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NisC Binds the FxLx Motif of the Nisin Leader Peptide

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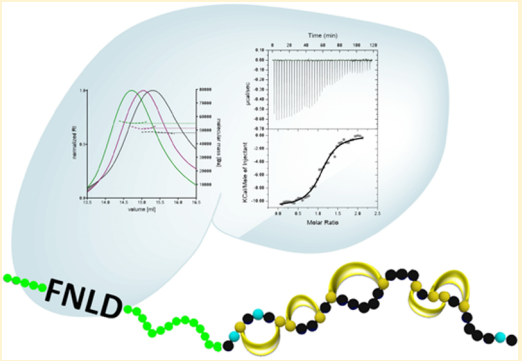
Supporting Information

ABSTRACT: Nisin is a model system for lantibiotics, a class of peptides displaying antimicrobial activity against various Gram-positive bacteria. After ribosomal synthesis, the precursor peptide is modified in two steps, of which the last one involves consecutive cyclization reactions mediated by the cyclase NisC. Here, we present a detailed *in vitro* study of the interaction between NisC and the nisin precursor peptide. Our results unravel a specific interaction of NisC with the leader peptide independent of the maturation state. Furthermore, mutagenesis studies identified a specific binding sequence within the leader. Two amino acids (F_{-18} and L_{-16}) within the highly conserved -FNLD- box of class I lantibiotics are essential for binding. They represent a potential general binding motif between leader peptides of a group of lantibiotics with their cyclase family. In summary, these *in vitro* data provide a new perception on the complexity of the lantibiotic modification machineries.



The ribosomally synthesized and post-translationally modified antimicrobial peptide nisin is produced by several *Lactococcus lactis* (*L. lactis*) strains.[1−3] Nisin was first discovered in 1928[4] and contains dehydrated amino acids, which become covalently linked to free cysteine thiols to form the characteristic (methyl)lanthionine rings.[3,5] These modifications classify nisin as a member of the lanthipeptide (lanthionine-containing peptides) superfamily, of which lantibiotics are a subclass with antimicrobial activity.[6,7] The N-terminal (methyl)lanthionine rings of nisin are crucial for its mechanisms of antimicrobial activities,[5,8] which are also present in other lantibiotics.[5,9,10] Another example is mersacidin from *Bacillus subtilis*, which displays activity against methilicin-resistant *Staphylococcus aureus* (MRSA).[11]

Nisin is the most intensively used and best-characterized lantibiotic and serves as a model system for these highly effective antibacterial peptides.[3,6,7] Nisin already has antimicrobial activity at nanomolar concentrations.[12]

Within *L. lactis*, a gene operon consisting of *nisABTCIPRKFEG* is responsible for nisin production. These 11 gene products are involved in nisin maturation (*nisBC*), secretion and processing (*nisTP*), regulation (*nisRK*), or immunity (*nisIFEG*).[13−15] Nisin is ribosomally synthesized as a precursor peptide consisting of 57 amino acids. The first 23 amino acids are defined as the leader peptide (residues −23 to −1), while the remaining 34 amino acids are called the core peptide (residues 1−34, Figure 1). The 34 amino acid part corresponds to the unmodified nisin, which by modification is converted in the (methyl)lanthionine-containing nisin.[16] After ribosomal synthesis, several posttranslational modifications occur, affecting the core peptide.[15,17] These reactions result in the formation of the dehydrated amino acids didehydroalanine (from serine) and didehydrobutyrine (from threonine) introduced by the nisin dehydratase NisB.[17−19] After dehydration, the double bond reacts with the thiol group of a cysteine yielding to a (methyl)lanthionine. This reaction is catalyzed by the nisin cyclase NisC in a regio- and stereospecific manner.[17,20]

Subsequent to the modification reactions, fully modified nisin is secreted across the Gram-positive cell membrane. An ABC transporter, NisT, which recognizes the leader peptide catalyzes this step.[21−23] After secretion, the peptide is converted into the active state by catalysis of a serine protease, NisP, which is anchored to the cell membrane and cleaves off the leader peptide C-terminal to the amino acid sequence PR.[24,25] This releases mature nisin into the extracellular space.

The first evidence for the function of the modification machinery was derived from knockout studies in a nisin-producing system *in vivo* indicating that NisB is responsible for dehydration and can function independently of NisC.[17,18] Very recently the *in vitro* activity of NisB was demonstrated, which revealed an additional step before dehydration. The serine and threonine residues become initially glutamylated by NisB before the dehydration reaction can take place.[19] Within an *in vivo* study of NisB, it was also demonstrated that the absence of *nisC* resulted in a peptide that was dehydrated but lacked any antimicrobial activity. This was the first experimental indication for a cyclization function of NisC.[17]

The formation of a potential NisB and NisC complex was investigated via two hybrid studies in yeast.[22] These results

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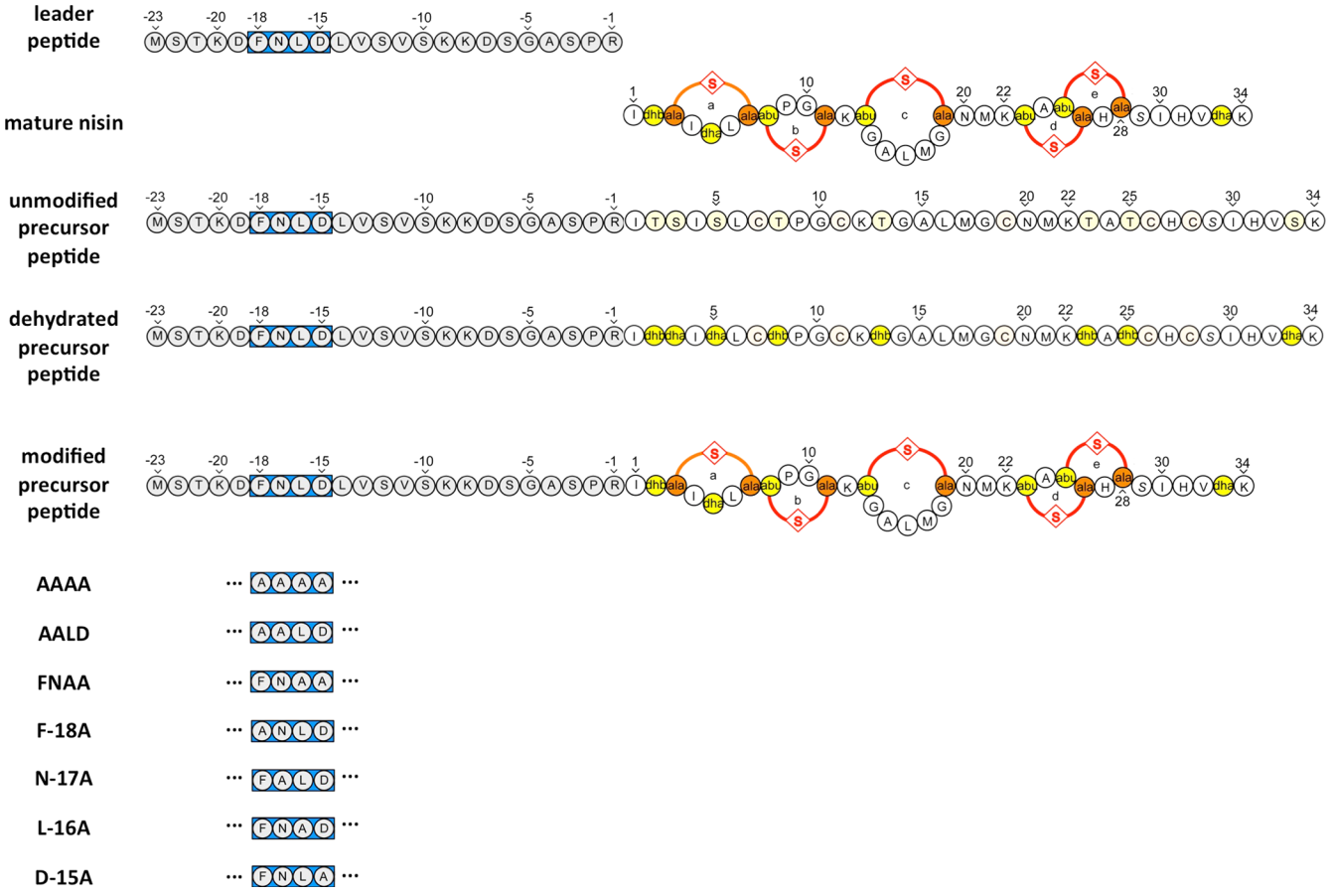

Figure 1. Scheme of the nisin precursor peptide variants and mature nisin used in this study. Mature nisin: final product after NisB, NisC, and NisP treatment possessing antimicrobial activity. Unmodified precursor peptide: ribosomally produced precursor peptide without any post-translational modifications. Dehydrated precursor peptide: unmodified precursor peptide after post-translational dehydrations catalyzed by NisB. Modified precursor peptide: fully modified precursor peptide after dehydration by NisB and cyclization by NisC. Leader peptide: the isolated polypeptide chain ranging from amino acid −23 to −1 of the precursor peptide. The following mutations are all based on the modified precursor peptide. -AAAA-: mutation in the leader peptide $F_{-18}A/N_{-17}A/L_{-16}A/D_{-15}A$. -AALD-: mutation in the leader peptide $F_{-18}A/N_{-17}A$. -FNAA-: mutation in the leader peptide $L_{-16}A/D_{-15}A$. The highly conserved -FNLD- box within the leader peptide is highlighted in blue, and specific serine and threonine residues are highlighted in yellow. The five (methyl)lanthionine rings are shown in red and orange, respectively.

together with mutational analysis within the core peptide led to the conclusion that the dehydration and cyclization reactions are related in space and time. It also implied directionality from the N- to the C-terminus and a direct interaction of NisB and NisC with the precursor peptide.[26]

A molecular understanding of the modification processes was given by an *in vivo* identification of a conserved box within the leader peptide of the lantibiotic precursor.[25] Sequence alignments of class I lantibiotic leader peptides identified a conserved -FNLD- box.[27] *In vivo* studies revealed that no posttranslational modifications were introduced into the core peptide in the case of an -FNLD-/-AAAA- mutation.[25] This suggested that at least NisB is interacting with the -FNLD- box.[25] However, a potential influence of mutations within the -FNLD- box on the activity of NisC is unknown.

NisC belongs to the enzyme family LanC, which is present in class I lantibiotic genetic operons. Examples of this family are the cyclases SpaC specific for subtilin,[28] EpiC specific for epidermin,[29] or EciC specific for Epicidin280.[30] On the basis of a phylogenetic study of lanthipeptides synthetases, members of this family can be clustered in a subgroup of the LanC clade.[31] The strict correlation of the presence of an -FNLD- box within the leader sequence and the presence of a LanC cyclase in the lantibiotic operon point towards a general recognition motif.

In this study, we focus on the second and last step of the nisin modification machinery, the cyclization by NisC. We analyzed the molecular mechanisms of interaction of the cyclase NisC with the precursor peptide *in vitro* to obtain a quantitative view on the mode of action. These studies provide the first *in vitro* analysis of the proposed NisC−nisin precursor interaction. Our thermodynamic data demonstrate that NisC is solely interacting with the leader peptide. More precisely, the recognition site of NisC was pinpointed to the -FNLD- box, in which two highly conserved amino acids (F_{-18} and L_{-16}) are essential for the interaction with the nisin cyclase.

MATERIALS AND METHODS

Cloning of pET-28b-AA-NisC. NisC was amplified out of *Lactococcus lactis* strain NZ9700[32] with the oligonucleotides NisCpet28for (CAAGAACTTTATTATTCAGGCT-AGCATGAGGATAATGAT) and NisCpet28rev (CACAATA-AGTATTAAATCTCGAGTCATTTCCTCT-TCCCTCCTTTC) containing restriction sites for NheI and XhoI, respectively. Digested PCR product and pET-28b (with NheI and XhoI, Fermentas) were ligated and transformed in

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Escherichia coli (*E. coli*) XL-1 Blue cells. The clones were sequenced, and positive hits were transformed in *E. coli* BL21 (DE3) cells for expression. All DNA kits were obtained from Qiagen.

Overexpression of NisC. *E. coli* BL21 (DE3) cells containing the pET-28b-AA-NisC plasmid were used for a 200 mL LB preculture containing 30 μg mL^{-1} kanamycin. Twelve liters of LB medium was supplemented with 100 μM zinc chloride, inoculated to an OD_{600} of 0.05, and grown at 37 °C with 180 rpm shaking to an OD_{600} of 0.8. At 18 °C, the culture was induced with 100 μM IPTG and grown overnight. After 20 h, cells were harvested at 6,000*g* and stored at −20 °C.

Purification of NisC. Cells were thawed on ice and resuspended in 50 mM HEPES-NaOH, 1 M NaCl, pH 8.0, and 10% glycerol. After adding small amounts of DNase, cells were lysed with a cell disruptor (IUL Instruments) and centrifuged at 4 °C with a low spin step (30 min, 14,5000*g*), followed by a high spin step (45 min, 90,000*g*). The resulting supernatant was supplemented with imidazole (final concentration 10 mM) and applied to an immobilized metal-ion affinity chromatography column (IMAC, Chelating HP column preloaded with Zn^{2+}, GE Healtcare). NisC was eluted with 50 mM HEPES-NaOH, pH 8.0, 1 M NaCl, 10% glycerol, and 150 mM imidazole in one step and further purified by size-exclusion chromatography (SEC) (Superdex 200 26/60) (GE Healthcare) pre-equilibrated with SEC buffer (50 mM HEPES-NaOH, pH 7.0, 1 M NaCl, and 10% glycerol). All fractions containing NisC were pooled and concentrated with an Amicon ultracentrifugation unit (30 kDa MWCO). The resulting NisC was digested with the Thrombin clean cleave kit (Sigma) to cleave off the N-terminal His_6-tag following the instructions of the manufacturer. After cleavage overnight, noncleaved and cleaved NisC were separated via IMAC following the procedure outlined above. Tag-free NisC was concentrated to 20 mg mL^{-1}, aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

Overexpression and Purification of Mature Nisin, Precursor Peptide, and Its Variants. Cloning, expression, and purification of mature nisin and the precursor peptide variants were previously described.[12,33] Compared to the published purification protocols, only the elution buffer of the cation exchange chromatography (cIEX) of the different precursor peptide derivatives was changed to 50 mM HEPES-NaOH, pH 7.0, 1 M NaCl, and 10% glycerol.

RP-HPLC Analysis of Mature Nisin, Precursor Peptide, and Its Variants. Peptides were analyzed by RP-HPLC with a LiChrospher WP 300 RP-18 end-capped column using an acetonitrile/water solvent system consisting of solvent A (10% acetonitrile/90% water/0.1% TFA) and solvent B (90% acetonitrile/10% water/0.1% TFA). After sample injection, the elution was performed by a linear gradient over 35 min to 60% solvent B at a flow rate of 1 mL min^{-1}.

Specific peptide concentrations were determined by the absorption at 205 nm. For calibration, known amounts of nisin (Sigma) and human insulin (Sigma) were injected, and the absorption integrals were determined with the software EZChrom Elite software V.3.3.1. and plotted against the known masses. A linear calibration line is calculated, and this equation was used to determine the specific concentrations from all used peptides.

Binding Experiments between NisC and the Precursor Peptides by Isothermal Titration Calorimetry (ITC). The binding parameters between NisC and the precursor peptides were determined by ITC. To circumvent dilution heat or heat resulting from the buffer mixture, the enzyme NisC and the substrate precursor peptide were separately dialyzed against SEC buffer. After dialysis, the concentration of NisC was adjusted to 400 μM and the concentration of the precursor peptide to 40 μM. Using an ITC200 (Microcal, GE Healtcare), the enzyme NisC, with a volume of 40 μL, was titrated to the different precursor peptide variants. Each experiment was performed at least in triplicate. The ITC measurements were performed at 25 °C with 40 injections (1 μL each). Only the first injection had a volume of 0.5 μL and was discarded from the isotherm. The other technical parameters were reference power = 5 μcal s^{-1}, stirring speed = 1000 rpm, spacing time = 180 s, and a filter period = 5 s. The control experiment (NisC in buffer) was consistently subtracted from each isotherm, and the resulting isotherm was fitted with a one site binding model using the Origin 7 Microcal software.

Complex Formation Analysis Using MALS-SEC. Twenty micromolar NisC was incubated with 200 μM mature nisin or precursor peptide variants for 1 h at 25 °C. Five hundred microliters were injected on a SEC column (Superdex 200 10/300 (GE Healthcare)) pre-equilibrated with MALS buffer (50 mM HEPES-NaOH, pH 7.0, 500 mM NaCl) with a flow rate of 0.4 mL min^{-1}. Absorption signal at 280 nm, light scattering signal (triple-angle light scatter detector miniDAWM TREOS, Wyatt Technology Europe), and differential refractive index (Optilab rEX detector (Wyatt Technology Europe) were monitored. Data were analyzed with the ASTRA software package (Wyatt Technology).

NisP Purification. The *L. lactis* strain NZ9000 with plasmid pNG-NisP8His allowed for the expression of the serine protease NisP. A 50 mL GM17 preculture was supplemented with 5 μg mL^{-1} chloramphenicol and grown overnight at 30 °C. Cells were harvested, and 500 mL of MM-medium was inoculated with a starting OD_{600} of 0.1. Expression was induced with nisin at a final concentration of 0.1 ng mL^{-1}. Cells were grown overnight at 30 °C and removed by centrifugation (15,000*g*, 30 min). The pH of the resulting supernatant was adjusted with 3 M Tris-HCl to pH 7.5 and applied to an IMAC HP column (GE Healthcare) preloaded with Co^{2+} at a flow rate of 4 mL min^{-1}. The column was intensively washed with buffer (50 mM HEPES-NaOH, pH 8.0, and 150 mM NaCl) until a stable baseline was reached, and NisP was eluted in a single step (50 mM HEPES-NaOH, pH 8.0, 150 mM NaCl, and 300 mM imidazole). Elution fractions were concentrated by ultracentrifugation (10 kDa MWCO) to 2.5 mL, and the buffer was exchanged with a PD10 column (GE Healthcare) to 50 mM HEPES-NaOH, pH 8.0, 150 mM NaCl, and 10% glycerol. The eluting fractions were concentrated to 250 μL, and NisP was aliquotted and stored at −80 °C.

Leader Peptide Preparation and Precursor Peptide Digestion. The nisin leader peptide (amino acids −23 to −1) was produced by digestion of the modified precursor peptide by the protease NisP. To this end, the purified modified precursor peptide was incubated with purified NisP in a molar ratio of 1000:1 at 30 °C overnight. The reaction mixture was applied to a RP-HPLC column, and the eluting leader peptide was fractionated and lyophilized. The corresponding leader peptide was dissolved in SEC buffer and used for ITC and MALS-SEC measurements.

MALS-SEC Analysis of NisC with NisP Digested Precursor Peptides. The unmodified, dehydrated, and modified precursor peptides were digested with NisP as described above. Before MALS-SEC analysis, NisP was

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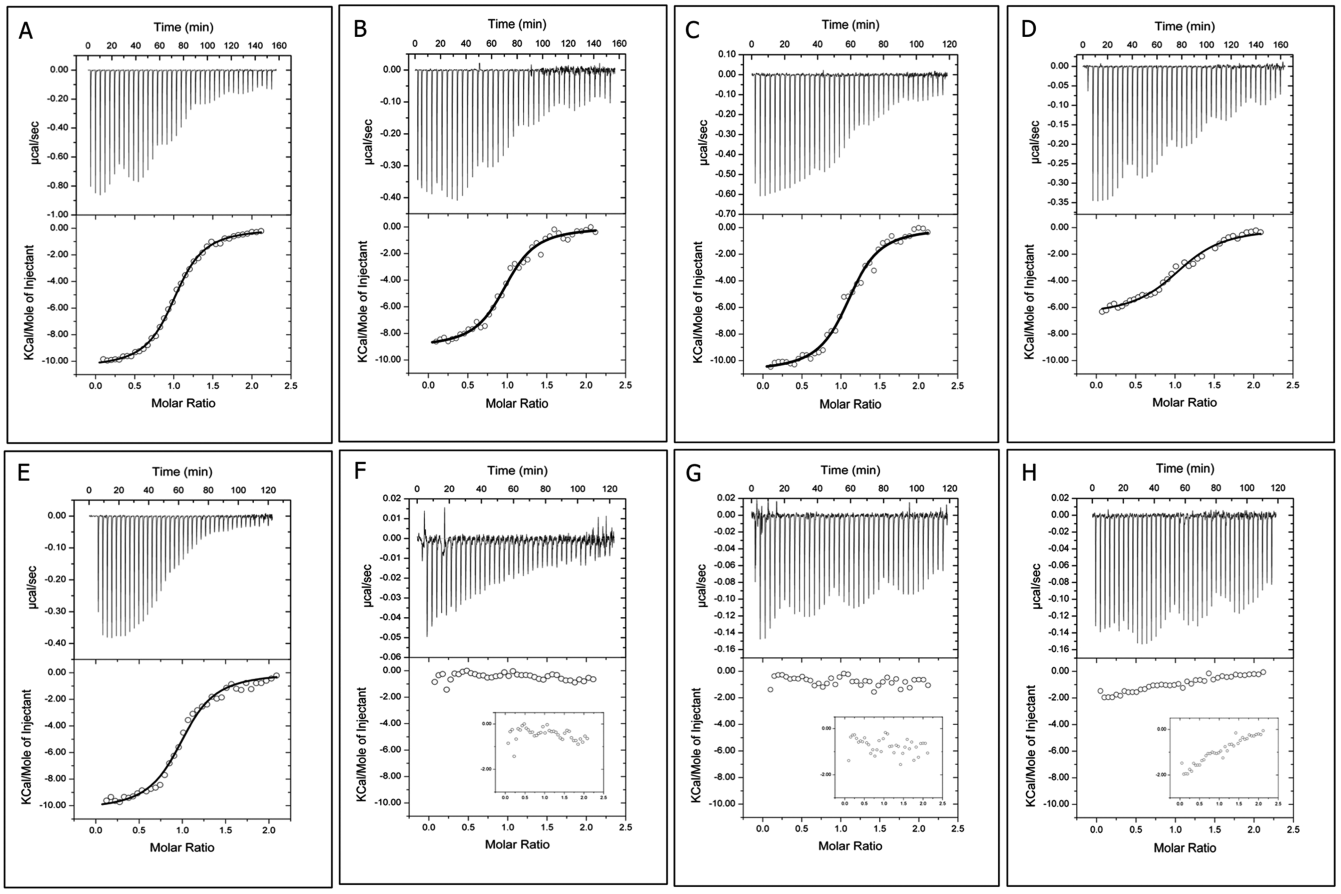

Figure 2. ITC experiments of NisC and different precursor peptide variants. A: unmodified precursor peptide. B: dehydrated precursor peptide. C: modified precursor peptide. D: leader peptide. E: $N_{-17}A$ variant. F: $F_{-18}A$ variant. G: -AAAA- variant. H: NisC is titrated into SEC buffer. All experiments were performed at least in triplicate. For detailed thermodynamic parameters, see Table 1.

extracted from the reaction mixture by binding to Ni^{2+} coupled magnetic beads (Qiagen) for 1 h at room temperature.

RESULTS

Purification of the Cyclase NisC. The enzyme NisC was isolated as described in detail in Materials and Methods. NisC was analyzed by SEC resulting in a single peak of NisC. His_6-NisC was purified to more than 95% purity as judged from SDS−PAGE analysis with a yield of roughly 10 mg L^{-1} of cell culture and stored at −80 °C until further use (Figure S1, Supporting Information). Prior to the interaction studies, the His_6-tag was removed using thrombin (see Materials and Methods). This was necessary because the His_6-tag inhibited the interaction of NisC with the precursor peptide (data not shown). Subsequently, the MALS-SEC analysis demonstrated that tag-less NisC is a monomer in solution (see below and Figure S2, Supporting Information, black line).

Purification of Mature Nisin and the Precursor Peptide Variants. All precursor peptide variants used in this study (Figure 1) were expressed in *L. lactis* and secreted using a two-plasmid system as previously described.[23,34,35] The isolation is described in detail in the Materials and Methods section.

All precursor peptide variants, mature nisin, and the leader peptides were analyzed by tricine SDS−PAGE (Figure S3, Supporting Information) and reached at least 90% purity. In contrast, the purity of the -AAAA- mutation of the -FNLD- box within the leader peptide was only around 70%, which is likely due to the low secretion level preventing an efficient purification.[25]

To perform an accurate and reproducible ITC experiment, the exact concentration of each interaction partner had to be known precisely. To determine the specific concentration of the precursor peptides, we employed analytical RP-HPLC. Different nisin and insulin samples of known concentrations were used to calibrate the RP-HPLC column. Here, the absorption of the peptide bond at 205 nm was monitored, which is independent of the exact amino acid composition. The resulting peaks were integrated and plotted against the known nisin and insulin concentrations. Figure S4 (Supporting Information) summarizes the obtained calibration line, which was used in all subsequent experiments to determine the concentration of the different nisin and nisin intermediate samples. For clarity, the RP-HPLC elution profiles are summarized in Figure S5 (Supporting Information).

Interactions of NisC with the Precursor Peptide Variants. We applied ITC to characterize potential interactions between NisC and nisin precursor peptides and variants. Because of the high solubility of NisC and the lower solubility of the precursor peptides, especially at high concentrations, the classic ITC setup was inverted. Here, the syringe was filled with a concentrated NisC solution (400 μM), and the measuring cell was filled with the corresponding precursor peptide (40 μM). Initially, the unmodified, dehydrated, and modified precursor peptides were measured (see Figure 2A−C). All three precursor peptides resulted in an

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Table 1. ITC Data of NisC and the Precursor Peptide Variants[a]

precursor peptide variant	N	K_D [μM]	$\Delta H°$ [kJ/mol]	$\Delta S°$ [J/mol·K]	$\Delta G°$ [kJ/mol]
unmodified	1.1 ± 0.1	2.4 ± 0.5	−44.1 ± 3.3	−40.1 ± 10.5	−32.1 ± 0.5
dehydrated	1.1 ± 0.1	2.0 ± 0.2	−46.0 ± 7.1	−62.6 ± 8.9	−27.3 ± 1.5
modified	1.0 ± 0.1	2.0 ± 0.5	−55.3 ± 2.2	−75.5 ± 5.6	−32.9 ± 0.9
leader peptide	1.0 ± 0.2	3.8 ± 0.6	−34.9 ± 4.6	−20.3 ± 7.3	−30.3 ± 1.3
-AAAA-	no binding observed in ITC measurements				
-AALD-	no binding observed in ITC measurements				
-FNAA-	no binding observed in ITC measurements				
-ANLD-(F_{-18}A)	no binding observed in ITC measurements				
-FALD-(N_{-17}A)	1.1 ± 0.2	1.4 ± 0.2	−41.2 ± 2.2	−25.8 ± 8.3	−33.5 ± 0.3
-FNAD-(L_{-16}A)	no binding observed in ITC measurements				
-FNLA-(D_{-15}A)	1.1 ± 0.1	7.1 ± 1.6	−32.9 ± 4.3	−17.5 ± 3.1	−29.0 ± 1.1
NisC	control experiment				

[a]All experiments were performed at least in triplicate, and the error represents the standard deviation of a minimum of three independent experiments. For experimental details, see Materials and Methods.

isotherm indicating specific binding. Furthermore, they displayed a similar equilibrium or dissociation constant (K_D) of 2.4 ± 0.5 μM for the unmodified, 2.0 ± 0.2 μM for the dehydrated, and 2.0 ± 0.5 μM for the modified precursor peptide (Figure 2A−C and Table 1). Within experimental error, a 1:1 stoichiometry of the individual complexes was determined (Table 1). This suggests that the maturation state of the core peptide does not influence the binding affinity of NisC to the precursor peptides. In other words, the leader peptide is sufficient for an effective interaction.

To validate this hypothesis, the leader peptide was produced by incubation of the modified precursor peptide with the purified protease NisP (see Materials and Methods and Supporting Information, Figure S6). The two products of the cleavage reaction, the leader peptide and the core peptide, were separated by RP-HPLC (Figure 3A). After purification, the interaction of the isolated leader peptide with NisC was analyzed by ITC (Figure 2D). A binding affinity of K_D = 3.8 ± 0.6 μM and a 1:1 stoichiometry of the leader peptide and NisC complex were observed (Table 1). A second technique, MALS-SEC, was used to verify this result. MALS-SEC allows for the determination of absolute masses of a protein or a protein complex in solution. Incubation of NisC with the precursor peptides resulted in a shift of the elution profile toward earlier retention times (Figure S2, Supporting Information, and Table 2). The molecular weight determined by MALS was 48550 ± 1085 Da for NisC. For the complex of NisC with unmodified, dehydrated, or modified precursor peptide, the analysis resulted in 55730 ± 1280 Da, 56310 ± 1730 Da, and 55260 ± 1100 Da, respectively (highlighted by the green dotted line in Figure S2 (Supporting Information) for the dehydrated precursor peptide; see Table 2). The difference in molecular mass of the protein−ligand complexes corresponds to the molecular mass of a single precursor peptide within experimental error. The reinjection of the NisC−precursor peptide complex resulted in an identical elution profile and identical difference in molecular mass (data not shown). This highlights that the NisC−precursor peptide complex is stable. Furthermore, a shift in retention time as well as a stable complex with a determined molecular mass of 51250 ± 1420 Da was observed by performing the analysis with the isolated leader peptide (highlighted by the orange line in Figure S7, Supporting Information, and Table 2). Subsequently, MALS-SEC analysis between NisC and mature nisin was performed. The observed mass of 49260 ± 1290 Da corresponded to the molecular mass of monomeric NisC (highlighted by the black lines in Figure 3B and Table 2). This shows that the leader peptide is responsible for binding to NisC.

Does the Leader Peptide Contain All Required Information for Interaction? These thermodynamic ITC data demonstrate that the affinity of the complex of NisC and the isolated leader peptide was slightly lower than the affinity of the complex of NisC and the isolated nisin intermediates. This difference was small but statistically reproducible from three independent experiments. One can explain these data by low affinity binding by the whole or by part of the core peptide. This low affinity binding would only become accessible after the leader peptide has formed a stable complex due to, for example, an induced conformational change.

To verify this hypothesis, the modified precursor peptide was digested with NisP resulting in the leader peptide and mature nisin (Figure 3A). This mixture was incubated with NisC and subsequently analyzed by MALS-SEC resulting in only a NisC−leader complex. (Figure S7, Supporting Information, and Table 2). Even a 4-fold access of mature nisin added to a preformed NisC−leader complex provided no indication of binding of the core peptide of mature nisin (Figure 3B and Table 2).

Identical results were obtained for the core peptides of unmodified and dehydrated nisin. Here, the core peptides were obtained by digestion of the corresponding nisin intermediates with NisP. A successful and quantitative cleavage was verified by MS analysis. The mixture was analyzed by MALS-SEC resulting only in a NisC−leader peptide complex (Figure S7, Supporting Information, and Table 2). Altogether, this shows that the leader contains the recognition motif for the cyclase NisC.

Specific Binding Motif within the Leader Peptide. To further investigate the binding site within the leader peptide, alanine mutagenesis within the highly conserved -FNLD- box was performed.[25] The seven precursor peptide variants used in this study are summarized in Figure 1 and were expressed and purified as the modified precursor peptides (see Materials and Methods).

The interaction of NisC with the precursor peptide was abolished in the -AAAA- variant of the -FNLD- box (Figure 2G and Table 1). The ITC isotherm resulted in a graph similar to that of the control experiment (Figure 2H and Table 1), where NisC was titrated into buffer only indicating no interaction. This result was verified by MALS-SEC analysis revealing only

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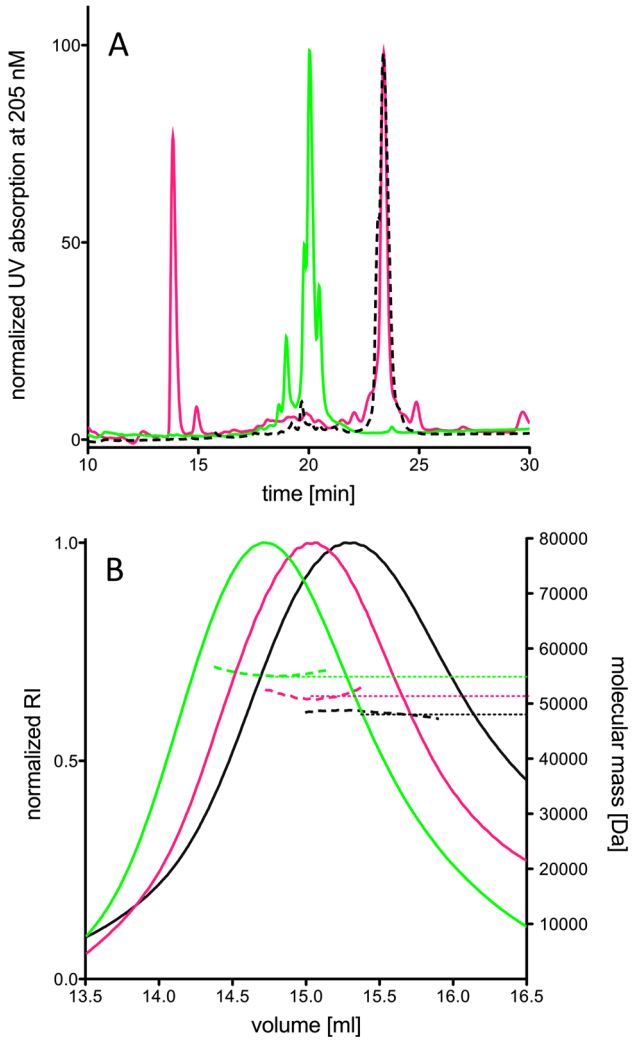

Figure 3. (A) NisP digestion of the modified precursor peptide observed by RP-HPLC. The modified precursor peptide (green) was digested with NisP and analyzed by RP-HPLC. The digested sample (pink) showed two peaks. One at an elution time of 14.5 min. This corresponded to the leader peptide, which was confirmed by MS. The second peak eluted at 24 min. The elution time is identical to that of mature nisin shown in black. All experiments were performed at least in triplicate. (B) MALS-SEC analysis of NisC and the modified precursor peptide before and after NisP digestion. Green line: elution profile of NisC incubated with the modified precursor peptide. The observed molecular mass of NisC in complex with the modified precursor peptide is shown in the green dotted line (the horizontal green dotted line indicates the expected molecular mass of a NisC–precursor peptide complex). Black line: elution profile of NisC incubated with mature nisin. The observed mass of NisC incubated with mature nisin shown in the black dotted line (the horizontal black dotted line indicates the expected molecular mass of a NisC monomer). Pink line: elution profile of NisC presaturated with a 2-fold excess of the leader peptide incubated with a 4-fold excess of mature nisin. The pink dotted line shows the observed molecular mass. The horizontal pink line represents the expected molecular mass of a NisC–leader peptide complex. All experiments were performed at least in triplicate. See Table 2 for a detailed summary of the observed molecular masses.

monomeric NisC without any precursor peptide bound (Table 2). Also, the variants -AALD- and -FNAA- were analyzed and did not show any interaction with NisC in the ITC experiment (Table 1).

Table 2. Overview of the MALS-SEC Analysis between NisC and Mature Nisin and Precursor Peptide Variants[a]

(pre)nisin	MALS-SEC analysis [Da]	difference to NisC [Da]
NisC	48550 ± 1086	
unmodified	55730 ± 1280	7180 ± 274
dehydrated	56310 ± 1730	7760 ± 911
modified	55260 ± 1100	6710 ± 20
mature nisin	49260 ± 1290	710 ± 288
leader peptide	51250 ± 1420	2700 ± 472
-AAAA-	48350 ± 1124	−200 ± 54
leader + modified core peptide (1:1)	51080 ± 1120	2530 ± 48
leader + modified core peptide (1:2)	50690 ± 1318	2140 ± 328
leader + unmodified core peptide (1:1)	50570 ± 1140	2020 ± 76
leader + dehydrated core peptide (1:1)	51220 ± 1060	2670 ± 37

[a]The first row summarizes the analyzed samples. The observed molecular masses obtained by MALS-SEC analysis are shown in row two. In the third row, all potential NisC–substrate complexes were subtracted by the mass of isolated NisC. These data indicate that one single precursor peptide, specifically one leader peptide molecule, is interacting with NisC within the inaccuracy of the MALS-SEC analysis. For experimental details, see Materials and Methods.

Within the -FNLD- box sequence of class I lantibiotics, the F_{-18} and the L_{-16} are highly conserved, whereas slight variations are observed at position −17 (N in nisin) and position −15 (D in nisin) (Figure 4). Single amino acid replacements by alanine showed no binding for F_{-18}A and L_{-16}A to NisC, whereas N_{-17}A and D_{-15} both were recognized by the cyclase NisC. N_{-17}A had a similar affinity of 1.4 ± 0.2 μM (Figure 2E) for NisC as the wild type precursor peptide and the D_{-15}A mutant showed a slighty lower affinity of 7.1 ± 1.6 μM (Table 1).

Taken together, these data show that the highly conserved amino acids F_{-18} and L_{-16} within the -FNLD- box are essential for binding to NisC. Both have to be present simultaneously with respect to the ITC result of the -AALD- and -FNAA- variants. The mutation of N_{-17} had no impact on the binding; similarly, D_{-15} played only a minor role in recognition as observed by a slightly increased K_D.

DISCUSSION

Lantibiotics are peptides, which possess antimicrobial activity against Gram-positive bacteria such as *Streptococcus pneumonia*, *Staphylococcus aureus*, *Enterococcus faecium*, and *Enterococcus faecalis*.[11,36] This activity is mediated by at least two modes of action: (i) by binding to the cell wall precursor lipid II, which inhibits cell wall synthesis, and (ii) by forming pores within the membrane.[2,37,38] All lantibiotics share post-translational modifications like dehydration and cyclization.[19] The dehydrated amino acids and more significantly the (methyl)lanthionine rings are essential for antimicrobial activity.[5] Furthermore, the characteristic (methyl)lanthionine rings protect the peptide against proteases and increase the stability of the lantibiotic.[24,39]

Within the nisin biosynthesis system, NisB and NisC, are responsible for the modification and maturation process.[17] The exact stoichiometry of the modification complex consisting of the dehydratase NisB and the cyclase NisC has not been investigated in detail. Recently, it was shown that NisB is a dimer in solution[33] and binds to a single precursor peptide.

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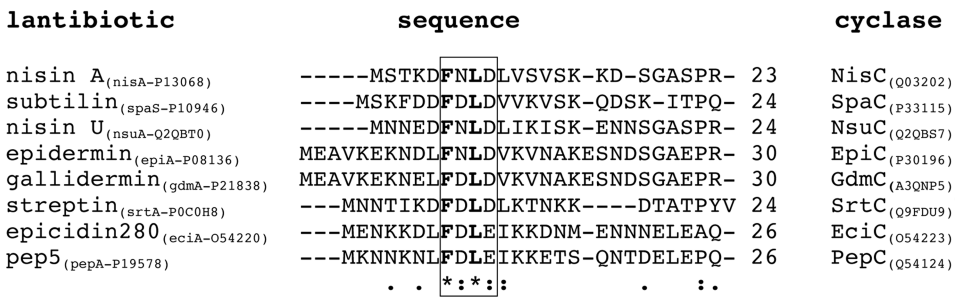

Figure 4. Sequence alignment of the -FNLD- box created with ClustalW2 based on the amino acid sequence taken from the UniProtKB database. The UniProtKB entry number is shown in parentheses. The asterisk (*) shows single fully conserved amino acids. Conserved residues are highlighted by (:) and similarity by (.).

Presumably, NisB is also dimeric in the NisBC complex purified directly from the cytosol of *L. lactis*,[40] where it has been suggested to have a stoichiometry of $NisB_2$/NisC/precursor peptide. Our *in vitro* data show that NisC is monomeric in solution and furthermore that NisC is interacting with a single precursor peptide as determined in ITC and MALS-SEC.

The X-ray structure of NisC was solved revealing a monomeric zinc-dependent protein.[20,31] The structure revealed an α,α barrel toroid domain consisting of 14 α-helices and an extended domain, which represents a SH2-like fold. The active site contained a zinc finger motif composed of two conserved cysteines and one conserved histidine residue, which are essential for catalysis. A putative reaction mechanism and a binding site for part of the core peptide were proposed by modeling. However, the specific binding sites for the leader as well as for the core peptide could not be identified.[20]

The importance of the leader peptide for both modification enzymes has been shown by fusion with therapeutic peptides in *L. lactis*. The adrenocorticotropic hormone (ACTH), which acts similar to cortisol in the adrenal cortex, the luteinizing hormone-releasing hormone (LHRH), or the cardiovascular peptide angiotensin was dehydrated and cyclized using this methodology, resulting in a higher stability and half-life time.[18,41,42] This shows that the leader peptide contains information, which is sufficient to initiate the modification process.

Here, we show that the leader peptide of the nisin precursor peptide is necessary and more importantly sufficient for an *in vitro* interaction with NisC. The precursor peptides used in this study are composed of two distinct parts, the N-terminal leader and a partially or fully modified C-terminal core peptide. ITC and MALS-SEC showed that only the leader peptide interacts with NisC, while no interaction was observed with the isolated core peptide, independently of the modification state. This is in line with the observation that NisC binds the precursor peptides with similar affinities, but is in contrast to NisB, which displays different affinities for these different forms.[33]

Our observed *in vitro* interaction of NisC with the leader peptide and the -FNLD- box has been suggested by previous *in vivo* data.[16,25,40] This -FNLD- box is conserved in all class I lantibiotics.[27] A sequence alignment of selected examples of class I lantibiotics including the corresponding LanC protein is shown in Figure 4. Here, F_{-18} and L_{-16} are highlighted, which are strictly conserved, while N_{-17} and D_{-15}, display variations. This suggests that the side chains of this motif might represent an interaction platform.

We also showed that the -FNLD- box is the major determinant for binding to NisC. Within this box, the highly conserved F_{-18} and L_{-16} are indispensible for this interaction. In light of these results, the variants -AALD- and -FNAA- emphasize that both amino acids, F and L, have to be present to ensure binding to NisC. Mutation of either one abolished binding completely. Thus, the identified recognition motif within the -FNLD- box of the leader peptide is FxLx. Within the crystal structure of NisC, a binding groove lined with hydrophobic and negatively charged residues was identified in close proximity to the Zn^{2+} binding site.[20] This groove was proposed to interact with the positively charged leader peptide of nisin. Our results would be in line with this suggestion. If this groove indeed represents the binding site of the leader peptide, the F and L would interact with the hydrophobic residues of this extended groove, while a repulsion would be generated between the negatively charged residues of the groove and N and D of the leader peptide. This might align the leader peptide in an orientation that would facilitate a Zn^{2+} mediated cyclization of the core peptide.

In vivo analysis of the above-mentioned double alanine mutants showed that one to two dehydrations were missing but that the resulting cleaved peptide still displayed antimicrobial active.[25] Also, for the single alanine variants full dehydration pattern and antimicrobial activity were observed. This implies that the *in vivo* situation is different, which is likely due to the presence of both, NisB and NisC, within the cell.

Interestingly, the -FNLD- box also forms the binding site for the dehydratase NisB.[33] This suggests a mechanism in which both modification enzymes compete for the -FNLD- box and that this motif could be the trigger for a handing over of nisin from one modification enzyme to the other, resulting in a sequential reaction mechanism as proposed by Lubelski et al.[26] Pulldown assays performed by Khusainov et al. resulted in predominantly NisB−precursor and only low amounts of the NisB−NisC−precursor complex. Additionally, an alanine mutagenesis study within the leader peptide revealed that that the NisC−precursor complex was not observed if NisB was not present.[43] This suggests that NisB has a tighter interaction with the precursor peptides than NisC[40] suggesting that NisB is responsible for the initial binding to the precursor peptide.[33,40] A ping−pong mechanism was already proposed,[26] and our *in vitro* data support such a scenario. After the modification reactions are completed, the affinities towards the modified precursor peptide become different for the two modification enzymes. Here, we show that NisC possesses a five times higher affinity for the fully modified precursor peptide when compared to NisB (K_D = 10.5 ± 1.7 μM).[33] This could indicate that NisB initiates the modification steps of the precursor peptide by binding to the unmodified precursor peptide, while the cyclase

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reaction of NisC is terminating the modification and a subsequent cycle of modifications can be initiated. After all modifications are terminated, the NisB/NisC/precursor complex disengages. This is supported by a wild type complex consisting of NisB, the precursor peptide, and NisC that was isolated in low amounts.[40] The complex was, however, stabilized, by introducing the H331A mutation inactivating NisC resulting in a higher yield.

In summary, our thermodynamic data demonstrate that NisC is interacting only with the nisin leader peptide independent of the core peptide. In addition and more precisely, NisC interacts with the -FNLD- box of the leader peptide. Especially, the highly conserved FxLx motif might represent a general recognition motif for the LanC protein family.

■ ASSOCIATED CONTENT

Supporting Information

SDS−PAGE of NisC, NisA, and NisP; MALS-SEC analysis of NisC with digested precursor peptides; RP-HPLC calibration and elution profiles of the precursor peptide variants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

RP-HPLC, reverse phase-high pressure liquid chromatography; ITC, isothermal titration calorimetry; IMAC, immobilized metal-ion affinity chromatography; MALS, multi angle static light scattering; SEC, size exclusion chromatography; Dha, dehydroalanine; Dhb, dehydrobutyrine

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