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Structural basis of lantibiotic recognition by the nisin resistance protein from *Streptococcus agalactiae*

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Lantibiotics are potent antimicrobial peptides. Nisin is the most prominent member and contains five crucial lanthionine rings. Some clinically relevant bacteria express membrane-associated resistance proteins that proteolytically inactivate nisin. However, substrate recognition and specificity of these proteins is unknown. Here, we report the first three-dimensional structure of a nisin resistance protein from *Streptococcus agalactiae* (SaNSR) at 2.2 Å resolution. It contains an N-terminal helical bundle, and protease cap and core domains. The latter harbors the highly conserved TASSAEM region, which lies in a hydrophobic tunnel formed by all domains. By integrative modeling, mutagenesis studies, and genetic engineering of nisin variants, a model of the SaNSR/nisin complex is generated, revealing that SaNSR recognizes the last C-terminally located lanthionine ring of nisin. This determines the substrate specificity of SaNSR and ensures the exact coordination of the nisin cleavage site at the TASSAEM region.

Antibiotics provide a great advantage in the treatment of infections caused by bacteria such as *Streptococcus pneumoniae* and *Streptococcus agalactiae*. However, due to their widespread use, the number of resistant bacterial strains is increasing¹, leading to an urgent need for the development of new antibiotics. Several approaches have been taken to identify new antibiotics where naturally occurring compounds are found to be the most promising ones². Here, small antimicrobial peptides such as lantibiotics are excellent candidates because they exhibit high effectivity against various Gram-positive human pathogenic bacteria including *Streptococcus pneumoniae* and several methicillin-resistant *Staphylococcus aureus* (MRSA) strains³.

Lantibiotics display antimicrobial activities in the very low nanomolar range^{4,5}. The anti-infective potency of lantibiotics such as nisin, mutacin, mersacidin and others has been recognized, and several are in the preclinical stages of medical application^{6,7}. After translation, lantibiotics are modified and contain unusual amino acids such as dehydroalanine (Dha) and dehydrobutyrine (Dhb), which are covalently linked to the side chain of cysteine residues forming the so-called lanthionine rings^{8,9}. The number as well as the exact location of the lanthionine rings vary within lantibiotics¹⁰. Lantibiotics have multiple modes of action, of which binding to lipid II, thereby inhibiting cell wall synthesis, and pore formation are the most predominant ones^{8,11}. Nisin produced by *Lactococcus lactis* (*L. lactis*) is one of more than 50 lantibiotics discovered so far¹² and is considered to be the role model. Active nisin consists of 34 amino acids and contains five lanthionine-based rings (Supplementary Fig. 1). The first three rings (A-C) are separated from the other two intertwined rings (D-E) by a flexible hinge region. The first two rings are able to bind lipid II¹³; the hinge region and the last two intertwined rings are able to flip into the membrane and create a pore^{14–16}.

Due to their multiple modes of action, hardly any resistance against lantibiotics has developed over the past decades. However, some bacterial strains have been reported to be congenitally resistant against nisin¹⁷ via various

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mechanisms such as cell wall modifications, biofilm formation or the expression of resistance proteins¹⁸. For the latter case, a *nsr* gene was identified in the *Streptococcus lactis* subspecies *diacetylactis* (DRC3) that encodes the nisin resistance protein, NSR^{17,19}. Similar genes were identified in other species^{17,20,21}, including several human pathogenic strains^{22,23}. NSR is a member of the S41 protease family, specifically the C-terminal processing peptidases (CTPs). NSR from *L. lactis* TS1640 has been shown to degrade nisin by cleaving the peptide bond between MeLan₂₈ in ring E and Ser₂₉. The resulting nisin₁₋₂₈ fragment displays a significantly lower bactericidal efficacy and reduced affinity towards cellular membranes²⁴. Furthermore, the NSR protein from *S. agalactiae* ATCC 13813 induced a 20-fold increased resistance towards nisin when expressed in *L. lactis*²².

NSR is localized within an operon comprising five genes, which encode for NSR, a two-component signaling system (NsrRK), and an ABC transporter (NsrFP). When expressed together, these proteins deliver full nisin resistance²². Interestingly, similar operon structures were also found to be associated with resistance against other lantibiotics^{18,23}. These operons resemble (auto)-immunity systems found in lantibiotic producer¹⁰ strains. Structures of SpaI from *B. subtilis*²⁵ conferring resistance against subtilin and MlbQ from the actinomycete *Microbispora* ATCC PTA-5024 conferring resistance against NAI-107²⁶ were resolved by NMR. However, no significant sequence identity is found between NSR and SpaI or MlbQ, suggesting a different mechanism for the defense against lantibiotics. Furthermore, most (auto)-immunity proteins do not cleave or manipulate the lantibiotic but rather shield the host's membrane from being harmed by its lantibiotic^{10,27}.

The ability of NSR to cleave nisin is impressive because it has been shown for several lantibiotics that they are not easily accessible for protease cleavage¹⁴. Here, the lanthionine rings are likely causing steric hindrance within the active site of proteases, thereby inhibiting proteolysis. Thus, notwithstanding the recent advances in this field, we still structurally know relatively little about lantibiotic resistance. In particular, the lantibiotic binding site in NSR and the mechanism how substrate specificity is conferred remains elusive. In this study, we report the first structure of a nisin resistance protein, NSR from *S. agalactiae* COH1 (*SaNSR*). Mutagenesis studies guided by molecular dynamics (MD) simulations reveal that *SaNSR* recognizes the lanthionine ring closest to the C-terminus of nisin and that this ring binds at one end of the catalytic tunnel, thereby determining the substrate specificity and ensuring the exact coordination of the nisin cleavage site at the catalytic site region.

Results

Crystal structure of *SaNSR*. Nisin has been shown to be quite resistant against proteolytic digestion in general¹⁴, supposedly due to the presence of lanthionine rings. Therefore, it is intriguing to understand the proteolytic resistance mechanism mediated by NSRs. To obtain a molecular view on this mechanism, we solved the structure of *SaNSR* by X-ray crystallography.

Through sequence analyses, it was predicted that the first 30 amino acids encode for a transmembrane helix²⁸. We deleted this N-terminal transmembrane helix and included a His₈-tag for purification purposes, resulting in soluble expression of *SaNSR*. After over-expression, two-step purification yielded 5 mg of pure *SaNSR* protein per liter of cell culture (Supplementary Fig. 2a). *SaNSR* is a monomer in solution as determined by multiple angle light scattering (MALS) (Supplementary Fig. 2b). *SaNSR* protein was crystallized and cubic crystals were obtained that diffracted up to 2.2 Å resolution²⁹. We solved the structure by Single Anomalous Dispersion (SAD) phasing, using crystals of selenomethionine-substituted protein (data and refinement statistics are shown in Table 1).

The asymmetric unit (Supplementary Fig. 2c) contained four copies of *SaNSR* that were virtually identical (root mean square deviation (RMSD) between the monomers = 0.15–0.5 Å over 300 amino acids). Therefore, the overall structure is described only for monomer A. The entire sequence of *SaNSR* could be fitted into the electron density, with the exception of the N-terminal His₈-tag that was disordered. The R_{work} and R_{free} values after refinement were 0.19 and 0.24, respectively.

A *SaNSR* monomer (Fig. 1) consists of eleven helices (α_1 – α_{11}) and eleven β -strands (β_a – β_k), which form three domains: an N-terminal helical bundle and two protease subdomains. Altogether, these domains form a hydrophobic tunnel of ~10 Å width (Fig. 1b), which could very well harbor the nisin molecule. The N-terminal helical bundle (Fig. 1b, represented in green) comprises 65 amino acid residues (Lys₃₁–Gly₉₆), which form helices α_1 – α_3 . This domain ends in a triple glycine motif (₉₄G₉₅G₉₆) before entering the protease cap domain (Fig. 1b, represented in red). The protease cap domain consists of helix α_4 and a β -hairpin structure formed by strands $\beta_{i,j}$. The protease cap forms a lid-like structure above the tunnel. The third domain is the so-called protease core domain (Fig. 1b, represented in grey), which adopts a 'protease fold' domain as observed in other S41 peptidases^{30–32}. The protease core domain is formed by six strands β_b – β_g and five helices α_5 – α_9 . It contains the highly conserved TASSAEM region that harbors the previously identified catalytically active serine at position 236²² (Fig. 1, represented in blue; Supplementary Fig. 3). The TASSAEM region lies in the tunnel between the two protease subdomains (Fig. 2a).

N-terminal helical bundle. A comparison of the N-terminal helical bundle with all available entries in the Protein Data Bank was performed using the Dali server³³. The Dali server identifies similarities in 3D structures irrespective of sequence similarities. A structurally similar helical bundle has been identified in the human Factor H (Z-score of 5.2), which is responsible for tight binding of the pneumococcal protein virulence factor CbpA (choline-binding protein A)³⁴. Furthermore, a similar helical bundle is present at the C-terminus of the 70 kDa human heat shock protein (HSP70) (Z-score of 5.0). This region is responsible for causing a structural switch during HSP70 allosteric activation, which is important for maintaining a proper conformation of the protein for binding to the J-domain and ATPase activity purposes³⁵. Finally, a dynamic helical region is present at the N-termini of staphylococcal complement inhibitors (SCINs) (Z-score of 4.7), which is responsible for binding to the substrate C3b and is also necessary for the formation of higher order complexes of C3b, which blocks phagocytosis³⁶. While these findings suggest that a certain degree of mobility of the found helical bundles is required for function, in three replicates of molecular dynamics (MD) simulations of a monomer of *SaNSR*

	Native SaNSR	SeMet SaNSR
Data collection		
Space group	$P2_12_12_1$	$P4_32$
Cell dimensions		
a, b, c (Å)	58.8, 137.2, 164.0	186.1, 186.1, 186.1
α, β, γ (°)	90, 90, 90	90, 90, 90
Wavelength	0.87260	0.97625
Resolution (Å)	100.0–2.21 (2.29–2.21)	100.0–2.80 (2.9–2.8)
R_{merge}	11.5 (63.8)	29.3 (110.5)
$\langle I / \sigma(I) \rangle$	8.67 (1.79)	20.33 (1.72)
Completeness (%)	99.6 (99.3)	99.8 (98.7)
Redundancy	4.5 (4.2)	75.6 (70.5)
Refinement		
Resolution (Å)	55.36–2.21 (2.28–2.21)	
No. reflections	303208 (27954)	
$R_{\text{work}}/R_{\text{free}}$	0.19 (0.27) / 0.24 (0.31)	
No. of atoms	9588	
Protein	9017	
Ligand/ion	48	
Water	523	
B-factors (Å ²)	40.5	
Protein	40.3	
Ligand/ion	68.1	
Water	41.2	
R.m.s deviations		
Bond lengths (Å)	0.008	
Bond angles (°)	1.09	

Table 1. Data collection, phasing and refinement statistics for SaNSR. *Values in parentheses are for highest-resolution shell.

Figure 1. Structural architecture of the SaNSR monomer. (a) Schematic illustration of the domain organization of SaNSR indicating the domain borders and catalytically important residues (His₉₈ and Ser₂₃₆). (b) The overall structure of a SaNSR monomer in a cartoon representation. The N-terminal helical bundle is depicted in green where the light green region represents the N-pep. The protease cap and core domains are highlighted in red and grey, respectively. The catalytically important residues and the highly conserved “TASSAEM” region are depicted in blue.

Figure 2. Surface representation of SaNSR. (a) The surface representation of SaNSR in white, highlighting the tunnel localized in between the protease cap and the core domain. The TASSAEM motif is colored in blue. (b) Surface representation of SaNSR with bound N-pep (colored in orange). (c) Stereo view on the active site architecture of SaNSR highlighting the N-pep that is bound within the tunnel as ball and stick representation. The corresponding $2F_o - F_c$ omit electron density map is calculated at $2.2 \text{ \AA}$ and contoured at 1.0σ . The water mediated interactions of N-pep (colored in orange) with residues of the protease cap (depicted in red) and the direct interactions with the residues of the protease core (grey color) are shown.

(termed NSR_{Apo}; see online methods for details), each of 500 ns length, the N-terminal helical bundle is rather immobile with respect to the protease core and cap domain (backbone root mean square fluctuations (RMSF) $< 2.5 \text{ \AA}$; Supplementary Fig. 4a).

N-pep bound to SaNSR. In the crystal structure, the hydrophobic tunnel is filled with the N-terminal residues ₃₁KNIYLLPP₃₈ of a neighboring SaNSR molecule (termed N-pep; Fig. 2b; shown in light green in Fig. 1). N-pep is predominantly bound to SaNSR via direct backbone interactions to amino acids ₁₆₇NNTGGN₁₇₂ of β -strand β_d , which is part of the protease core domain and is structurally located on the opposite site of the TASSAEM sequence motif (Fig. 2c). In addition, N-pep is stabilized via water-mediated hydrogen bonds between backbone atoms of Asn₃₂, Tyr₃₄, Leu₃₅ and residues Asn₂₆₅ and Thr₂₆₇ of the protease cap domain (Fig. 2c). The presence of N-pep within the tunnel is clearly an induced artifact of the crystallization procedure, since in the full-length SaNSR protein, another 30 amino acids are attached at the N-terminus of the N-pep sequence that form a transmembrane helix. Yet, during MD simulations of 500 ns length of a SaNSR monomer complexed with N-pep (termed NSR_{tail}; see online methods for details), N-pep remains stably bound within the hydrophobic tunnel (mean backbone RMSD $< 1.6 \text{ \AA}$; Supplementary Fig. 4b). The predominance of backbone interactions of N-pep with the protease core and cap domains could explain why N-pep binds into the putative binding region of nisin despite its sequence being very dissimilar to the one of nisin.

Substructures of nisin determining its molecular recognition. To investigate the substrate specificity of SaNSR and determine substructures of nisin important for its recognition by the protein, we used different

Figure 3. Influence of wild type SaNSR and its mutations against nisin and its variants. (a) Growth inhibition experiment of SaNSR with nisin. The activity of SaNSR is determined using the *L. lactis* NZ9000 strain, where the plasmid encoding the SaNSR wildtype and the mutations were transformed, and the IC₅₀ against nisin was determined. As a control, the empty vector was transformed and used in the IC₅₀ study (termed NZ9000Erm). Black lines represent the NZ9000Erm (filled Δ) and NZ9000-SaNSR (filled $\blacklozenge$) strains, respectively. The black dotted lines represent the NZ9000-SaNSR-His₉₈Ala ($\square$) and NZ9000-SaNSR-Ser₂₃₆Ala ($\circ$) strains. The data were fitted and evaluated as described in ref. 51. The difference in the growth exhibited by the strains was used to calculate the percentage of activity. Each experiment was performed at least in triplicates. (b) Graphical representation of the fold of resistance exhibited by SaNSR with nisin and different nisin variants (CCCCA, CCCAA, nisin₁₋₂₂ and nisin₁₋₂₈). The NZ9000Erm and NZ9000SaNSR strains were used to determine the activity of all the nisin variants. The error bars indicate the standard error of at least three independent experiments. (c) The activity of SaNSR and its mutations is determined using the *L. lactis* NZ9000 strain. A normalization of the IC₅₀ values were done by setting the values exhibited by the empty vector (NZ9000Erm) and NZ9000SaNSR to 0% and 100%, respectively. The error bars indicate the standard error of at least three independent experiments.

nisin variants. In order to test the influence of rings D and E located next to the cleavage site of nisin, we genetically replaced the last or the last two cysteine(s) in nisin by alanine, resulting in the expression of active nisin containing only rings A-D (termed CCCCCA) or A-C (termed CCCAA), respectively²⁷. Similarly, we removed the last six amino acids of nisin (termed nisin₁₋₂₈), resulting in the product of the proteolysis reaction mediated by SaNSR. Furthermore, a truncated variant (nisin₁₋₂₂) was expressed, which contained the rings A-C but lacked the rest of the C-terminus of nisin²⁷. Since all variants show a different activity against the nisin sensitive *L. lactis* NZ9000Erm strain (Supplementary Table 1a), we analyzed them with respect to the fold of resistance mediated by the expression of the SaNSR protein in the NZ9000SaNSR strain. From current and previous work, it is known that SaNSR confers a 20-fold increased resistance against wildtype nisin (Fig. 3a)²².

For CCCCCA as well as CCCAA, the resistance mediated by SaNSR decreased to roughly 1.4–1.7 fold when comparing the IC_{50} values of the different strains (Fig. 3b; Supplementary Table 1a). For the truncated variants, nisin₁₋₂₂ and nisin₁₋₂₈, no resistance was observed anymore as the IC_{50} values dropped to the levels observed for the NZ9000Erm strain. Thus, the lanthionine ring E is clearly important for the recognition by SaNSR.

Structural model of nisin binding to SaNSR. Despite intensive trials, we were not successful in obtaining a crystal structure of a SaNSR/nisin complex. Thus, we resorted to generating a structural model by integrative modeling and validating it by mutagenesis studies. Initially, we structurally aligned the backbone of residues 31–36 of nisin to the backbone of N-pep such that the nisin cleavage site between ring E and Ser₂₉ was oriented towards the catalytically active Ser₂₃₆ in SaNSR. Rings D and E were then manually placed in three orientations at the tunnel entrances such that they showed good complementarity with the SaNSR surface. This resulted in three structural models of SaNSR/nisin complexes, two (termed NSR_{Nisin,1}, NSR_{Nisin,2}) where rings D and E are located close to Asn₁₇₂, Met₁₇₃, and Ile₁₇₄, and one (termed NSR_{Nisin,3}) where nisin is oriented oppositely with respect to the tunnel axis such that Tyr₂₆₁ stacks onto rings D and E (Supplementary Fig. 5a). The three models were subjected to MD simulations³⁷ of 500 ns length, with three replicate simulations each.

The average distances between the side chain oxygen of Ser₂₃₆, previously identified as the catalytically active serine²², and the carbonyl carbon of Ser₂₉ at the nisin cleavage site²⁴ are 3.71 Å, 4.13 Å, and 7.74 Å for NSR_{Nisin,1}, NSR_{Nisin,2} and NSR_{Nisin,3}, respectively (Fig. 4a). This strongly indicates that a nucleophilic attack of the side chain of Ser₂₃₆ at the nisin cleavage site as a first step in the catalytic mechanism²⁴ is possible for the first two models but not for the third, suggesting that NSR_{Nisin,1} and NSR_{Nisin,2} represent the most likely orientation of nisin within the SaNSR tunnel. Thus, we focused further analyses on the NSR_{Nisin,1} and NSR_{Nisin,2} models.

Visual inspection of the MD trajectories and computations of the backbone RMSF identified residues Lys₂₂, His₃₁, Val₃₂, Dha₃₃, and Lys₃₄ of nisin as highly mobile (RMSF values up to 6.39 ± 0.49 Å) (Fig. 4b). In contrast, the core region (Nisin_{Core}) composed of the rings D and E, and residues Ser₂₉ and Ile₃₀ revealed RMSF values $< 1.85 \pm 0.24$ Å (Fig. 4b) suggesting a tightly bound Nisin_{Core} region. This was corroborated by a per-residue decomposition of effective binding energies computed by the MM-PBSA approach³⁸. Here, rings D and E (treated as one residue in the energy decomposition) and Ile₃₀ are identified as essential for nisin binding (residue-wise effective binding energies in the range from -4.26 kcal mol⁻¹ to -8.63 kcal mol⁻¹) (Fig. 4c). In contrast, for Ser₂₉, a smaller contribution to the effective binding energy of -1.64 kcal mol⁻¹ (-0.70 kcal mol⁻¹) for SaNSR_{Nisin,1} (SaNSR_{Nisin,2}) was found (Fig. 4c). Overall, this suggests that the rings D and E as well as Ile₃₀ form a binding motive, that way ensuring that also Ser₂₉ at the nisin cleavage site is correctly positioned within the catalytic site.

In Figure 4d, a representative set of six nisin structures within the SaNSR tunnel is shown. For this, the structure with the smallest backbone RMSD to the average structure was extracted from each of the NSR_{Nisin,1} and NSR_{Nisin,2} MD trajectories. The set shows that the location and orientation of rings D and E, Ser₂₉, and Ile₃₀ agree well in all cases, with RMSD values with respect to the average structure for the Nisin_{Core} ranging from 0.80 Å to 2.27 Å (Supplementary Fig. 5b). Thus, both NSR_{Nisin,1} and NSR_{Nisin,2} models were considered equivalent and used to identify residues in SaNSR important for catalysis and nisin binding for mutagenesis studies. The remaining residues of nisin show large structural deviations, in agreement with the above analyses (Supplementary Fig. 5b, Fig. 4b,c).

The TASSAEM region and His₉₈ form the active site. The NSR superfamily contains a highly conserved sequence motif “TASSAEM” (Supplementary Fig. 3) located at the rear end of the protease core domain. Within this TASSAEM region, Ser₂₃₆ has been previously identified as the catalytically active serine²². This serine is in close proximity to the strictly conserved His₉₈ residue, which is localized at the end of the N-terminal helical bundle directly next to the ₉₄GGG₉₆ motif (Fig. 1) and is in hydrogen bonds distance with the side chain of Ser₂₃₆. In the NSR_{Nisin,1} and NSR_{Nisin,2} MD simulations, hydrogen bonds were found in up to ~23% of all conformations (Supplementary Fig. 6a), which indicates that both residues likely interact also in the nisin-bound state. Based on the interactions of Ser₂₃₆ and His₉₈ and the absence of any other lysine or aspartate residue localized nearby, we presume that SaNSR acts via a catalytic dyad mechanism as observed for some other serine proteases^{39,40}. The NZ9000SaNSR-Ser₂₃₆Ala strain displayed a low background activity as observed by an IC_{50} value of 12.6 ± 0.7 nM (Supplementary Table 1b), which relates to a SaNSR residual activity of 14% (Fig. 3c). The His₉₈Ala mutation displayed a similar IC_{50} value of 12.3 ± 1.5 nM and a residual activity of 14% (Fig. 3c, Supplementary Table 1a). The residual activity displayed by both variants is likely due to the binding of nisin to that particular SaNSR variant such that a higher concentration of nisin is required to kill the corresponding nisin sensitive NZ9000Erm *L. lactis* strain.

Within the TASSAEM sequence, a second serine residue, Ser₂₃₇, is present. In the NSR_{Nisin,1} and NSR_{Nisin,2} MD simulations, the mean distance between the side chain oxygen and the carbonyl carbon of ring E is < 5.7 Å (Supplementary Fig. 6b). However, the distance to the δ -nitrogen of His₉₈ is > 9 Å (Supplementary Fig. 6c), and no hydrogen bonds were detected between both residues, making a proton shift between Ser₂₃₇ and His₉₈ unlikely. Instead, we observed hydrogen bond formation between the side chain of Ser₂₃₇ and the backbone of Gly₁₇₁ of the protease core in at least 46% of the conformations that may be relevant for nisin recognition (see section “Residues involved in nisin recognition and SaNSR specificity”) but not for catalytic activity. Thus, Ser₂₃₇ is not expected to be involved in the catalytic mechanism. In accordance, a Ser₂₃₇Ala mutation does not have a pronounced effect on the activity of SaNSR (residual activity 74%; see Fig. 3c, Supplementary Table 1b).

The next residue in the TASSAEM motif is Glu₂₃₉, which is pointing away from the active site. In the crystal structure, the Glu₂₃₉ side chain interacts with backbone atoms of Gly₂₆₀ and Tyr₂₆₁ via hydrogen bonds, and during the NSR_{Nisin,1} and NSR_{Nisin,2} MD simulations this interaction is present in at least 82% of all conformations (Supplementary Fig. 6a). Additionally, we found hydrogen bond interactions between Glu₂₃₉ and Ser₂₃₆ in at least 25% of the cases (Supplementary Fig. 6a). These interactions are likely important for the correct positioning of the TASSAEM region. This is in line with the drastically lowered activity of the Glu₂₃₉Ala mutant (IC_{50} value

Figure 4. Structural and energetic analysis of MD simulations of SaNSR/nisin model complexes.

(a) Distance between the side chain oxygen of Ser₂₃₆ and the carbonyl carbon of ring E at the nisin cleavage site (black dotted line in the upper right panel) in NSR_{Nisin, {1, 2, 3}} during 500 ns of MD simulations; lines were smoothed by cubic splines. Mean values and standard error of the mean (SEM; in parentheses) are shown in the legend. The mean distance over all three MD simulations is shown in the lower right panel (SEM < 0.1 Å and not shown). (b) Mean backbone RMSF (SEM indicated as error bars) for NSR_{Nisin, {1, 2}} models over three trajectories each of 500 ns length. Rings D and E, Ser₂₉, and Ile₃₀ compose the Nisin_{Core}. (c) Mean effective binding energy per residue for NSR_{Nisin, {1, 2}} models. Error bars indicate SEM over three trajectories. (d) Superimposition of six close-to-average structures (based on the backbone RMSD) of nisin (ball-and-stick models each colored differently), extracted from three independent MD simulations each of NSR_{Nisin,1} and NSR_{Nisin,2} within the tunnel of SaNSR (white surface representation). Ser₂₃₆ of the catalytic dyad is colored in blue. For clarity, the N-terminal helical bundle and part of the cap region of SaNSR have been omitted. (e) Representative nisin model (orange and green ball-and-stick model) within the tunnel of SaNSR (white cartoon representation with transparent surface). Residues Asn₁₇₂, Met₁₇₃, and Ile₁₇₄ that bind to rings D and E are colored in magenta.

of 17.1 ± 0.7 nM; residual activity of 22%; Fig. 3c, Supplementary Table 1b). Furthermore, we found stabilizing hydrogen bonds between Thr₂₆₃ and His₉₈ in up to ~28%, and between Asn₂₆₅ and His₉₈ in up to ~20% of all cases (Supplementary Fig. 6a). These interactions likely ensure a correct orientation of His₉₈ as the mutations Thr₂₆₃Ala and Asn₂₆₅Ala decreased the residual activities of SaNSR to 20% and 30%, respectively, (Fig. 3c) with associated IC₅₀ values of 16.0 ± 0.3 and 22.1 ± 1.1 nM (Supplementary Table 1b). Taken together, the TASSAEM sequence is crucial for the activity of SaNSR and contains the catalytically active serine as well as a glutamic acid residue, which is likely responsible for a correct positioning of the TASSAEM helix.

Residues involved in nisin recognition and SaNSR specificity. Next, we investigated nisin recognition by SaNSR. Residue-wise effective binding energies were computed for both the NSR_{Nisin,1} and NSR_{Nisin,2} MD trajectories to identify SaNSR residues likely to be important for nisin binding (Supplementary Fig. 7a). Considering energies < -0.8 kcal mol⁻¹ resulted in seven candidates (Leu₁₀₂, Leu₁₃₇, Asn₁₇₂, Met₁₇₃, Ile₁₇₄, Glu₂₆₆, Ala₂₇₇). Our model (Fig. 4d) suggests that the hydrophobic residues Leu₁₀₂, Leu₁₃₇, Met₁₇₃, Ile₁₇₄, Ala₂₇₇ and the polar/charged ones Asn₁₇₂ and Glu₂₆₆ bind to rings D and E in nisin. Asn₁₇₂, Met₁₇₃, and Ile₁₇₄ form a pocket that harbors rings D and E in our model (Fig. 4e). The Asn₁₇₂Ala mutant displayed an activity of 47% (IC₅₀ value of 33.5 ± 2.9 nM) (Fig. 3c, Supplementary Table 1b). Furthermore, when mutating the strictly conserved Met₁₇₃ residue (Supplementary Fig. 3), a reduced activity of 42% compared to the wild type value was observed (IC₅₀ value of 30.3 ± 1.4 nM). Additionally, the Ile₁₇₄Ala mutant exhibited an activity of 33% (IC₅₀ value of 24.1 ± 2.2 nM) (Fig. 3c, Supplementary Table 1b).

Moreover, we found hydrogen bonds between backbone atoms of Thr₁₆₉ and Gly₁₇₁ with the Nisin_{Core} residues (Supplementary Fig. 7b). These interactions are reminiscent to those found for N-pep (Fig. 2c) and likely ensure a proper placement of the Nisin_{Core} within the binding site. Additional stabilizing hydrogen bonds were observed between Asn₁₆₈ and Gly₁₇₀ (Supplementary Fig. 7b), which could contribute to nisin binding indirectly. A similar indirect effect was found for Glu₂₆₆, for which we observed salt-bridge formation with Arg₅₄ from the N-terminal helical bundle (Supplementary Fig. 7c; mean distance < 3.4 Å). We also found water-mediated hydrogen bonds between backbone atoms of rings D and E, and Asn₂₆₅ and Thr₂₆₇, respectively (Supplementary Fig. 7d), again mimicking what was observed for the bound N-pep (Fig. 2c). Accordingly, the mutations Asn₂₆₅Ala (see above) and Thr₂₆₇Ala decreased the residual activity of SaNSR to 30% and 71%, respectively, (Fig. 3c) with associated IC₅₀ values of 22.1 ± 1.1 and 48.5 ± 0.6 nM (Supplementary Table 1b).

Role of the protease cap domain in SaNSR. Other S41 peptidases also contain a protease cap domain comprising a helix and a β -hairpin structure, where the helix appears to open and close depending on the presence of the peptide substrate: once a peptide is bound, the cap closes and seals the active site. As such, the protease CtpB from *Bacillus subtilis* has been crystallized in an open and closed state with the helix of the protease cap moving by 10–15 Å towards the active site once the peptide was bound³⁰. In SaNSR, helix α_4 (103SKETVRRDITLDS₁₁₄) was identified as the protease cap helix, localized directly after the N-terminal helical bundle. Out of all residues of this helix, only the side chain of Asp₁₁₀ is intruding into the tunnel, which neither forms an interaction to N-pep in the crystal structure nor in the NSR_{Tail} MD simulations. This suggests that the protease cap is not adopting a fully closed state, rather an intermediate state. MD simulations show a salt-bridge formation between Asp₁₁₀ and Arg₂₇₅ of the protease cap domain for both NSR_{Nisin,1,2} models (Supplementary Fig. 8a). In those cases where the salt-bridge formation is weak (mean distance is > 10 Å), a loss of the secondary structures of helix α_4 is observed (Supplementary Fig. 8b). The Asp₁₁₀Ala mutant of SaNSR is still active although with a lower IC₅₀ value of 32.8 ± 2.1 nM (residual activity of 46%; Fig. 3c, Supplementary Table 1b). The Arg₂₇₅Ala mutant revealed an identical IC₅₀ value of 33.6 ± 2.3 nM (residual activity of 48%). Taken together, this suggests that a proper secondary structure of helix α_4 is required for SaNSR function, and that Asp₁₁₀ contributes to the stability of the secondary structure.

Discussion

The present study reveals that the lanthionine ring E of nisin determines substrate specificity of the nisin resistance protein (NSR) and contributes to the coordination of the nisin cleavage site at the catalytic center. These results are based on the first structure of a nisin resistance protein from *S. agalactiae* COH1 (SaNSR) at 2.2 Å resolution and subsequent integrative modeling and mutagenesis studies. The SaNSR structure consists of an N-terminal helical bundle, a protease cap domain, and a protease core domain (Fig. 1). The core domain harbors the highly conserved TASSAEM motif, which contains the catalytically important Ser₂₃₆ residue, in a hydrophobic tunnel formed by all three domains. In this tunnel, an N-terminal peptide from another SaNSR protomer (N-pep) in the asymmetric unit is bound predominantly by direct and water-mediated backbone hydrogen bonds (Fig. 2). A very similar binding pattern is found for the C-terminal lanthionine rings D and E, and residues Ser₂₉, and Ile₃₀ of nisin in our model of the SaNSR/nisin complex (Fig. 5a,b; Supplementary Fig. 7d). According to this model, lanthionine ring E binds at one end of the hydrophobic tunnel (Fig. 5a,b) and ensures the exact coordination of the nisin cleavage site at the highly conserved TASSAEM region (Fig. 5a,b).

In contrast to some other C-terminal processing proteases^{30,32}, the active center of SaNSR consists of a catalytic dyad formed by residues Ser₂₃₆²², which is part of the TASSAEM motif, and His₉₈ as determined by mutational analysis and also described for some other proteases⁴¹ (Fig. 5a–c). Mutational analysis and geometric parameters in the crystal structure and during MD simulations exclude that the neighboring Ser₂₃₇ participates in the catalytic step. Residues Glu₂₃₉, Gly₂₆₀, Tyr₂₆₁ and Thr₂₆₃ form hydrogen bonds with either Ser₂₃₆ or His₉₈ during all-atom MD simulations of the SaNSR/nisin complexes (Supplementary Fig. 6a, Figure 5a–c) and, thus, likely stabilize the catalytic residues, as also indicated by alanine mutations of these residues that lead to a decrease in SaNSR activity (Fig. 3c).

Figure 5. Nisin/SaNSR binding model. (a) Representative structure of nisin (residues 22–34; extracted from the NSR_{Nisin,1} model) bound to the crystal structure of SaNSR (cartoon representation with transparent surface; each domain is colored differently). Orange spheres with one-letter/three letter amino acid code indicate nisin residues 1–21 not considered for modeling studies (abbreviations: abu = aminobutyric acid; dha = dehydroalanine; dhb = dehydrobutyrine; ala-S-X = lanthionine derivatives). (b) Close up view of nisin binding to SaNSR residues important for nisin recognition (left), residues with catalytic function (middle),

and residues with a regulatory function (right). Amino acids of interest are depicted as ball-and-stick model; residues for which experimental data is reported in this study are, additionally, shown in transparent surface representation. (c) Schematic representation of the Nisin_{core} bound to SaNSR residues (residue numbers according to the crystal structure described here). Residues that compose the catalytic site are colored in blue, residues that contribute to nisin binding in magenta, residues that have an indirect effect on binding in black-magenta, and residues with a supposedly regulatory function in SaNSR in red. For residues with colored background, SaNSR activity information for alanine mutants is available (see Fig. 3c). Residues marked with a star form the catalytic dyad. In panels a, b, and c, the nisin structure is depicted as orange ball-and-stick model.

Since all our efforts to obtain crystals of SaNSR with bound nisin were unsuccessful, we generated a model (Fig. 5a–c) of the SaNSR/nisin complex by integrative modeling and subsequent site-directed mutagenesis studies and activity measurements for validation. The modeling step was guided by exploiting the knowledge on the location of N-pep in the SaNSR crystal structure as well as on the substructures of nisin determining its molecular recognition. As to the latter, we focused on the C-terminus of nisin (nisin_{22–34}) where NSR from *L. lactis* TS1640 has been shown to cleave²⁴. As a result, nisin variants in which the bulky lanthionine rings D and E, or only E, were replaced by a linear sequence (CCCCA, CCCAA) showed a large drop in the fold of resistance comparable to those exhibited when the last 12 or 6 residues of nisin (nisin_{1–22}, nisin_{1–28}) were missing (Fig. 3b). These results demonstrated that ring E is essential for nisin recognition by SaNSR.

Initial models of SaNSR/nisin complexes were generated in which the linear, C-terminal sequence (sequence Lys₂₂ - Lys₃₄) were placed at the location of the backbone trace of N-pep and where rings D and E showed a good complementarity with the SaNSR surface at the tunnel entrance. We considered that no *a priori* knowledge on the direction of nisin with respect to the tunnel axis was available by generating models with both possible directions. By subsequent all-atom MD simulations, we could exclude one of the possibilities (NSR_{Nisin,3}) as in this case the distance between Ser₂₃₆ and the nisin cleavage site was too large as to allow for a nucleophilic attack of the serine side chain (Fig. 4a). In contrast, for the other direction (NSR_{Nisin,1}, NSR_{Nisin,2}), such an attack is very likely according to distances that are only slightly larger than the sum of van der Waals radii of oxygen and carbon. This model of a SaNSR/nisin complex is further supported by rather immobile residues of the core region of nisin (rings D and E, Ser₂₉ and Ile₃₀), which is considered to facilitate a nucleophilic attack, in contrast to the more mobile C-terminal residues 31–34 (Fig. 4b), and by a residue-wise decomposition of the effective binding energy, which identified rings D and E as well as Ile₃₀ as major contributors to the binding affinity (Fig. 4c).

The model (Fig. 5a–c) reveals that SaNSR binding to rings D and E of nisin is dominated by hydrophobic interactions (Fig. 5b,c). Within the protease core Asn₁₇₂, Met₁₇₃, and Ile₁₇₄ form a pocket that harbors both rings D and E (Figs 4e,5b). In agreement with this model, mutation of these residues reduces the activity of SaNSR. Furthermore, water-mediated hydrogen bonds between backbone atoms of rings D and E and side chains of Asn₂₆₅ and Thr₂₆₇, respectively, were identified, mimicking interactions with N-pep. Asn₂₆₅Ala and Thr₂₆₇Ala mutations decreased the residual activity of SaNSR (Fig. 3c). Finally, along the tunnel, hydrogen bonds between backbone atoms of Thr₁₆₉ and Gly₁₇₁ of SaNSR with Ser₂₉ and Ile₃₀ of nisin were found (Fig. 5b,c; Supplementary Fig. 7b), which likely contribute towards the correct orientation of the nisin cleavage site at the catalytic center and are again reminiscent of interactions observed for N-pep in the crystal structure.

N-pep and the C-terminus of nisin are not similar on the amino acid level. Together with the above findings of similar interactions along the tunnel between backbone atoms of SaNSR and the two peptides, respectively, this suggests that the tunnel's role in peptide binding is not to confer substrate specificity but rather to “rope in” the peptide while establishing these interactions. In the case of nisin, this “roping in” is stopped when the lanthionine ring E starts interacting with SaNSR, thereby acting as a plug on the tunnel (Fig. 5c). These interactions are highly relevant for the molecular recognition of nisin and the substrate specificity of SaNSR, as shown by a decrease in the fold of resistance for the nisin variants CCCCCA and CCCAA (Fig. 3b) and a decrease in the activity of SaNSR mutants Asn₁₇₂Ala, Met₁₇₃Ala, and Ile₁₇₄Ala (Fig. 3c). In addition, rings D and E are highly likely relevant for a proper placement of the nisin cleavage site with respect to the catalytic Ser₂₃₆, as only with nisin a distance to this residue compatible with a nucleophilic attack and, simultaneously, hydrogen bonds with His₉₈ are found in the MD simulations. In contrast, during MD simulations of NSR_{Tail}, no hydrogen bond formation between Ser₂₃₆ and His₉₈ was detected. This may explain why N-pep binds to SaNSR but is not cleaved.

Previously, an “inhibiting role” of lanthionine rings has been recognized in that they protect lantibiotics from degradation by standard proteases⁴², likely because of their bulky 3D structure which prevents a proper placement in the substrate binding regions of proteases evolved to cleave linear peptides. In turn, the findings in this study for the first time reveal a significant “fostering role” of the lanthionine rings D and E in nisin for the highly specific cleavage of this lantibiotic by SaNSR. These findings and our structural model of the SaNSR/nisin complex open up a new avenue in the understanding of lantibiotic resistance by human pathogens. They may also facilitate the development of therapeutics to overcome nisin resistance.

Methods

Cloning, expression and purification of SaNSR. The *nsr* gene from *Streptococcus agalactiae* COH1 was cloned into pET28b and purified as previously described. For details see Supplementary Information.

Multiple angle light scattering. For HPLC-MALS analysis, a Bio SEC-5 HPLC column (Agilent Technologies Deutschland GmbH, Böblingen, Germany) with a pore size of 300 Å was equilibrated with 25 mM MES pH 6.0, 150 mM NaCl for HPLC using a system from Agilent Technologies connected to a triple-angle light-scattering detector (miniDAWN TREOS, Wyatt Technology Europe GmbH, Dernbach, Germany) followed

by a differential refractive index detector (OPTILab T-rEX, Wyatt Technology). Typically, 100 μ l of purified SaNSR (2.0 mg/ml) was loaded onto the Bio SEC-5 HPLC column, and the obtained data were analyzed with the ASTRA software package (Wyatt Technology).

Crystallization, data collection and structure determination of SaNSR. Crystals were obtained and optimized as described in the Supplementary Information. X-ray diffraction data were collected at the ID23eh2 or ID29 beamlines of the European Synchrotron Radiation Facility (ESRF), Grenoble. All the data sets were processed and scaled using XDS and XSCALE software package⁴³. Data sets from native crystals were collected at a wavelength of 0.872 Å at 100 K. For selenomethionine-substituted crystals, the ID29 beamline (ESRF Synchrotron, Grenoble)⁴⁴ was used for anomalous diffraction data collection, done at 100 K. The structure was solved by single-wavelength anomalous dispersion (SAD) from a single selenomethionine derivative crystal measured at 0.976 Å, which diffracted up to 2.7 Å. The Auto-Rickshaw program⁴⁵ was then used to phase the protein and build an initial model, which was further manually build and refined using COOT⁴⁶ and phenix.refine from the Phenix package⁴⁷. This model was then used to phase the native data set at a resolution of 2.2 Å. After molecular replacement, automatic model building was performed with the program ARP/wARP⁴⁸, followed by manual iterative cycles of model refinement using the program phenix.refine⁴⁷. Manual adjustments between the refinement cycles were done with the program Coot⁴⁶ and Ramachandran validation was done using MolProbity⁴⁹. Almost all residues (96.3%) were in the preferred regions of the Ramachandran plot, and the remaining 3.7% were in the additionally allowed regions. The data collection and refinement statistics are listed in Table 1. The images of the models were prepared using MacPyMOL⁵⁰.

IC₅₀ determination of nisin and its variants. Cells from the different expressing strains were grown overnight in GM17 media supplemented with 5 μ g ml⁻¹ erythromycin in the presence of 1 ng ml⁻¹ nisin. The diluted cells (final OD₆₀₀ of 0.1) were incubated with a serial dilution of nisin or its variants in a 96-well plate. The total volume in each well was 200 μ l, consisting of 50 μ l nisin or its variants and 150 μ l GM17 containing the corresponding *L. lactis* strain. The plate was then incubated at 30 °C and after 5 hours, the optical density was measured at 600 nm via 96-well plate reader BMG. The IC₅₀ value was determined as previously described⁵¹.

Molecular dynamics simulations. In order to investigate nisin recognition by SaNSR we performed molecular dynamics (MD) simulations of an unbound SaNSR monomer (NSR_{Apo}), a SaNSR monomer bound to the N-terminal part of SaNSR (residues 31–36; in the following named “Tail”) from an adjacent subunit (NSR_{Tail}) in the crystal structure (see Fig. 1), and a SaNSR monomer bound to the C-terminal part (residues 22–34; Supplementary Fig. 2c) of nisin (NSR_{Nisin}). Initial coordinates for NSR_{Apo} and NSR_{Tail} were taken from the crystal structure described here. Since no structural information is available for nisin bound to SaNSR, we generated models as starting structures for MD simulations by structurally aligning the nisin part to the Tail using the program Moloc. The nisin cleavage site between ring E and Ser₂₉ was oriented towards the catalytically active Ser₂₃₆ in SaNSR²². Rings D and E were manually placed in three orientations within the binding site such that they showed good complementarity with the SaNSR surface, resulting in three different models of SaNSR/nisin complexes (NSR_{Nisin,1}, NSR_{Nisin,2}, and NSR_{Nisin,3}, Supplementary Fig. 4a).

For the MD simulations, structures of NSR_{Apo}, NSR_{Tail}, and NSR_{Nisin,1-3} were prepared, relaxed, and thermalized as described in detail in the Supporting Information. Three independent production runs of MD simulations of 500 ns length in the canonical (NVT) ensemble at 300 K were then conducted for each of the five systems, leading to a total simulation time of $5 \times 3 \times 500$ ns = 7.5 μ s; see Supporting Information for details.

The trajectories were analyzed with respect to distances, root mean square fluctuations (RMSF) and deviations (RMSD) as a measure for mobility and structural similarity, respectively, and hydrogen bonds defined by a distance between the two donor and acceptor atoms < 3.2 Å and an angle (donor atom, H, acceptor atom) between 120° and 180° using *cpptraj*⁵². Salt-bridge interactions are defined by a distance < 4.0 Å between the center of mass of both charged groups. The set of structural models binding to SaNSR (see section “Structural model of nisin binding to SaNSR”) was generated by structurally aligning SaNSR and subsequent RMSD calculations for the nisin peptide.

Calculation of the effective binding energy. In order to identify amino acids in SaNSR that contribute most to nisin binding, we computed the residue-wise contribution to binding effective energies by the “single trajectory” molecular mechanics Poisson-Boltzman area (MM-PBSA) approach^{53–55}. To determine the per-residue contribution, the decomposition scheme⁵⁶ as implemented in the *mm_pbsa.pl* script in Amber 14³⁷ was applied. The calculations were performed with the ff99SB force field^{57,58}. The polar part of the solvation free energy was determined by applying the PBSA solver using a dielectric constant of 1 (solute) and 80 (solvent) together with Parse radii⁵⁹. The conformational ensemble consists of 10,000 snapshots and was extracted from the 1–200 ns interval of each of the NSR_{Nisin,1-2} trajectories. Prior to the MM-PBSA computations, counter ions and water molecules were stripped from the snapshots. For the computations, we considered the SaNSR protein the receptor, whereas the nisin C-terminus was considered the ligand. All residues in SaNSR and nisin were considered for per-residue decomposition. Rings D and E in nisin were treated as one residue.

PDB Deposition

The final model has been deposited in the PDB database under the accession code: 4Y68.

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Author Contributions

S.K., A.H., D.K. performed the biochemical and structural experiments. B.F., D.M. performed molecular modeling and M.D. simulations. S.K., B.F., H.G., S.S. designed the experiments, evaluated the data, and wrote the manuscript. All authors reviewed the manuscript.

Additional Information

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