Role of the Leader and Structural Regions of Prelantibiotic Peptides as Assessed by Expressing Nisin-Subtilin Chimeras in Bacillus subtilis 168, and Characterization of their Physical, Chemical, and Antimicrobial Properties*

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Biosynthesis of lantibiotics such as nisin and subtilin involves post-translational modifications, including dehydration of serines and threonines, formation of thioether cross-linkages, translocation, cleavage of a leader sequence, and release into the medium. We have studied the cellular machinery that performs the modifications by constructing and expressing nisin-subtilin chimeric prepeptides in a strain of Bacillus subtilis 168 that possesses all of the cellular machinery for making subtilin except for the presubtilin gene. The chimeras consisted of a normal subtilin leader region (SL), fused to nisin-subtilin chimeric structural regions, one of which was S1–Nis1–11–Sub12–32, in which the N-terminal portion of the structural region was derived from nisin, and the C-terminal portion derived from subtilin. This chimera was accurately and efficiently converted to the corresponding mature lantibiotic, as established by reverse phase high performance liquid chromatography profiles, proton NMR spectroscopy, mass spectral analysis, and biological activity. A succinylated form of the chimera was also produced. Another chimera was in the reverse sense, with subtilin sequence at the N terminus and nisin sequence at the C terminus of the structural region (S1–Sub1–31–Nis12–34). It was processed into a heterogeneous mixture of products, none of which had the characteristics of a correctly processed polypeptide, but did contain a minor component that was active, with a specific activity that considerably exceeded nisin itself. These results, together with results published earlier, establish that processing requires specific recognition between the prelantibiotic peptide and the processing machinery, and in order for the processing to occur correctly, there must be an appropriate combination of the N-terminal part of the leader region and the C-terminal part of the structural region of the prepeptide.

Nisin (produced by Lactococcus lactis) and subtilin (produced by Bacillus subtilis) are the most thoroughly studied examples of lantibiotics, which are ribosomally synthesized antimicrobial peptides that are characterized by the presence of unusual lanthio and dehydro residues. Their structures are shown in Fig. 1. Their biosynthesis involves several post-translational modifications: dehydration of serines and threonines, formation of thioether cross-linkages between dehydro residues and cysteines, translocation, removal of a leader sequence, and release of the mature antimicrobial peptide into the extracellular medium (reviewed in Refs. 1–3). Gene-encoded antimicrobial peptides constitute a family of natural products, whose known members are expanding rapidly in number and diversity and are produced by many kinds of organisms, ranging from bacteria to eukaryotes, including mammals (1, 4–6).

Their ubiquity among widely diverged organisms implies that they have had an opportunity to explore many strategies for achieving their antimicrobial properties, some of which are quite different from the mechanisms of classical antibiotics such as penicillin. They may therefore be able to supplement the arsenal of therapeutic antimicrobial agents that has been depleted as a result of the evolution of resistance among microbes. An advantage that is unique to gene-encoded antimicrobial peptides is that their structures can be readily manipulated by mutagenesis, which provides a facile means for constructing and producing the large numbers of structural analogs needed for structure-function studies and rational design. Whereas this advantage is shared by all gene-encoded antimicrobial peptides, the lantibiotics are unique in possessing the unusual dehydro and lanthio residues, which are absent from magainins (7–9), defensins (10–13), or oecropins (14, 15). This means that the lantibiotics offer chemical, physical, and hence biological properties that are not attainable by polypeptides that lack these residues. For example, the dehydro residues (Dha1 and Dhb) are electrophiles, whereas none of the ordinary amino acids is electrophilic. The thioether cross-linkages are more resistant to breakage than the disulfide bridge and can better survive reducing conditions and extremes of pH and temperature (16).

A concern when making mutants of lantibiotics is the effect of the mutations on the post-translational modification process, because a mutation that disrupts processing makes the biosynthesis of the corresponding mature lantibiotic peptide impossible. All known lantibiotic prepeptides contain an N-terminal region that is cleaved during maturation, and for the Type A lantibiotics (e.g. nisin, subtilin, and epidermin), this leader region is highly conserved (17), and its participation in the orchestration of post-translational modification and secretion has been proposed (17, 18). Certain mutations in the leader region of the nisin prepeptide have rendered the cell incapable of nisin production (19), whereas many mutations in the structural region of several lantibiotics that do not disrupt processing have been reported (e.g. Refs. 20 and 21). When the complete nisin prepeptide consisting of the nisin leader region and the nisin structural region (N1–Nis1–34) was expressed in a subtilin-producing cell, no nisin-related peptide products were

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made the downstream was modified in that it contained 2% sucrose and 10
leader region, SL nisin-producing cell, the nisin structural region contained the
attributed to the formation of incorrect thioether cross-linkages
region had been correctly cleaved and contained a full comple-
active nisin-like peptide was produced in which the leader
region (SL-Nis1–34) was expressed in a subtilin-producing cell (23).

These results imply that the subtilin processing machinery in B. subtilis is not capable of recognizing the nisin prepeptide (which is ordinarily expressed in Lactococcus lactis) and converting it to nisin. However, the subtilin machinery will perform the modification reactions on the nisin structural peptide if it is attached to a subtilin leader region, although the modifications seem to be misdirected so that active nisin is not produced. Finally, the subtilin machinery will produce active nisin if the leader region is an appropriate combination of subtilin leader and nisin leader sequences. In this work, we explore the contribution of the structural region and its relationship to the leader region by the construction and expression of nisin-subtilin chimeras, which contain chimeric nisin-subti-
lisin structural regions fused to the subtilin leader region. We have discovered that chimeras in which the C-terminal portion of the structural region correspond to subtilin are processed correctly and give active products, whereas those in which the C-terminal portion of the structural region corresponds to nisin produces a heterogeneous mixture of products, most of which, but not all, are inactive.

MATERIALS AND METHODS

Bacterial Strains, Cloning Vectors, and Mutagenesis—B. subtilis 168 strains and cloning vectors used were LH45ermAS (21), pTZ19U (Life Technologies, Inc.), pSMcat (21), and pACat (this work). Structural mutants of subtilin were constructed and expressed using the cassette mutagenesis system as described previously (21). Synthetic oligonucleo-
tides were annealed, filled in using Klenow fragment, restricted with EcoRI and HindIII, and cloned into the EcoRI-HindII site of pTZ19U.
The cloned fragment, which contained the mutation, was cut out with appropriate restriction enzymes and cloned into the corresponding site of the pSMcat vector or the pACat vector. The mutated sequence was confirmed by sequence analysis of the cloned insert using the Seque-

Culture Conditions and Purification of Chimeric Peptides—Strains producing the mutant peptides were grown in Medium A (21, 25) that was modified in that it contained 2% sucrose and 10 \mu g/ml chloram-
photin. The culture was incubated with vigorous aeration for 25–35 h at 35 °C, acidified to pH 2.5 with phosphoric acid, and heated to 121 °C for 3 min to inactivate proteases. A 0.5 volume of n-butanol was added, stirred at 4 °C for 2 h, allowed to stand at 4 °C for 2 h, and centrifuged; 2.5 volumes of acetone were added to the supernatant, allowed to stand at −20 °C for 16 h, and centrifuged. The pellet was lyophilized and resuspended in 20% acetonitrile with 0.05% trifluoroacetic acid. This was immediately purified on a C-18 reverse phase HPLC column using a trifluoroacetic acid (0.05%)-water-acetonitrile gradient in which the acetonitrile varied from 0 to 100% over 30 min at a rate of 1.2 ml/min, unless indicated otherwise.

RESULTS

Inspection of the structures of nisin and subtilin in Fig. 1 reveals that the number and locations of the thioether rings and the Dha residues are conserved. Each has one Dhb residue, but its position is not conserved. The N-terminal region is relatively conserved, except for three non-conservative differences out of the first 11 residues. Nisin has isoleucine at position 1, whereas subtilin has a bulky aromatic tryptophan. Subtilin has a positively charged lysine at position 2, whereas nisin has an unusual Dha residue. Finally, subtilin has a negatively charged glutamate at position 4, whereas nisin has a neutral aliphatic isoleucine. In previous work, we changed the Glu of subtilin to the Ile of nisin, and obtained a mutant with enhanced chemical stability and activity (21). We won-
dered what would happen if we changed the other two residues as well, to give a subtilin analog with a nisin-like N terminus. This analog would have only hydrophobic residues at the N terminus, as well as a fourth hydroxyl residue at a location that...
is unfamiliar to the subtilin processing machinery, and if it were unable to process it properly, the entire processing pathway could abort. Since the subtilin machinery cannot process the S₅-Nis₁⁻¹⁻Sub₁₂⁻³⁴ prepeptide to an active product (22), it is difficult to predict how the machinery would handle the S₅-Nis₁⁻¹⁻Sub₁₂⁻³⁴ prepeptide. We therefore constructed and expressed this prepeptide and examined its products.

Construction and Expression of the Nis₁⁻¹⁻Sub₁₂⁻³⁴ Chimera—Using the mutagenesis strategy shown in Fig. 2, residues 1, 2, and 4 in the subtilin structural region were changed to those of nisin. This chimeric gene was integrated into the chromosomal subtilin (spa) operon (21) at the site from which the natural subtilin gene has been deleted. The sequence of the Nis₁⁻¹⁻Sub₁₂⁻³⁴ chimera and the nucleotide sequence that encodes it is shown (top), in which the 32-residue mature Nis₁⁻¹⁻Sub₁₂⁻³⁴ sequence is numbered. Immediately below are the mutagenic oligonucleotides used to construct this sequence. The sequence of the S₁⁻¹⁻Nis₂⁻³⁴ chimera and the oligonucleotides used to produce it are at the bottom.

Construction and Properties of Nisin-Subtilin Chimeras

Mutagenesis was performed in the plasmid pSMcat, which is a cassette-mutagenesis plasmid that contains a copy of the subtilin structural gene upstream of a cat gene (21). When this plasmid is transformed into the B. subtilis 168 host LH45ermS and selected on chloramphenicol, the subtilin gene is integrated into the chromosomal subtilin (spa) operon (21) at the site from which the natural subtilin gene has been deleted. The appearance of subtilin that has been succinylated at its N terminus (29) indicates that succinylated subtilin is significantly less active than the normal unsuccinylated subtilin. We therefore subjected the succinylated subtilin to an assay using the same procedures as for the normal subtilin. The succinylated subtilin showed very little activity, while the normal subtilin showed strong activity. This result indicates that the succinylation of subtilin is not a normal post-translational modification.

Early Peak and Late Peak—In an attempt to attain a higher yield of material, the culture was allowed to incubate into late stationary phase, resulting in the appearance of a new peak on the HPLC column (inset), with the original peak (Early Peak) and the new peak (Late Peak). These peaks were separated by only 1 min, so were chromatographed using a shallower gradient (35–60% acetonitrile over 45 min, center), whereupon the early peak and late peak were separated by 4 min. The results of halo assays are shown above the center HPLC profile, with arrows indicating the positions in the profile from which the samples used for halo assays had been derived.

Detect any activity in the late peak, but when higher concentrations were tested, its activity was found to be approximately 10-fold lower than the early peak (data not shown). This is consistent with the observation that B. subtilis 6633 (the natural producer of subtilin) and LH45 (a subtilin-producing derivative of B. subtilis 168) produce two forms of subtilin (28); when B. subtilis 6633 is incubated into late stationary phase, there is an accumulation of subtilin that has been succinylated at its N terminus (29). The succinylated subtilin was significantly less active than the normal unsuccinylated subtilin. We therefore suspected that the late peak is the succinylated form of the early peak. This was confirmed by mass spectral analysis (Fig. 5), showing that the early peak consists mainly of a species with an Mᵦ = 3185.98 (panel A), which conforms exactly to the expected 3186-Da mass of the mature Nis₁⁻¹⁻Sub₁₂⁻³² chimer.

The Nis₁⁻¹⁻Sub₁₂⁻³² chimera was constructed as shown in Fig. 2 was expressed, isolated, and subjected to HPLC chromatography. Samples were collected at 1-min intervals and assayed for activity using the halo assay. The major peak contained the only activity. A portion of this peak sample was subjected to SDS-polyacrylamide gel electrophoresis and silver-stained. The stained gel is shown in a panel beside the peak (sample, left lane, size standard, right lane). Standards were 2.5-kDa myoglobin fragment (F3), 6.2-kDa myoglobin fragment (F2), and 8.1-kDa myoglobin fragment (F1). The expected mass of the Nis₁⁻¹⁻Sub₁₂⁻³² chimera is 3186 Da, which is consistent with the position of the band in the sample lane.
The Biological Activity of the Nis1–11-Sub12–32 Chimera

The Biological Activity of the Nis1–11-Sub12–32 Chimera—

Nisin and subtilin can inhibit spore-forming food spoilage bacteria from undergoing outgrowth from spores to the vegetative state, as well as inhibit cells that are in the vegetative state (30). The mechanism of inhibition of these types of cells is different, as it has been shown that the Dha5 residue is critical for subtilin to inhibit spore outgrowth, but not for subtilin to inhibit vegetative cells (31). The activity of the two purified forms of Nis1–11-Sub12–32 were therefore measured against outgrowing spores and vegetative cells, and compared to nisin. Since the activities of subtilin and E4I-subtilin have previously been compared to nisin (21), the relative activities among all forms can be inferred in terms of relative nisin units. The activity of Nis1–11-Sub12–32 against spore outgrowth was estimated by the halo assay and the liquid assays, and against vegetative cells by the liquid assay. The chimera was active against both spore outgrowth and vegetative growth, and the specific activities of the chimera and nisin were so similar that they could not be distinguished in either their ability to inhibit spore outgrowth or to inhibit vegetative cells (data not shown). Accordingly, one sees inhibition of spore outgrowth at about 0.2 μg/ml, and against vegetative cells at about 2 μg/ml, with both the chimera and nisin. Based on previous measurements (21, 31, 32), this means that the Nis1–11-Sub12–32 chimera is about 2-fold more active than E4I-subtilin and about 6-8 times more active than natural subtilin.

Stability of the Dehydro Residues in the Nis1–11-Sub12–32 Chimera during Incubation in Aqueous Solution—The chemical and biological instability of subtilin have been correlated with the tendency of residue Dha5 to spontaneously undergo chemical modification, which results in disappearance of the Dha5 peak in the NMR spectrum and loss of biological activity (21, 28, 33). This instability of residue Dha5 has been attributed to the participation of the carboxyl group of Glu4 of subtilin in the modification process. Accordingly, changing Glu4 to Ile4 (E4I-subtilin) dramatically enhanced the chemical stability of the Dha5 residue (21), with the chemical half-life of the Dha5 residue increasing nearly 60-fold, from less than a day to 48 days. Since the Nis1–11-Sub12–32 chimera has additional changes in the vicinity of the Dha5 residue, the chemical stability of the dehydro residues was examined by taking the NMR spectrum of a sample that was incubated in aqueous solution for an extended period of time. A 3-mg amount of the Nis1–11-Sub12–32 chimera (consisting of a mixture of the early peak and late peak as defined in Figs. 4 and 5) was dissolved in D2O at a pH of 6.0, placed in a closed NMR tube, and incubated in the dark at room temperature for 2.5 months. The NMR spectrum of this sample was determined from time to time, with the results shown in Fig. 6. The resonances of the dehydro residues changed very little during the course of the 72-day incubation period. The slight differences that are seen are readily attributable to variations introduced during base-line correction during computations with the spectral data. In contrast to the 0.8-day half-life of the Dha5 residue in natural subtilin and its 48-day half-life in E4I-subtilin, the half-life of the Dha5 residue in the Nis1–11-Sub12–32 chimera is so long that it cannot be estimated from the 72-day time point. Longer incubation times were not performed. We conclude that the dehydro residues in the Nis1–11-Sub12–32 chimera are extremely stable.

We conclude from these results that the Dha5 residue is subject to profound changes in its chemical reactivity, ranging...
from the most reactive state observed in natural subtilin, to the least reactive state observed in the Nis1–11-Sub12–32 chimera; with E4I-subtilin being in between. Somewhat surprisingly, the biological activity displayed by these structural variants varies inversely with the reactivity, with the unstable subtilin having the lowest activity, and the highly stable Nis1–11, Sub12–32 chimera displaying the greatest activity. The fact that the chemical reactivity of Dha5 varies inversely with biological activity argues that role of the Dha5 residue in the antimicrobial machinery is not related to its chemical reactivity in a simple fashion, and that other factors, such as the specificity imposed by the peptide sequence surrounding the dehydro residue, are also important.

Properties of the Sub1–11-Nis12–34 “Reverse Chimera”—An important feature of the S6-Nis1–11-Sub12–32 chimeric prepeptide is that the subtilin processing machinery was able to correctly recognize and process it into its corresponding mature form. Since the same machinery cannot successfully process S6-Nis1–34, it is clear that there is something in the Nis12–34 region that disturbs the subtilin processing machinery. If this is the case, we reasoned that the machinery would not be able to correctly process a chimera that contained this Nis12–34 region. We accordingly constructed a chimera that was a reverse (S6-Sub1–11-Nis12–34) of the previous one, in that it contained subtilin sequence at the N terminus of the structural region and nisin sequence at the C terminus. This chimera was constructed using the strategy described in Fig. 2, which was integrated into the chromosome and expressed. The corresponding polypeptide was recovered from the culture supernatant using the butanol-acetone extraction method, and further purified by RP-HPLC as shown in Fig. 7. A major peak emerged somewhat earlier than expected for the Sub1–11-Nis12–34 chimera, but it was devoid of activity. Moreover, mass spectral analysis showed it to have an M, = 3544.47, which is 56.47 mass units greater than the expected 3488 Da. It is clear that something has gone wrong in the processing of the prepeptide. Following this large peak was a small peak that showed activity in the halo assay, also shown in Fig. 7. The amount of material in this peak is quite small, and the mass spectral analysis shows it to be very heterogeneous, consisting of at least a half-dozen species; none of which corresponded to the mass expected for the Sub1–11-Nis12–34 chimera. Instead of an expected mass of 3488 Da, values of 3079 (expected ~ 408, 13% of total), 3193 (~ 295, 27%), 3322 (~ 166, 12%), 3437 (~ 51, 27%), and 4174 (~ 686, 21%) were obtained. None of these masses are readily explained in terms of simple processing defects, such as the dehydrations to give Dha5 and Dha33 (there is no Dhb6). Therefore, it is likely that the specific activity of whatever is responsible for the inhibitory activity is much higher than nisin itself. To show this, the total area of the active peak consists of no more than 10 μg of peptide, of which 0.13 μg was used for the halo assay shown in Fig. 7. This possesses an activity equivalent to 0.5 μg of nisin (data not shown). If all of the components in the peak were equally active, they would be about 4-fold more active than nisin. The amount of the various components in the peak ranges from about 12% to 27% of the total. If all of the activity is due to just one of the components, then this component would be about 15–35 times as active as nisin, depending on whether it is a major or minor component. Determining the actual active species and its activity will require that the active component be purified to homogeneity and studied further. Although we do not know what contributes to this high activity, this observation may constitute a serendipitous path to the design of lantibiotic analogs with superior antimicrobial properties.

**Discussion**

Our ability to incorporate the unusual dehydro and lanthio type amino acids into lantibiotic analogs and non-lantibiotic polypeptides depends on the ability of the lantibiotic processing machinery to cope with foreign precursor sequences. Our working hypothesis is that the leader region is primarily responsible for engaging the prepeptide with the processing machinery, and, once engaged, serines and threonines are dehydrated with little regard for the sequence in which they reside. Cysteines then react with particular dehydro partners in accordance with the forces of folding and conformation that exist within the polypeptide in a manner that is reminiscent of the specific selection of disulfide bond partners in polypeptides such as ribonuclease A and insulin (34). Although the results presented here do not prove this hypothesis, they are consistent with it and therefore support it. There are now several known instances in which prelantibiotic peptides undergo processing reactions, but give rise to inactive products. These are summarized in Table I. Examples are the S6-Nis1–34 chimera, which produces a processed (22) but inactive (22, 23) product when expressed in a cell that possesses the subtilin machinery, and the S6-Sub1–11-Nis12–34 chimera (this paper), which produces a heterogeneous mixture of products that are mainly inactive, although at least one active form is produced. This means that although N2-Nis1–34 is an authentic lantibiotic precursor, the subtilin machinery seems incapable of coping with it, and its gene products have not been detected (22, 23). However, if you place the subtilin leader in front of the nisin structural region to give S7-Nis1–34, a processed, but inactive, product is produced by the subtilin machinery (22). From this, we conclude that the subtilin leader is competent in engaging the processing machinery, but there is something about the conformational and folding interactions between the leader and structural region in the S7-Nis1–34 construct that causes some of the processing reactions, perhaps the partner selection in thioether
formation, to malfunction. The fact that the Sl-NiS1–11-NiS2–34 construct is processed properly to give active nisin (23) argues that critical conformational interactions are restored when an appropriate N-terminal sequence element from the nisin leader region is combined with a C-terminal sequence element of the subtilin leader. However, this combination of leader sequence elements must be appropriately complemented by the structural region. Whereas the Sl-NiS1–11-Sub12–32 construct is processed correctly (this work), the Sl-Sub1–11-NiS12–34 construct is not. Moreover, the processing reactions for the latter construct really run amok, giving a complex mixture of mainly inactive products. Surprisingly, at least one component in this mixture was active. Since none of the components had the mass of a correctly processed product, this activity must be due to an incorrectly processed component, and its specific activity was at least 4-fold and as much as 35-fold higher than nisin itself. We would like to know more about what is responsible for such high activity, because it may provide insight about how to design lantibiotics that are dramatically more effective than the natural forms. We conclude from these results that correct processing of the prelantibiotic peptide requires specific conformational communication between the N-terminal portion of the leader region and the C-terminal portion of the structural region.

These results also provide new insight about the relationship between the structure of lantibiotics and their chemical properties and biological activity. Subtilin and nisin are highly disparate in their chemical stability and specific activity, with nisin being superior to subtilin in both categories. The NiS1–11-Sub12–32 chimera has the superior properties of nisin, showing that the 3 residues that differ in the N-terminal regions of nisin and subtilin are primarily responsible for the disparity between nisin and subtilin. We note that NiS1–11-Sub12–32 has a very hydrophobic N-terminal region, which may facilitate insertion of the lantibiotic into the membrane, which is its target of action (26, 35–37). However, another possible explanation for its elevated activity is the presence of a second dehydro residue (Dhb) at position 2 in the NiS1–11-Sub12–32 chimera. One might expect that the Dhb2 would have a dramatic effect on the antimicrobial properties of the chimera, since it is so close to the critical Dha5 and might cooperate in reacting with its microbial target; however, if it does, there could be no more than a 2-fold effect. This illustrates a frustrating aspect of our knowledge about lantibiotics. The ubiquitous occurrence of the unusual residues among the many known lantibiotics argues that they are conserved because they have important functions. Except for the critical role of Dha5 in inhibition of spore outgrowth, functions that clearly justify this ubiquitous occurrence.

Table I

| Prepeptide sequence | Strain in which expressed | Prepeptide is processed | Peptide is secreted into extracellular medium | Secreted peptide is active | Ref. |
|---------------------|---------------------------|-------------------------|---------------------------------------------|---------------------------|-----|
| Sl-S1–32            | B. subtilis 6633          | Yes                     | Yes                                         | Yes                       | 18  |
|                    | B. subtilis 168           | Yes                     | Yes                                         | Yes                       | 21  |
| Nl-NiS1–34          | L. lactis 11454           | Yes                     | Yes                                         | Yes                       | 17  |
|                    | B. subtilis 6633          | No                      | No                                          | NA*                       | 22, 23 |
|                    | B. subtilis 168           | No                      | No                                          | NA                        | Unpublished |
| Sl-NiS11–34         | L. lactis 6633            | Yes                     | Yes                                         | No                        | 24  |
|                    | B. subtilis 6633          | No                      | No                                          | NA                        | 22, 23 |
| Sl1–7-Nl8–23-NiS1–34| B. subtilis 6633          | Yes                     | Yes                                         | Yes                       | 23  |
| Sl1–11-Nl12–32      | B. subtilis 6633          | Yes                     | Yes                                         | Yes                       | This work |
| Sl-Sub1–11-NiS12–34 | B. subtilis 168           | Heterogeneous           | Yes                                         | Partially                 | This work |

* NA, not applicable.
rence have yet to be identified. This suggests that there are some very important attributes of lantibiotics that we know nothing about.

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