Identification of distinct nisin leader peptide regions that determine interactions with the modification enzymes NisB and NisC

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Nisin is the most prominent and applied bacteriocin that serves as a model for class I lantibiotics. The nisin leader peptide importantly determines interactions between precursor nisin and its modification enzymes NisB and NisC that mature nisin posttranslationally. NisB dehydrates serines and threonines, while NisC catalyzes the subsequent coupling of the formed dehydroamino acids to form lantibiotics. Currently, little is known about how the nisin leader interacts with NisB and even less is known about its interactions with NisC. To investigate the nisin leader peptide requirements for functional interaction with the modification enzymes NisB and NisC, we systematically replaced six regions, of 2–4 amino acids each, with all-alanine regions. By performing NisB and NisC co-purification studies with these mutant leader peptides, we demonstrate that the nisin leader regions STKDI(22–19), FNLD(18–15) and PR(2–1) importantly contribute to the interactions of precursor nisin with both NisB and NisC, whereas the nisin leader region LVSV(14–11) additionally contributes to the interaction of precursor nisin with NisC.

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1. Introduction

Nisin is a posttranslationally modified antimicrobial peptide encoded as precursor with a characteristic N-terminal leader and a C-terminal core peptide part. Nisin is produced by some strains of Lactococcus lactis and shows significant antimicrobial activity against a wide range of Gram-positive bacteria, including Staphylococcus aureus, Bacillus cereus and the food borne pathogens Listeria monocytogenes and Clostridium botulinum. More than 50 years of usage of nisin in food applications has led to little antimicrobial resistance [1,2]. Nisin contains unusual amino acids: dehydroalanines (Dha), dehydrobutyryl- lones (Dhb), lanthionines and methyllanthionines, i.e. thioether bridged amino acids (Ala–S–Ala; and Abu–S–Ala). The lanthionine rings provide conformational rigidity to parts of the peptide and resistance against breakdown by peptidases [3,4]. Posttranslational modification of precursor nisin is performed by the dedicated enzymes NisB and NisC. NisB dehydrates Ser and Thr in the core peptide part to yield Dha and Dhb, respectively, and NisC covalently couples thiol groups of Cys to Dha or Dhb (Fig. 1). In this way, the five lanthionine rings of precursor nisin are formed (Fig. 1). Dehydration and cyclization reactions for classes I and II lantibiotics (see below) have been shown to proceed from the N-terminal to C-terminal direction [5,6] and alternating activity of NisB and NisC has been proposed [5,7]. The molecular weight of NisB is 117.5 kDa and an N-terminal degradation product of about 50 kDa has been reported [8,9]. Full length precursor nisin consists of 57 amino acids, and comprises a 23 amino acid N-terminal leader peptide followed by a 34 amino acid C-terminal modifiable core peptide part. The nisin leader contributes to interactions with the modification enzymes NisB and NisC, determines interactions with the nisin transporter, NisT, and is cleaved off by a protease, NisP [10,11]. Non-lantibiotic peptides can be modified by the NisRTC machinery, provided the nisin leader peptide is present at the N-terminus [12–14]. This demonstrates the broad substrate specificity of the modification enzymes (see also some recent reviews on lantibiotics biosynthesis, regulation and engineering) [4,15–17].

Significant progress has also been made with respect to the elucidation of the structural organization of the nisin modification enzyme machinery. Notably, a complex of nisin modification enzymes has been isolated consisting of NisB, NisC and NisT [9]. Moreover, NisB has been shown to have stronger interactions with precursor nisin than NisC has [9]. The nisin leader plays an important role, since

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without the leader only extremely low amounts of NisB could be co-purified with precursor nisin and no isolation of the enzyme complex was possible [18]. Based on the leader sequences and the number of modification enzymes, lantibiotics can be divided into two classes, i.e. class I and class II. Class I prelantibiotics contain a nisin-like leader peptide with a conserved FNLDL-like consensus sequence and are modified by two modification enzymes, LanB and LanC. On the other hand, class II prelantibiotics possess a different leader peptide [19] and are modified by one bifunctional enzyme LanM that is able to perform both the dehydation and the cyclization reaction. From the engineering point of view, the advantage of class I lantibiotics lies in the possibility to dissect the dehydation reaction from the cyclization reaction. Hence peptides with dehydroamino acids can be produced by bacteria [20] followed by in vitro cyclization catalyzed by LanC [21].

Some progress has also been made with respect to the identification of the functional domains of the nisin leader peptide. The importance of the FNLD(-18-15) region for the functionality of the leader has been well established [9,10,19]. This region determines the initial recognition and binding of precursor nisin to NisB [22]. Thus far no relevance of the C-terminal part of the leader peptide for interaction with NisB has been discovered. Since even a 6His-tag could be introduced at residues -13 to -8 without loss of functionality, a spacer function has been proposed for leader peptide region -14 to -1 [19]. Notably, a minimal leader peptide, consisting of MSTKDFNL, has been proposed and has been shown to be partial functional, resulting in up to 3- and 4-fold dehydrated precursor nisin [19].

Here we investigate the relevance of short regions covering the whole nisin leader peptide for interaction with either NisB or NisC, as measured by our previously reported pull-down assay [9]. Importantly, it is currently unknown which particular regions of the leader are involved in which modification process. By constructing region-specific substitutions of four consecutive amino acids in the nisin leader and performing co-purification studies with the resulting precursor nisin leader mutants, we here identify leader regions important for functional precursor nisin–NisB and NisC interactions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strain *L. lactis* NZ9000 [23] was used as an expression host in this study (Table 1). In brief, cells were grown as described previously [9] at 30 °C in M17 medium (Difco) supplemented with 0.5% (w/v)
2.2. Recombinant DNA techniques

Standard genetic manipulations were essentially performed as described by Sambrook et al. [24]. The round PCR method with 5′-phosphorylated primers was performed as described earlier [25]. Plasmid isolation was performed by means of the Plasmid DNA Isolation Kit (Roche Applied Science). Restriction analysis was performed with restriction enzymes from Fermentas. DNA ligation was performed with T4 DNA ligase (Fermentas) and round PCR amplification was done with Phusion DNA polymerase (Finnzymes).

2.3. Protein overexpression

Cells were grown until an OD_{600} of 0.6 and induced with 0.5 ng ml⁻¹ nisin. Subsequently, cells were grown for 2 h, the OD was normalized and cells were harvested by centrifugation, and washed once with 50 mM Tris–HCl, pH 7.4. Cells were resuspended in the same buffer and treated with lysozyme (10 mg ml⁻¹). After 30 min at 30 °C, 10 mM MgSO₄ and 100 μg ml⁻¹ of DNase were added, the suspension was passed twice through a French press cell (15,400 psi). Cell debris was removed by two centrifugation steps at 13,000 × g (15 min at 4 °C).

2.4. Ni-NTA purification

For Ni-NTA purification 1.5 ml of 50% superflow Ni-NTA column resin (Qiagen) was equilibrated twice with 38.5 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8) in a 50 ml tube by mixing on a rotor for 30 min. Subsequently, column material was resuspended in a 4–8 ml cytoplasmic fraction, transferred into a 15 ml tube, lysis buffer was added to a final volume of 12 ml and His-tagged protein was allowed to bind to the column material on a rotor in the cold room at 4 °C for 2 h. Subsequently, the column was washed twice with 35 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8). Elutions were collected in four fractions of 0.5 ml each with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8). Fractions were analyzed by SDS–PAGE and Western blots.

2.5. SDS–PAGE and Western blot

Western blots were performed using anti-NisB, anti-NisC or anti-leader antibodies. SDS–PAGE was performed using standard molecular biology techniques. Samples were not boiled before applying to SDS–PAGE.

2.6. 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 ZipTipped (C18 ZipTip, Millipore), essentially as described before [9]. In short, ZipTips were equilibrated with 100% acetonitrile and washed with 0.1% trifluoroacetic acid. Subsequently, the supernatant containing the peptides was mixed with 0.1% trifluoroacetic acid and applied to a ZipTip. Bound peptides were washed with 0.2% trifluoroacetic acid and eluted with 50% acetonitrile and 0.1% trifluoroacetic acid. The eluent was mixed in a 1:1 ratio with matrix (10 mg ml⁻¹ α-cyano-4-hydroxy-cinnamic acid) and 1 μl was spotted on the target and allowed to dry. Mass spectra were recorded with a Voyager-DE Pro (Applied Biosystems) MALDI-time-of-flight mass spectrometer. In order to increase the sensitivity, external calibration was applied with six different peptides (Protein MALDI-MS Calibration Kit, Sigma).

3. Results

To investigate which regions of the nisin leader are relevant for the interaction with NisB and NisC, we substituted five regions of four amino acids and one region of two amino acids with alanine residues (Fig. 1, Table 2). Substitutions were made in leader sequences which are genetically fused to the wild type core peptide with the glucose and antibiotics 5 μg ml⁻¹ chloramphenicol and erythromycin 5 μg ml⁻¹ where appropriate. When both antibiotics were used simultaneously, 4 μg ml⁻¹ chloramphenicol and 4 μg ml⁻¹ erythromycin were applied.
C-terminal extension GSEGRHHHHHHH. All the nisin leader mutants were successfully expressed (Fig. 2A). The previously described nisin-modification enzyme binding assay, based on this His-tagged precursor nisin [9], was used to pull down NisB and NisC through co-purification with NisA-H6 leader mutants.

3.1. Co-purification of NisB with the nisin leader mutants

In a first step we evaluated the interaction of the leader region precursor mutants with NisB on the basis of the amount of NisB co-purified in His-tagged precursor nisin pull down experiments (Fig. 2A and B). The substitution in the nisin leader peptide region NisA-H6 SGAS(-6-3) (lane 5) by 4 Ala residues resulted in wild-type amounts of NisB co-purified. The leader region mutants NisA-H6 LSVV(-14-11) (lane 3) and NisA-H6 SKKD(-10-7) (lane 4), also by 4 Ala residues each, resulted in lower amounts of purified NisB and on the basis of the amount of co-purified NisB, the order of binding of the leader mutants to NisB was the following: wild-type Δ-mutant NisA-H6 SGAS(-6-3)AAAA = mutant NisA-H6 SKKD(-10-7)AAAA = mutant NisA-H6 LSVV(-14-11)AAAA. The other leader mutants NisA-H6 STKD(-22-19), NisA-H6 FNLD(-18-15) and NisA-H6 PR(-2-1) hardly show any co-purification of NisB. Taking together the results of the SDS–PAGE and Western blot analysis (Fig. 2A and B), the nisin leader regions that mainly determine the interaction with NisB are the regions NisA-H6 STKD(-22-19), the region NisA-H6 FNLD(-18-15) and the region NisA-H6 PR(-2-1), since their corresponding alanine substitutions resulted in highly decreased levels of NisB co-purification.

3.2. Co-purification of NisC with the nisin leader mutants

Interestingly, the nisin pull-down assay, together with the anti-NisC immunoblot analysis, indicated that only the substitution in the nisin leader region NisA-H6 SGAS(-6-3)AAAA allows co-purification of NisC (Fig. 2C). Hence only this region of the leader peptide does not significantly contribute to interaction with NisC. Extremely low amounts of NisC were co-purified applying the nisin leader region NisA-H6 SKKD(-10-7)AAAA mutant. No detectable levels of NisC are co-purified with the nisin leader alanine substitutions in the regions NisA-H6 STKD(-22-19), NisA-H6 FNLD(-18-15), NisA-H6 LSVV(-14-11) and NisA-H6 PR(-2-1) (Fig. 2C), indicating that all these regions play a significant role in the interaction with NisC, or that NisC is not co-purified when no NisB can be bound. In the latter case only region -14-11 can be directly responsible for the loss of NisC binding.

3.3. Influence of the leader regions on the dehydration efficiency

The wild-type precursor nisin is efficiently modified by the nisin modification enzymes NisB and NisC. Initial methionine (Met 1) is occasionally cleaved off by methionine aminopeptidase. In case of NisA-H6, an additional Ser in the C-terminal extension GSEGRH is also dehydrated resulting in peaks corresponding to 8 and 9 dehydrations on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS). The leader substitutions resulted in partially modified precursor nisin (Fig. S1, Table 3). In some cases, i.e. the leader mutants NisA-H6 STKD(-22-19)AAAA, NisA-H6 SKKD(-10-7)AAAA, NisA-H6 SGAS(-6-3)AAAA and NisA-H6 PR(-2-1)AAAA both partially modified precursor nisin and much less intense peaks of fully modified precursor nisin were observed (Fig. S2B and E–G). The NisA-H6 mutants FNLD(-18-15)AAAA and LSVV(-14-11)AAAA resulted only in partially modified precursor nisin (Fig. S2C and D).

4. Discussion

Lantibiotics represent an important and promising class of antibiotics for the replacement of conventional antibiotics. Knowledge on the biosynthesis of lantibiotics is of crucial importance for generating novel lantibiotics. In particular, it will enable the rational engineering of therapeutically relevant lantionine-containing peptides. Delineating the functions of different regions of the nisin leader may allow the subtle modulation of dehydration and/or cyclization reactions. Here, by substitution of leader regions and co-purification studies, we reveal regions within the nisin leader peptide that are important for the interactions with the nisin modification enzymes NisB and NisC.

Studies on the nisin leader [10,19], the PepS leader [26], the nukacin A leader [27], the mutacin II leader [28], and the lacticin 481 leader [26] demonstrated that apart from a few conserved residues, single point mutations are well tolerated by lantibiotic modification enzymes. Our approach to simultaneously replace all residues within a certain region by alanines successfully distinguishes the importance of various regions within the nisin leader peptide.

The FNLD region has already been shown to be important for interactions with both the nisin modification enzymes NisB and NisC [9,19,22]. This study indicates that in addition to the FNLD(-18-15) region, the substitutions NisA-H6 STKD(-22-19)AAAA and NisA-H6 PR(-2-1)AAAA lead to significantly decreased interactions with NisB and completely abolished interactions with NisC, demonstrating the relevance of the corresponding regions for their interactions with NisB and NisC. The region -14 to -11 (LSVV) appeared to be relevant for interaction of the leader with NisC. However, in a previous study replacement of residues -13 to -8 (SVSKK) by a 6His-tag still allowed production of functional nisin [19]. Together this might suggest that the Leu at -14 is also rather important, so that FNLD rather than FNLD determines interaction of the leader with NisC.

Co-purification assays (Fig. 2) together with the MALDI–TOF MS
Table 2
The nisin leader mutants used in this study and the amounts of the co-purified nisin medication enzymes NisB and NisC.

<table>
<thead>
<tr>
<th>The nisin leader mutant</th>
<th>Substitution</th>
<th>Interactions with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NisB</td>
</tr>
<tr>
<td>Wild type leader</td>
<td>MVKDFNLDSLVSNSKLDSGASPR</td>
<td></td>
</tr>
<tr>
<td>Leader 1 mutant</td>
<td>MAAASTKDNLDSNKLDSGASPR</td>
<td>STKD(-22-19)AAAA</td>
</tr>
<tr>
<td>Leader 2 mutant</td>
<td>MAAASTKDNLDSNKLDSGASPR</td>
<td>FNLD(-18-15)AAAA</td>
</tr>
<tr>
<td>Leader 3 mutant</td>
<td>MAAASTKDNLDSNKLDSGASPR</td>
<td>LVSV(-14-11)AAAA</td>
</tr>
<tr>
<td>Leader 4 mutant</td>
<td>MAAASTKDNLDSNKLDSGASPR</td>
<td>SKKD(-10-7)AAAA</td>
</tr>
<tr>
<td>Leader 5 mutant</td>
<td>MAAASTKDNLDSNKLDSGASPR</td>
<td>SGAS(-6-3)AAAA</td>
</tr>
<tr>
<td>Leader 6 mutant</td>
<td>MAAASTKDNLDSNKLDSGASPR</td>
<td>PR(-2-1)AAAA</td>
</tr>
</tbody>
</table>

± denotes the reduced levels of co-purification of the nisin modification enzymes NisB and NisC.

Table 3
Dehydration of the leader mutants by NisB in L. lactis containing pIL3BTC, isolated by Ni-NTA purification and analyzed by MALDI–TOF mass spectrometry.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Dehydration extent</th>
<th>Observed (Da)</th>
<th>Calculated (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NisA-H6 (wild type)</td>
<td>9</td>
<td>7091.45</td>
<td>7092.28</td>
</tr>
<tr>
<td>NisA-H6 STKD(-22-19)AAAA (leader 1)</td>
<td>9</td>
<td>7075.18</td>
<td>7076.34</td>
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<tr>
<td>NisA-H6 FNLQ(-18-15)AAAA (leader 2)</td>
<td>8</td>
<td>7064.76</td>
<td>7049.34</td>
</tr>
<tr>
<td>NisA-H6 LVSV(-14-11)AAAA (leader 3)</td>
<td>8</td>
<td>7026.56</td>
<td>7027.42</td>
</tr>
<tr>
<td>NisA-H6 SKKD(-10-7)AAAA (leader 4)</td>
<td>4</td>
<td>7097.32</td>
<td>7080.08</td>
</tr>
<tr>
<td>NisA-H6 SGAS(-6-3)AAAA (leader 5)</td>
<td>3</td>
<td>7062.59</td>
<td>7062.08</td>
</tr>
<tr>
<td>NisA-H6 PR(-2-1)AAAA (Leader 6)</td>
<td>4</td>
<td>7022.85</td>
<td>7026.08</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7003.24</td>
<td>7008.08</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7234.30</td>
<td>7236.30</td>
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<tr>
<td></td>
<td>1</td>
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<td>7218.30</td>
</tr>
<tr>
<td></td>
<td>4</td>
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<td>7164.30</td>
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<td>5</td>
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<td></td>
<td>7</td>
<td>7107.16</td>
<td>7105.30</td>
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<tr>
<td></td>
<td>8</td>
<td>7089.70</td>
<td>7092.30</td>
</tr>
<tr>
<td>NisA-H6 (wild type)</td>
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<td>7145.28</td>
<td>7143.12</td>
</tr>
<tr>
<td>NisA-H6 (wild type)</td>
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<td>7129.08</td>
<td>7125.13</td>
</tr>
<tr>
<td>NisA-H6 (wild type)</td>
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<td>7087.43</td>
<td>7089.13</td>
</tr>
<tr>
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<td>4</td>
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<td>7071.13</td>
</tr>
<tr>
<td>NisA-H6 (wild type)</td>
<td>7</td>
<td>7019.38</td>
<td>7017.13</td>
</tr>
<tr>
<td>NisA-H6 (wild type)</td>
<td>8</td>
<td>6991.88</td>
<td>6999.13</td>
</tr>
</tbody>
</table>

Data (Table 3, Fig. S2) indicate that the N-terminal part of the nisin leader is crucial for binding to the modification enzymes and the subsequent posttranslational modifications. This suggests that shorter versions of the leader, containing only the N-terminal part of the leader, are functional, consistent with the functionality of an N-terminal leader fragment observed by Plat et al. [19]. However, to prevent any possible adverse effect of a shorter leader sequence, we preserved the length of the leader intact. Our results demonstrate that for efficient modification, the full-length leader without long substitutions in its sequence is optimal.

The substitution of the nisin leader STKD(-22-19) to AAAA leads to very low amounts of co-purified NisB (Fig. 2A and B), but still results in 8 and 9-fold dehydrated precursor nisin (Table 3, Fig. S2). This suggests that the STKD(-22-19) in the leader sequence is important for the binding of NisB and possibly complex formation, but not for the dehydration reaction itself. It is tempting to speculate that the STKD(-22-19) is the initial sequence that interacts with NisT and possibly NisB is recruited to the nisin biosynthesis complex through NisT which would explain the relatively low amounts of NisB co-purified in the absence of the STKD motif (Fig. 2A and B). The interactions of NisB and NisT are in line with previous studies, where it has been shown by the yeast two-hybrid system that NisB and NisT form a complex [29] and second, the presence of NisT significantly enhances export by NisT [30]. The region PR(-2-1) also results in low amounts of co-purified NisB (Fig. 2A and B) and in a combination of partially and fully modified precursor nisin. The Pro at the position -2 is conserved (Fig. S1) and has been demonstrated to play an important role in nisin biosynthesis [10]. The FNLQ motif is a highly conserved region in class 1 lantibiotics (Fig. S1). It has been well described and shown before to be important for nisin biosynthesis [9,10,19,22]. As expected, the substitution of this region results in very low amounts of co-purified NisB (Fig. 2A and B) and only partially dehydrated precursor nisin (Table 3, Fig. S2). For the regions LVSV(-14-11), SKKD(-10-7) and SGAS(-6-3)AAAA by Ala, the higher efficiency of binding to NisB is concomitant with higher efficiency of in vivo dehydration (Table 3, Fig. S2).

Notably, the alterations we applied to the leader sequence have a more prominent effect on the co-purification of NisC than that of NisB. NisB seems to be less affected by the leader substitutions since at least three regions LVSV(-14-11), SKKD(-10-7) and SGAS(-6-3) could be replaced with alanines, while still resulting in significant amounts of NisB co-purified (Fig. 2B).

Previously, it has been suggested that the leader might interact with the modification enzymes through its secondary structure [26]. Introduction of stretches of alanines into the leader sequence might change a possible secondary structure of the nisin leader and thus might decrease the interactions with the nisin modification enzymes NisB and NisC. However, by NMR studies no secondary structure was observed in any of the studied lantibiotics in aqueous solutions, although α-helix formation in trifluoroethanol has been observed [31].
Analogously, interaction with the modification enzymes might induce a functional structure into the leader peptide.

Dissection and manipulation of the recognition motifs within the leader peptide that direct the interaction of precursor nisin with the nisin modification enzymes can ultimately guide the modification of non-lantibiotic peptides such as medically important peptide hormones. The data obtained in our study provide novel insights into nisin peptide maturation and provide the basis for subtle modulation of the interaction of precursor lantipeptides with lantihione-introducing modification enzymes.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.job.2013.05.001.

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