In vitro activity of the nisin dehydratase NisB

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The biosynthesis of several classes of ribosomally synthesized and posttranslationally modified peptides involves dehydration of serine and threonine residues. For class I lantibiotics, thiopeptides, and goadsporin, this dehydration is catalyzed by lanthionine biosynthetic enzyme B (LanB) or LanB-like proteins. Although LanB proteins have been studied since 1992, in vitro reconstitution of their dehydration activity has been elusive. We show here the in vitro activity of the dehydratase involved in the biosynthesis of the food preservative nisin (NisB). In vitro, NisB dehydrated its substrate peptide NisA eight times in the presence of glutamate, ATP, Mg²⁺, and the ribosomal/membrane fraction of bacterial cell extract. Mutation of 23 highly conserved residues of NisB identified a number of amino acids that are essential for dehydration activity. In addition, these mutagenesis studies identified three mutants, R786A, R826A, and H961A, that result in multiple glutamylations of the NisA substrate. Glutamylation was observed during both *Escherichia coli* coexpression of NisA with these mutants and in vitro assays. Treatment of the glutamylated substrate with WT NisB results in dehydrated NisA, suggesting that the glutamylated peptide is an intermediate in dehydration. Collectively, these studies suggest that dehydration involves glutamylation of the side chains of Ser and Thr followed by elimination. The latter step has precedent in the virginiamycin resistance protein virginiamycin B lyase. These studies will facilitate investigation of other LanB proteins involved in the biosynthesis of lantibiotics, thiopeptides, and goadsporin.

Lantibiotics are ribosomally synthesized and posttranslationally modified peptide antibiotics (1). Nisin is the best known family member and has been used in the food industry for over 40 y to combat food-borne pathogens (2). These polycyclic compounds are generated from a linear precursor peptide that is generically termed LanA (3). The precursor peptides consist of an N-terminal leader peptide and a C-terminal core peptide where all post-translational modifications take place (4). Lantibiotics are biosynthesized by dehydration of Ser and Thr residues in the core peptide, yielding dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively (e.g., Fig. 1A for nisin). Subsequent Michael-type addition of cysteine thiols to the dehydrated residues results in the formation of cyclic thioether bridges termed lanthionine and methylanthionine (Fig. 1A). Lantibiotics are a subset of the lanthipeptides (1), which also include lanthionine-containing peptides that do not have antimicrobial activities. Lanthipeptides have been divided into four classes based on distinct biosynthetic enzymes that carry out the dehydration and cyclization reactions (3). The biosyntheses of class II, III, and IV lanthipeptides have all been reconstituted in vitro (5–7), but in vitro reconstitution of the biosynthesis of class I lanthipeptides that include nisin has been elusive.

The dehydration and cyclization reactions of class I lanthipeptides are carried out by two enzymes generically called lanthionine biosynthetic enzymes B (LanB) and LanC, respectively. The cyclization reaction catalyzed by NisC, the LanC involved in nisin biosynthesis, has been reconstituted in vitro (8) (Fig. 1B), and genetic and bioengineering studies have shown that the LanB for nisin biosynthesis (NisB) carries out the dehydration reaction and does not need any other lanthipeptide biosynthetic enzymes for activity (9, 10). However, despite considerable effort in multiple laboratories, reconstitution of the activity of LanB dehydratases has been unsuccessful (11–15), and hence, no information is

available about their mechanism of catalysis. The LanB dehydratases are 115–120 kDa proteins that do not share homology with the dehydratases from classes II–IV lanthipeptides or other proteins in the databases. Genes encoding homologous LanB-like enzymes are also present in the gene clusters of nonlantibiotic peptide natural products, such as the thiopeptides (16–19) and goadsporin (20). These proteins are also believed to be dehydratases.

We recently reported the successful preparation of nisin in *Escherichia coli* by coexpression of the precursor peptide NisA with NisB and NisC (21). Hence, the NisB protein must be functional in *E. coli*, prompting renewed attempts at establishing an in vitro reconstituted activity. In this study, we report generation of active NisB and the requirements for in vitro activity. We propose a mechanism for the dehydration reaction.

Results

In Vitro Reconstitution of Dehydration Activity of NisB. The successful production of nisin in *E. coli* was achieved by coexpression of hexahistidine-tagged NisA (His₆-NisA) and untagged NisB from a pRSFDuet-1 vector and untagged NisC from a pACYC vector (21). For the purpose of in vitro studies on NisB, these constructs were altered such that the gene for a hexahistidine-tagged NisB (His₆-NisB) was cloned into the multiple cloning site 1 (MCS-1) of pRSFDUET-1 and the gene for His₆-tagged precursor peptide NisA was inserted into MCS-2. Using this plasmid, His₆-NisA and His₆-NisB were coexpressed in *E. coli*, and analysis of the cell extracts indicated full dehydration of the His₆-NisA peptide, indicating that the His₆-tag on NisB did not interfere with in vivo NisB activity. His₆-NisB was then purified from the cytoplasmic fraction of the *E. coli* cells using nickel affinity chromatography followed by gel filtration chromatography. Curiously, incubation of the purified enzyme with His₆-NisA in the presence of ATP and Mg²⁺ did not result in any dehydration activity. Omitting the gel filtration step also did not provide active enzyme, and dehydrated peptide could not be detected after the addition of His₆-NisA to purified His₆-NisB in the presence of crude cell extracts of the *E. coli* cells that produced His₆-NisB and fully dehydrated His₆-NisA. These results are similar to the previously reported unsuccessful attempts to achieve in vitro activity of LanB enzymes (11–15).

Because the His₆-tagged NisB did dehydrate His₆-NisA in *E. coli*, we reasoned that some property of the intracellular environment of the bacteria must contribute to activity. After evaluating a host of different possibilities, such as addition of ribosomes and crowding agents without success, conditions used to optimize in vitro protein biosynthesis (22) were assessed. When glutamate (175 mM), the most abundant metabolite in *E. coli* (23), and spermidine (1.5 mM), a polyamine involved in modulating various cellular functions, were added to His₆-NisB (5 μM), His₆-NisA (200 μM), ATP (5 mM),

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any exogenous lantibiotic genes did result in full dehydration. Furthermore, spermidine was not essential for NisB activity, because it could be omitted from the assay without affecting the dehydration activity. Because the cell extract contained significant amounts of glutamate, its importance could not be determined at this stage, but it was later shown to be required (see below).

Required for Dehydration Activity. The cell extract was fractionated into small-molecule and macromolecule fractions using two methods. First, cell extract was passed through a 3-kDa amicon ultracentrifugation filter. Full dehydration activity was observed when the retentate was used in the assay mixture instead of cell extract, whereas no activity was seen when the flow-through was used (Fig. S3A and Fig. S24). Second, the cell extract was also passed through a Sephadex G-25 PD10 column, and the macromolecule fraction was collected separately from the small-molecule fraction. Again, dehydration activity was observed by adding the macromolecule fraction to the NisB activity assay, whereas no activity was observed with the small-molecule fraction. The observation that the macromolecule fraction contained the ingredients needed for activity also allowed a test of the requirement for glutamate. Addition of the macromolecular fraction of the gel filtration experiment to the activity assay in the absence of glutamate did not result in dehydration of NisA (Fig. S1), whereas inclusion of Glu (175 mM) again resulted in full dehydration activity.

Fig. 2. In vitro reconstitution of dehydration activity of NisB. MALDI mass spectrum of His₆-NisA incubated with His₆-NisB, ATP, Mg²⁺, glutamate, and *E. coli* cell extract.

Fig. 3. Investigation of the active ingredients in cell extract. (A) Full dehydration activity was observed when using the retentate of an ultrafiltration experiment with a membrane with a 3 kDa molecular mass cutoff for the *in vitro* NisB assay. (B) MALDI mass spectrum of His₆-NisA incubated with His₆-NisB, ATP, Mg²⁺, Glu, and the crude membrane fraction of an ultracentrifugation experiment at 200,000 $\times$ g.

Alanine Substitution of Highly Conserved Residues in NisB. A previous study on the effects of mutagenesis of five conserved amino acids in NisB on the dehydration reaction was performed in a *L. lactis* heterologous host (25). In the current study, we interrogated the role of 23 additional highly conserved residues with side chains that could be involved in the dehydration activity of NisB (Figs. S3, S4, and S5), including several residues identified in a previous bioinformatics study (26). Because of ease of manipulation, the previously described *E. coli* expression system (21) was used as an initial test. Mutation of residues Arg575, Ser581, and Asp648 of His₆-NisB to Ala did not affect the dehydration activity, because the product was eightfold-dehydrated His₆-NisA. Mutation of Glu44, Lys68, Lys116, Glu133, Arg505,

Lys741, Glu823, Ser958, and Glu975 to Ala resulted in partially dehydrated products, suggesting that these residues are important but not essential for dehydration (Fig. S4). Conversely, individual replacement of residues Arg14, Arg83, Arg87, Thr89, Asp121, Asp299, Arg464, and Arg966 with Ala resulted in abolishment of dehydration (Fig. S5), suggesting that they are important for catalysis. The most interesting observations were made with the NisB mutants R786A, R826A, and H961A. MALDI-MS analysis of the His₆-NisA peptides that were purified after coexpression with these mutants showed multiple adducts of 129 Da (Fig. 4). The inactive mutants and the mutants that led to adducts of 129 Da were selected for additional *in vitro* investigation. They were expressed with an N-terminal hexahistidine tag and purified using nickel affinity column chromatography and gel filtration chromatography, resulting in similar yields as WT protein. Thus, the observed loss of activity or generation of adducts was not a consequence of poor expression or solubility.

Mechanistic Investigation of NisB. To investigate the identity of the 129 Da adducts, the His₆-NisA peptide that was modified by the mutant NisB-R786A in *E. coli* was purified using nickel affinity chromatography and reversed phase (RP)-HPLC. The purified peptide was digested with trypsin, and analysis of the resulting fragments indicated that all of the +129 Da adducts were present in the core peptide. The observed masses are reminiscent of polyglutamylation of eukaryotic proteins, which involves consecutive additions of glutamate to the side chain of Glu residues in proteins through amide linkages between the γ -carboxy and α -amino groups of Glu (27). However, unlike such polyglutamylation, the 129 Da adducts on NisA could be readily removed by treatment with base, suggesting that, if the 129 Da adducts are associated with glutamylation, the linkage is more likely to involve an ester, thioester, or anhydride. To confirm that Glu was the source of the adducts, *in vitro* assays were performed. Incubation of His₆-NisB-R786A with His₆-NisA, ATP, Mg²⁺, cell extract, and [2,3,3,4,4-*d*₅]-Glu and subsequent MALDI-MS analysis showed multiple +134 adducts on NisA (Fig. 5). Thus, glutamate was confirmed as the source of the adducts that are produced during coexpression in *E. coli* and *in vitro* assays.

To test whether the adducts could be intermediates in the dehydration process, His₆-NisA obtained from coexpression with NisB-R786A was again purified, and the modified peptide containing the multiple 129 Da adducts was incubated with purified WT His₆-NisB. MALDI-MS analysis showed conversion of NisA containing the adducts to dehydrated peptide (Fig. S6). None of the other components of the *in vitro* NisB assay mixture that are important for dehydration (glutamate, cell extract, Mg²⁺, or ATP) were required for glutamate elimination. We also tested the *in vitro* glutamate elimination activity of the His₆-NisB mutants, which were all expressed in *E. coli* and purified. The alanine mutants of Arg83, Arg87, Thr89, Asp121, Asp299, Arg966, and Arg464 were confirmed to be inactive with respect to dehydration of His₆-NisA, but they had similar glutamate elimination activity as the WT protein on glutamylated His₆-NisA (Fig. S7). The alanine mutants of Lys116 and Glu97 displayed partial dehydration activity, but they again had similar glutamate elimination activity as WT NisB. The only mutants that exhibited very low glutamate elimination activity were the same mutants that, in *E. coli*, resulted in observation of glutamylated NisA: Arg786Ala, Arg826Ala, and His961Ala (Fig. S6).

When the products of the glutamate elimination assay containing glutamylated His₆-NisA and WT NisB were incubated with L-glutamic acid dehydrogenase and nicotinamide adenine dinucleotide (NAD⁺), a clear increase in absorbance at 340 nm was observed (Fig. S8), suggesting oxidation of glutamate that had been released from the NisA peptide by WT NisB. Attempts to determine the sites of glutamylation within the core peptide were unsuccessful, because the Glu adducts were removed in tandem MS experiments before any fragmentation of the peptide

Fig. 4. Modification of His₆-NisA by NisB-R786A, R826A, and H961A in *E. coli*. MALDI mass spectra of His₆-NisA coexpressed with (A) NisB-R786A, (B) NisB-R826A, and (C) NisB-H961A.

bonds. This observation once again suggests that the linkage of Glu to NisA is not by an amide bond. Moreover, the observed production of dehydrated peptide on treatment with WT NisB is most consistent with an ester linkage to Ser/Thr.

Discussion

NisB was initially isolated from membrane vesicles of the nisin producer *L. lactis* in 1992 (11) and recently also purified in soluble form (15), but reconstitution of its activity in vitro has remained elusive. In this study, NisB activity was successfully reconstituted and required the membrane fraction of cell extract, ATP, Mg²⁺, and glutamate. A direct role of glutamate in the physiological dehydration process is suggested by several experimental observations. First, multiple glutamate adducts to the NisA substrate are observed both in vitro and in *E. coli* with the NisB mutants R786A, R826A, and H961A. Second, small amounts of +129 Da adducts are also observed with WT NisB (Fig. 2). Adducts of 130/131 Da were also present in the MALDI-MS spectra of NisA products generated by the truncated NisB mutants Δ887–892 and Δ838–851 in a previous study in *L. lactis* that showed that these truncated proteins were unable to dehydrate (25). Khusainov et al. (25) suggested that the M+130/131 Da peaks were associated with NisA that still contained the N-terminal Met, but no explanation was offered for the presence of ions of M+ (2 × 130/131) Da, which cannot be explained by incomplete removal of Met. Given that these truncations were in the same C-terminal region of NisB as the single mutants that generated glutamylated products in this study, we suggest that the observed peaks in *L. lactis* were glutamylated peptides. The difference between the observed mass adducts of 130/131 Da and the calculated 129 Da adducts for glutamylation could be a consequence of the experimental error in MALDI spectra of peptides of this size.

A key observation in this study was that WT NisB converted glutamylated NisA to dehydrated NisA, showing that glutamylation generates a chemically competent intermediate. Because none of the required ingredients for the dehydration reaction (ATP, Mg²⁺, Glu, and cell extract) were needed for glutamate elimination, a process that involves hydrolysis of the ester followed by dehydration of the regenerated Ser/Thr residues can be ruled out. Collectively, we believe that these data provide support for glutamylation of Ser/Thr as a required step for dehydration. His961, Arg786, and Arg826 are clearly important for elimination of glutamate, because alanine substitutions at these positions resulted in greatly diminished elimination activity in vitro as well as in vivo. The His961Ala mutant of His₆-NisB was also the least active in glutamylation of His₆-NisA (Fig. 4), suggesting that either the mutation may have a general deleterious effect on protein structure or this residue is important for both glutamylation and elimination.

On the basis of our results, we propose that one of the carboxylic acid groups of glutamate is activated by ATP and subsequently reacts with the side chains of serine and threonine residues that are targeted for dehydration, resulting in glutamate esters (Fig. 6A). On deprotonation of the α-proton of the glutamylated Ser or Thr residues, elimination of glutamate results in dehydroalanine/dehydrobutyrine residues. It is possible that glutamylation and elimination occur in different domains, which is also observed for class III and class IV lanthipeptides that contain separate kinase and lyase domains (7, 28). If so, then the C terminus of NisB contains the likely lyase domain. The leader peptide binding site has been previously shown to not be located in this C-terminal domain (25). The proposed activation mechanism for LanB dehydratases stands in contrast to the activation of the hydroxyl groups of Ser and Thr by phosphorylation during the dehydration process of classes II, III, and IV lanthipeptides (6, 7, 29). Why glutamylation of Ser/Thr would evolve as an alternative means of Ser/Thr activation is unclear. One possibility is that it is a remnant of the use of activated Glu for other chemistry by the ancestors of LanB proteins. Although unexpected in the context

Fig. 5. In vitro activity assays of NisB-R786A with unlabeled and deuterium-labeled glutamate. MALDI mass spectra of His₆-NisA incubated with NisB-R786A, ATP, Mg²⁺, cell extract, and (A) glutamate or (B) d₅-glutamate.

of Ser and Thr dehydration, the elimination of a carboxylate from an ester linkage to the side chain of Thr in peptides has precedent in the resistance protein to the cyclic hexadepsipeptide virginiamycin (30, 31) as well as related streptogramin antibiotics (32, 33). The virginiamycin B lyase (Vgb) from *Staphylococcus aureus* catalyzes an elimination reaction of the macrolactone that is formed by an ester bond between the hydroxy group of a Thr and the C-terminal carboxylic acid (Fig. 6B). This antielimination reaction generates a Z-dehydrobutyryne (30), the same stereochemistry that is also generated by NisB in nisin (34, 35). Vgb requires a Mg^{2+} for its elimination reaction (30), whereas NisB catalyzes the elimination of Glu in the absence of Mg^{2+} . The Vgb crystal structure illustrates that the Mg^{2+} binds a 2-hydroxytypicolinic acid group in virginiamycin B (31) that is not present in the substrates for NisB.

providing an explanation as to why Mg^{2+} is not needed for the elimination step in NisB.

Whether NisB activates Glu itself or whether this activation is carried out by an enzyme in the membrane fraction of the *E. coli* cell extract is currently not known, because attempts to detect Glu activation in the absence of cell extract have thus far been unsuccessful. Because of the required presence of cell extract, we also have not been able to determine which of the two carboxylates of Glu is activated and whether this activation involves adenylation or phosphorylation. Nevertheless, this study provides the long-sought in vitro activity of a LanB protein and a unique mode of Ser/Thr activation that provides the basis for additional investigation. By providing the conditions needed for in vitro activity, this study will also aid research on several classes of natural products that use LanB proteins (1).

Materials and Methods

General Materials. *SI Text* details the sources of the reagents used.

Construction of the pRSFDuet-1/*His*₆-*NisB*-*NisA* Expression Plasmid. The PCR amplification of *nisA* and *nisB* was performed by PCR using the following cycle: denaturation (98 °C for 20 s), annealing (60 °C for 20 s), and extension (72 °C for 30 s for *nisA* and 120 s for *nisB*) with genomic DNA of *L. lactis* 6F3 as the DNA template. The PCR included 1× Phusion GC buffer (Finnzymes), DMSO [2% (vol/vol)], Phusion hot start high-fidelity DNA polymerase (0.05 U/μL), and primers (1 μM each) (Table S1). The amplifications were analyzed on a 1% agarose gel, and the products were purified using a QIAquick Gel Extraction Kit (QIAGEN). The amplified *nisA* DNA fragment and the pRSFDuet-1 vector were digested with NdeI and XhoI (MCS-2) at 37 °C for 12 h. The reaction mixtures were run on agarose gel, and the desired bands were extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting double-digested *nisA* DNA insert was ligated with the digested vector at 4 °C for 15 h using T4 DNA ligase (Invitrogen). *E. coli* DH5α cells were transformed with the ligation product using heat shock, plated on LB-kanamycin agar plates, and grown at 37 °C for 15 h. Colonies were picked and incubated in 5 mL LB-kanamycin medium at 37 °C for 10 h followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The desired sequences of the resulting plasmids (pRSFDuet-1-*NisA*) were confirmed by DNA sequencing using the appropriate primers by the Biotechnology Center of the University of Illinois at Urbana-Champaign. pRSFDuet-1-*NisA* containing the *nisA* gene was digested with BamHI and NotI (MCS-1) and ligated with *nisB* PCR product digested with BamHI and NotI. The ligation and transformation of *E. coli* DH5α cells were performed as described above. Colonies were analyzed for the presence of insert, and plasmid was isolated and sequenced.

Fig. 6. General mechanism for dehydro amino acid formation by carboxylate elimination. (A) Proposed mechanism for NiSb-catalyzed elimination of glutamate resulting in Dha/Dhb residues. (B) Reaction catalyzed by Vgb. Although not explicitly shown, the overall reaction may occur with an enolate intermediate (39).

Purification of His₆-NisA Modified by WT and Mutant NisB. The plasmid pRSFDuet-His₆-NisA/His₆-NisB was constructed using the same procedure as described above, except that *nisA* was inserted in MCS-1 and *nisB* was inserted in MCS-2. To introduce a hexahistidine tag at the N terminus of NisB, an oligonucleotide sequence encoding hexahistidine was incorporated in the 5' end of the primer immediately before the start codon of *nisB*. Site-directed mutagenesis was carried out on pRSFDuet-His₆-NisA/His₆-NisB to replace conserved polar residues in NisB with alanine using Phusion DNA polymerase and the primers listed in Table S1. Chemically competent BL21 (DE3) cells were transformed with pRSFDuet-His₆-NisA/His₆-NisB containing the *nisB* variant genes. An overnight culture grown from a single-colony transformant was used as inoculum for 1.5 L terrific broth medium containing 50 µg/L kanamycin and 25 µg/L chloramphenicol, and the culture was grown at 37 °C until the OD₆₀₀ reached about 0.6. The incubation temperature was then changed to 18 °C, and the culture was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside. The induced cells were shaken continuously at 18 °C for an additional 15 h. The cells were harvested by centrifugation (11,900 × g for 10 min; JLA-10.500 rotor; Beckman). The cell pellet was resuspended in 45 mL start buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, containing two tablets of protease inhibitor mixture from Roche Applied Science) and lysed with a MultiFlex C3 Homogenizer (Avestin). The sample was centrifuged at 23,700 × g for 30 min at 4 °C. The pellet was homogenized using a sonicator (35% amplitude, 4.4-s pulse and 9.9-s pause for a total of 20 min) in start buffer to remove any soluble proteins. The

pellet from this step was resuspended in 30 mL denaturing buffer (6 M guanidine hydrochloride, 20 mM NaH₂PO₄, 500 mM NaCl, pH 7.5). The insoluble portion was removed by centrifugation, and the supernatant was loaded onto a 5-mL HiTrap HP nickel affinity column. The column was washed with denaturing buffer containing 30 mM imidazole and eluted with 15 mL denaturing buffer containing 1 M imidazole. Desalting of the eluent from both supernatant and pellet fractions was performed using preparative-scale RP-HPLC using a Waters Delta-pak C4 PrepPak Cartridge (15 µm, 300 Å, 25 × 100 mm). A gradient of 2–100% (vol/vol) of solvent B [0.086% TFA in 80% (vol/vol) acetonitrile/20% (vol/vol) water] was used. Dehydrated His₆-NisA eluted at 52% (vol/vol) B. The fractions containing modified NisA were lyophilized and analyzed by MALDI-TOF MS.

In Vitro Dehydration Activity Assay. The activity assay contained 200 µM His₆-NisA, 5 µM His₆-NisB, 50 mM Hepes, pH 7.5, 10 µL *E. coli* cell extract, 1 mM tris(2-carboxyethyl)phosphine, 10 mM MgCl₂, 1.5 mM spermidine, 175 mM glutamate, and 5 mM ATP. The activity assays were incubated at 30 °C for 4–6 h with gentle shaking. The cell extract of *E. coli* BL21 codon-plus RIL was prepared as reported previously (24).

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