenges in the 21st century is to combat the increasing spread of multidrug-resistant bacteria. Promising candidates to tackle this problem are lantibiotics, which form a class of natural polycyclic agents with high antimicrobial activity. They are ribosomally synthesized and post-translationally modified peptides with unique structures, that is, dehydrated amino acids and lanthionine rings. The nisA gene encodes prenisin, the precursor peptide that consists of an N-terminal leader sequence and a C-terminal modifiable propeptide part. Dehydrated amino acids are introduced enzymatically by NisB-mediated dehydration of Ser and Thr residues in the modifiable propeptide part of prenisin, to dehydroalanines and dehydrobutyrines, respectively.[1] (Figure 1). Subsequently, Cys residues are coupled to some of the dehydro residues by the cyclase NisC, and thus lanthionine rings are formed. A dedicated transporter, NisT, transports modified prenisin out of the cell, and a protease, NisP, cleaves off the N-terminal 23 amino acid leader peptide to release the 34 amino acid bioactive nisin.[2] Modified nisin can also be structurally divided into an N-terminal part (rings A, B, C) and a C-terminal part (inter-twined rings D and E; Figure 1D). Both the N-terminal and the C-terminal parts are quite rigid and are connected by a flexible hinge region. To exert full antimicrobial activity all five (methyl)lanthionine rings must be present in nisin.[3] Analysis of truncated nisin variants, obtained by proteolytic cleavage, has shown that the presence of at least the three N-terminal rings (ABC) is necessary for nisin variants to exert some antimicrobial activity.[4] The nisin leader is believed to be crucial for nisin biosynthesis, and several functions have been proposed for the leader sequence: 1) it is required for keeping the peptide biologically inactive;[4,5] 2) it is required for recognizing the trans-acting nisB gene product;[5] and 3) it is an important site of interaction between prenisin and the modification enzymes NisB and NisC.[6] Several recent studies have shown that when the leader peptide is followed by dehydratable residues of non-nisin origin, they too can be modified.[6–8] The importance of the FNLD region (–18 to –15) for functionality of the leader has been described in several papers[5, 9] and, specifically, it has been shown to play a role in the initial recognition and binding of prenisin to NisB.[9] In a recent study, NisB and NisC were isolated in a relatively stable complex of nisin modification enzymes with a special prenisin mutant NisA-H6 (NisA followed by the C-terminal extension GSIEGR HHHHH, which appears to stabilize the interactions).[10] It was demonstrated that prenisin has higher affinity to NisB than to NisC has.

Based on the leader sequence and the number of modification enzymes, lantibiotics can be divided into two groups: class I and class II.[11] Nisin is a class I lantibiotic as it possesses two modification enzymes, NisB and NisC, as well as a class I-like leader peptide that is rich in aspartates and contains the highly conserved FNLD motif and the typical PR cleavage sequence.[12] Characteristic of class II lantibiotics, one bifunctional enzyme, LanM, performs both the dehydration and the cyclization reactions, and the class II leader, which is also rich in as...
partates, has a characteristic double glycine cleavage site.\textsuperscript{[13]} LanB/LanC and LanM enzymes recognize a wide variety of substrates; this suggests that lantibiotic modification enzymes have relaxed substrate specificity. Moreover, the class II enzyme LctM has been demonstrated to tolerate nonproteinogenic amino acids for incorporation into the propeptide in vitro.\textsuperscript{[14, 15]} Furthermore, for class II enzymes, the possibility to perform in vitro experiments has been exploited extensively, and it has been shown that LctM can modify a substrate when the leader and the propeptide parts are separated by three alanines.\textsuperscript{[14]} LctM was also able to modify a substrate with a nonproteinogenic amino acid incorporated into the leader sequence.\textsuperscript{[16]} The follow-up to this experiment was probing a construct lacking the leader, and surprisingly, in vitro incubation of this construct with purified LctM in the presence of ATP and Mg\textsuperscript{2+} resulted in three out of four possible modifications.\textsuperscript{[16]} This study indicated that the presence of the leader is not an absolute prerequisite for the dehydrations in class II enzymes in vitro. However, in vitro studies can suffer from artificial conditions, such as high concentrations of enzyme, substrate, and cofactors, and thus possess a risk of being not fully indicative of in vivo processes.

In vivo investigations into the effect of the absence of the leader on lantibiotic biosynthesis have not been performed. For class I lantibiotics the leader has been assumed to be crucial for the biosynthesis.\textsuperscript{[5]} Here, we demonstrate that the class I nisin modification enzymes NisB and NisC can partially modify a leaderless prenisin variant in vivo. Our results demonstrate that without the leader, dehydrations still happen in vivo, though to a much lesser extent, and that these dehydrations range from zero to five. When expressing the leader and propeptide parts in \textit{trans}, we observed a major peak of leaderless prenisin with five dehydrations, thus demonstrating that the nisin leader acts in \textit{trans} in vivo and suggesting that the separated leader peptide alone is able to stimulate the nisin biosynthesis enzymes, in particular for the N-terminal modification of prenisin.

**Results**

The nisin leader peptide is not absolutely necessary for dehydration

To investigate whether the leader is absolutely required for the dehydration reaction, a prenisin variant NisA(24–57)–H\textsubscript{6} (lacking

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**Figure 1.** Post-translational modifications of prenisin. A) Serines and threonines in the propeptide part are dehydrated by NisB to dehydroalanines (Dha) and dehydrobutyrines (Dhb), respectively (dark gray). B) Dehydrated residues are specifically coupled by NisC to cysteines to form five thioether rings (one lanthionine and four methylanthionines) in the fully modified prenisin. C) Fully modified prenisin is transported by a dedicated ABC transporter, NisT. D) The leader is cleaved off by the extracellular protease NisP, thereby liberating active nisin.
the leader sequence) was produced and was shown to interact with NisBTC. Mass spectra of the leaderless prenisin NisA(24–57)-H₆ (Figure 2A) showed a range of zero- to fivefold dehydrated leaderless prenisin species, thus clearly indicating that the leaderless prenisin can be dehydrated in vivo (Figure 3A, Table S1).

NisC is able to form rings in the absence of the leader

The NisBTC-modified and subsequently Ni-NTA-purified leaderless NisA(24–57)-H₆, when applied on an agar plate containing the indicator strain Lactococcus lactis NZ9000, showed significant antimicrobial activity (Figure S1 in the Supporting Information, well A). The medium-sized halo indicates that at least some of the rings had formed in some of the molecules, thus suggesting NisC’s ability to modify leaderless prenisin without the presence of covalently attached N-terminal leader. In a previous study, antimicrobial activity was shown to be present only if (at least) rings A, B, and C are present,[15] this suggests that at least the first three lanthionine rings had formed in our experiment.

**In trans expression of the nisin leader and the propeptide**

By expressing the nisin leader and the propeptide parts from separate genes (i.e., in trans; Figure 2B) and analyzing the obtained product by MALDI-TOF MS, we observed a single fivefold dehydrated leaderless prenisin peak (Figure 3B, Table S1).

The presence of an inhibition zone (significantly larger than in the previous case, without the leader in trans) in an antimicrobial well-plate assay (Figure S1, well B), shows that NisC is able to form the three lanthionine rings (A, B, C), when the nisin leader and the propeptide part are expressed in trans. As five dehydrated residues were found and it is known that the modifications take place from the N to the C terminus, it is highly likely that exactly three rings must have formed, with the second part of nisin unmodified.

**Influence of the leader on dehydration efficiency**

The wild-type prenisin molecule is highly efficiently modified by the nisin modification enzymes NisB and NisC. Wild-type NisA results in predominantly 7 and 8 dehydration peaks in MALDI-TOF MS. In this study, a NisA-H₆ nisin variant [10] that contains a C-terminal extension GSIEGR (Figure 2) and a His tag was used. As an additional serine is present in the C-terminal extension, the maximum number of dehydrations was 9, as confirmed by MALDI-TOF MS analy-
sis (Figure 3 C). The propeptide part, when expressed without the leader, resulted in nisin species with different numbers of dehydrations (0 to 5 dehydrations; Figure 3 A, Table S1). Interestingly, when the leader and the propeptide parts were expressed in trans, we observed a single major peak of leaderless prenisin with five dehydrations, thus indicating a more efficient dehydration (Figure 3 B) that stopped at a distinct position. As the modification process has been shown to proceed from N to the C terminus, the five dehydrations were expected to be found in the N-terminal part of the propeptide. For in trans expression, although the nisin leader was present, it was not physically attached to the propeptide. Higher amounts of dehydration were reached, thus indicating that the propeptide part (expressed without the leader) interacts with NisB more efficiently. Increase in the efficiency of dehydration/cyclization is additionally supported by the higher inhibition zone observed following in trans expression (Figure S1, well B).

Here, we show, for class I lantibiotics, that although the nisin leader is important for the biosynthesis process, it is not absolutely required for the modifications to occur in vivo, although it is meaningful that the dehydration process appears to stop after the first five dehydrations, thus suggesting a role for the covalently attached nisin leader to position the C-terminal Ser/Thr residues at the active site of NisB.

Discussion

The leader peptide of class I lantibiotics has so far been assumed to be crucial for the dehydration and modification processes. Our study, performed in vivo, shows that the nisin leader peptide is not absolutely necessary for lantibiotic biosynthesis. This is the first evidence that dehydration and cyclization of lantibiotics can occur in vivo in the absence of the leader. The presence of partially modified nisin demonstrated that the leader is not absolutely required for the modifications to occur, and that the nisin modification enzymes NisB and NisC still have affinity for the propeptide part. In a previous report, pull-down of NisB and NisC required full-length substrate prenisin, this suggests that these enzymes interact with the propeptide part as well as the leader peptide.

Interestingly, the prenisin variant lacking the leader sequence resulted in a range of zero- to fivefold dehydrated leaderless prenisin species; this suggests that without the leader the efficiency of dehydration is severely compromised. In contrast, in trans expression of the leader resulted in exactly five dehydrations, thus demonstrating that the leader acts in trans in vivo, and suggesting that the leader partly restores the efficiency of dehydration in the N-terminal part of nisin (Figure 4).

When the leader is absent, various premature nisin species with different numbers of dehydrated residues are observed, thus suggesting very loose binding of the substrate and allowing relatively frequent escape from the complex. However, only variants containing at least the three N-terminal ABC rings (and thus at least five dehydrated residues) possess antimicrobial activity. The observed antimicrobial activity indicates the presence of at least the three N-terminal rings ABC and the ability of NisC to partially modify these substrates, present in at least a small proportion of the substrates. Extremely low amounts of NisB were copurified in the absence of the leader (Figure S2); this supports interaction of the propeptide with NisB and loose binding when the leader is not present.

The previously published N- to C-terminal directionality of dehydration, together with the notion that for antimicrobial activity the presence of the three N-terminal rings ABC is necessary, suggests that the observed five dehydrations, both in the leaderless and in systems with the leader and propeptide part expressed in trans, happen in the N-terminal part of the propeptide, with Thr13 being the last modified residue. This observation suggests that to dehydrate the remaining dehydratable residues (in the C-terminal part), a pulling action is required that could protrude prenisin through the active center of LctM and that no “protrusion effect” is involved. However, the dehydratable residues in lacticin 481 are located in relatively close proximity to each other; thus, it is possible that the presence of a single binding region allows all four dehydrations. In this case of nisin, we observed up to five dehydrations when the leader was absent or expressed in trans. For this reason, we propose that the protrusion effect is valid, at least...
for longer lantibiotics. In this context, it is tempting to speculate that the leader, when attached covalently to the propeptide, imposes a superior binding state that allows the peptide to remain associated with the enzymes NisB and NisC during the successive dehydration and cyclization events. The most likely region that would facilitate the increased affinity is the leader FNLD region (−18 to −15). Substitution of FNLD with AAAA, reported in a previous study,\textsuperscript{10} resulted in highly decreased affinity to NisB, as well as a range of zero to four dehydrations, thus suggesting a decrease in the efficiency of dehydration. This result is in line with our findings here, and suggests that the FNLD region is responsible for the tight binding to the modification enzymes NisB and NisC.

The increase in the dehydration capacity when the leader was expressed in \( \text{trans} \) indicates that the nisin leader is important in terms of keeping the nisin modification enzymes NisB and NisC active, possibly by inducing conformational changes and most likely by pulling the substrate through the active site region of the modification complex. On the other hand, in the absence of the leader, the propeptide part can still be dehydrated, thus indicating low activity of NisB. Therefore, it is more likely that the leader peptide shifts NisB from partially inactive to a more-active state, in line with a very recent study where the leader of lacticin 481 (class II) was fused to its modification enzyme (LctM) with a linker.\textsuperscript{19}

Based on these and our latest findings, we propose a model in which 1) a complex is formed between prenisin and the modification enzymes NisB and NisC; 2) during consecutive dehydration and cyclization events the leader is constantly bound, most probably to both enzymes, by forming a complex; 3) the propeptide part flips between NisB and NisC, supported by an alternating action of NisB and NisC\textsuperscript{17,20} and 4) to dehydrate the C-terminal part of prenisin, prenisin is protruded through the active centers of NisB and NisC, by pulling at the nisin leader, in agreement with a previously proposed model.\textsuperscript{17} In the in \( \text{trans} \) expression, the leader peptide and the propeptide part are not covalently attached, thus the protrusion of the C-terminal part of the propeptide through the active centers of NisB and NisC cannot happen, thereby explaining the observation that only five dehydrated residues are present (most likely the N-terminal ones). The next challenge will be to determine where exactly the leader interacts with the NisB and/or NisC enzymes.

### Experimental Section

**Construction of the bicistronic construct for the expression of the leader and the propeptide part in \( \text{trans} \):** A plasmid encoding NisA\textsuperscript{H} was taken as a template. Primers were designed to separate the leader and propeptide parts. By applying the "round-PCR" method, several random nucleotides and a ribosome binding site were introduced between the regions encoding the leader and propeptide (see the Supporting Information), thereby resulting in the construct for expression of the leader and the propeptide parts in \( \text{trans} \).

**Bacterial strains and growth conditions:** \( L. \text{lactis} \) N29000 was used as an expression host in this study. In brief, cells were grown as described previously\textsuperscript{10} at 30 °C in M17 medium (Difco) supplemented with glucose (0.5 %, w/v) chloramphenicol (5 \( \mu \text{g mL}^{-1} \)), and erythromycin (5 \( \mu \text{g mL}^{-1} \)) where appropriate. When the antibiotics were used simultaneously, each was at 4 \( \mu \text{g mL}^{-1} \).

**Recombinant DNA techniques:** Standard genetic manipulations were performed as described by Sambrook et al.\textsuperscript{22} The round-PCR method with 5' phosphorylated primers was performed as described earlier.\textsuperscript{23} Plasmid isolation was performed by means of the Plasmid DNA Isolation Kit (Roche Applied Science). Restriction digest analysis was performed with restriction enzymes from Fermentas (Thermo Scientific). DNA ligation was performed with T4 DNA ligase (Fermentas), and round PCR amplification was performed with Phusion DNA polymerase (Finnzymes/Thermo Scientific).

**Protein overexpression:** Cells containing plasmid pIL3BTC and plasmids encoding NisA variants were grown to \( \text{OD}_{660} \) = 0.6 and induced with 0.5 \( \text{ng mL}^{-1} \) nisin. Subsequently, cells were grown for 3 h, the OD was normalized, and cells were harvested by centrifugation, and washed once with Tris-\( \text{HCl} \) (50 \( \text{mM}, \text{pH} 7.4 \)). Cells were resuspended in the same buffer and treated with lysozyme (10 \( \text{mg mL}^{-1} \)) for 30 min at 30 °C. MgSO\(_4\) (10 \( \text{mM} \)) and DNase (100 \( \mu \text{g mL}^{-1} \)) were added, and the suspension was passed twice through a French press cell (106 MPa). Cell debris was removed by two centrifugation steps (13,000g, 15 min, 4 °C).

**Ni-NTA purification:** Ni-NTA purification was performed as described previously.\textsuperscript{10} Briefly, superflow Ni-NTA column resin (50 %, 1.5 mL; Qiagen) was equilibrated twice with lysis buffer (38.5 \( \text{mL} \), NaH\(_2\)PO\(_4\) (50 \( \text{mM}, \text{pH} 8 \)), NaCl (300 \( \text{mM} \)), imidazole (10 \( \text{mM} \))) in a 50 \( \text{mL} \) polystyrene tube (Greiner Bio-One, Frickenhausen, Germany) by mixing on a rotor (30 min). Subsequently, the column material was resuspended in 4–8 \( \text{mL} \) of the cytoplasmic fraction, transferred into a 15 \( \text{mL} \) Falcon tube (BD Biosciences, Franklin Lakes, NJ), lysis buffer was added (final volume 12 \( \text{mL} \)), and His-tagged protein was allowed to bind to the column material on a rotor (4 °C, 2 h). Subsequently, the column was washed twice with wash buffer (35 \( \text{mL} \), Na\(_2\)HPO\(_4\) (50 \( \text{mM}, \text{pH} 8 \)), NaCl (300 \( \text{mM} \)), imidazole (20 \( \text{mM} \))). Elutions were collected in four fractions (0.5 \( \text{mL} \) each) with elution buffer (NaH\(_2\)PO\(_4\) (50 \( \text{mM}, \text{pH} 8 \)), NaCl (300 \( \text{mM} \)), imidazole (250 \( \text{mM} \))). Fractions were analyzed by SDS-PAGE and western blot.

**SDS-PAGE and western blot:** Western blots were performed by using anti-NisB, anti-NisC or anti-leader antibodies.\textsuperscript{10} SDS-PAGE was performed by using standard molecular biology techniques. Samples were not boiled before applying to SDS-PAGE.

**Antimicrobial activity assay:** The indicator strain \( L. \text{lactis} \) N29000 bearing plasmid pNZnisPT was used for the expression of the protease, NisP, grown overnight (ON) in M17 medium supplemented with glucose (0.5 %). The next day, this inoculate was inoculated into fresh M17 medium containing nisin (0.5 \( \text{ng mL}^{-1} \)) for the induction expression of NisP. When the \( \text{OD}_{660} \) reached 0.6, the culture (100 \( \mu \text{L} \)) was added to 100 \( \text{mL} \) of liquid M17 agar at 40 °C. Plates were dried, and wells were made. Ni-NTA-purified samples were applied to the wells, and the plates were left ON at 30 °C.

**Mass spectrometry analysis:** Mass spectrometric analysis of the produced peptides was performed with crude supernatants or Ni-NTA-purified fractions. Prior to the analysis, samples were desalted by Zip-Tip (C18 ZipTip, Millipore), essentially as described before.\textsuperscript{10} In short, ZipTips were equilibrated with acetonitrile (100 %) and washed with trifluoroacetic acid (0.1 %). Subsequently, the supernatant containing the peptides was mixed with trifluoroacetic acid.
and applied to a ZipTip column. Bound peptides were washed (0.1 % trifluoroacetic acid) and eluted (acetonitrile (50 %) and trifluoroacetic acid (0.1 %)). The eluent was mixed in a 1:1 ratio with matrix (10 mg mL\(^{-1}\) \(\alpha\)-cyano-4-hydroxycinnamic acid) and 1 \(\mu\)L was spotted on the target and allowed to dry. Mass spectra were recorded with a Voyager-DE Pro MALDI-TOF mass spectrometer (Applied Biosystems) in positive linear mode. In order to increase the sensitivity, external calibration was applied with six different peptides (Protein MALDI-MS Calibration Kit, Sigma–Aldrich).

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