Structural Features of the Final Intermediate in the Biosynthesis of the Lantibiotic Nisin. Influence of the Leader Peptide

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ABSTRACT: The antimicrobial membrane-interacting polypeptide nisin is a prominent member of the lantibiotic family, the members of which contain thioether-bridged residues called lanthionines. To gain insight into the complex biosynthesis and the structure/function relationship of lantibiotics, the final intermediate in the biosynthesis of nisin A was studied by nuclear magnetic resonance spectroscopy. In aqueous solution the leader peptide part of this precursor adopts predominantly a random coil structure, as does the synthetic leader peptide itself. The spatial structure of the fully modified nisin part of the precursor is similar to that of nisin in water. The leader peptide part does not interact with the nisin part of the precursor molecule. Thus, these two parts of the precursor do not influence each other's conformation significantly. The conformation of the precursor was also studied while complexed to micelles of dodecylphosphocholine, mimicking the primary target of the antimicrobial activity of nisin, i.e. the cytoplasmic membrane. The location of the molecule relative to the micelles was investigated by using micelle-inserted spin-labeled 5-doxylstearic acid. It was observed that the N-terminal half of the nisin part of the precursor interacts in a different way with micelles than does the corresponding part of mature nisin, whereas no significant differences were found for the C-terminal half of the nisin part. In this model system the leader peptide is in contact with the micelles. It is concluded that the strongly reduced in vivo activity of the precursor molecule relative to that of nisin is not caused by a difference in the spatial structure of nisin and of the corresponding part of precursor nisin in water or by a shielding of the membrane interaction surface of the nisin part of the precursor by the leader peptide. Probably a different interaction of the N-terminal part of the nisin region with membranes contributes to the low activity by preventing productive insertion. The residues of the leader peptide part just next to the nisin part are likely to contribute most to the low activity of the precursor.

A number of bacterial polypeptides contain post-translationally modified amino acid residues such as lanthionine and 3-methyllanthionine. These substances have been termed lantibiotics and are subdivided in types A and B (Kellner et al., 1988; Sahl et al., 1995). Lantibiotics of type A are elongated molecules that primarily act by membrane perturbation, whereas the globular type B lantibiotics appear to inhibit enzyme function (Sahl et al., 1995). A well-known example of type A lantibiotics is nisin, which is produced by strains of the food grade organism *Lactococcus lactis* subsp. *lactis*. Two natural variants, nisin A (His27) and nisin Z (Asn27), with almost identical antimicrobial properties, have been isolated (Mulders et al., 1991; De Vos et al., 1993). Nisin A is referred to as nisin in this paper. The molecule possesses bactericidal activity against a broad spectrum of Gram-positive bacteria or their spores (Hurst, 1981; Chan et al., 1996). The primary target is the cytoplasmic membrane of sensitive bacteria, in which membrane-potential-dependent transient pores can be formed, leading to an efflux of small molecules and a collapse of the protonmotive force, eventually resulting in cell death (Ruhr & Sahl, 1985; Henning et al., 1986; Kordel & Sahl, 1986; Sahl et al., 1987; Driessen et al., 1995). The activity of nisin against food spoilage bacteria such as *Clostridia* led to its application in a variety of food products, especially dairy products (Delves-Broughton et al., 1996). Also potential pharmaceutical and veterinary applications are under investigation.

One-third of the 34 residues in nisin are of a type not encountered in regular proteins and include dehydroalanine (Dha[1]), dehydrobutyrine (Dhb), lanthionine, and 3-methyllanthionine (Figure 1). The α,β-unsaturated residues Dha and Dhb are formed by dehydration of Ser and Thr residues, respectively (Ingram, 1970). Subsequently, cysteine thiol groups can undergo an addition reaction with Dha and Dhb, resulting in lanthionine and 3-methyllanthionine, respectively. The (3-methyl)lanthionines form five ring structures in the nisin molecule (Figure 1) (Gross & Morell, 1971).

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[1] Abbreviations: Ala^{*}_{S}, 3-methylalanyl moiety of (2*S*,3*S*,6*R*)-3-methyllanthionine; Ala_{S}, D-alanyl moiety of *meso*-lanthionine; $_{S}Ala$, L-alanyl moiety of *meso*-lanthionine or of (2*S*,3*S*,6*R*)-3-methyllanthionine; cmc, critical micelle concentration; δ, chemical shift; 1/2/3D, one-/two-/three-dimensional; Dha, dehydroalanine; Dhb, dehydrobutyrine; DPC, dodecylphosphocholine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; P.COSY, purged correlated spectroscopy; ppb, parts per billion; ppm, parts per million; ROE, rotating-frame nuclear Overhauser enhancement; ROESY, ROE spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TOCSY, total correlation spectroscopy.

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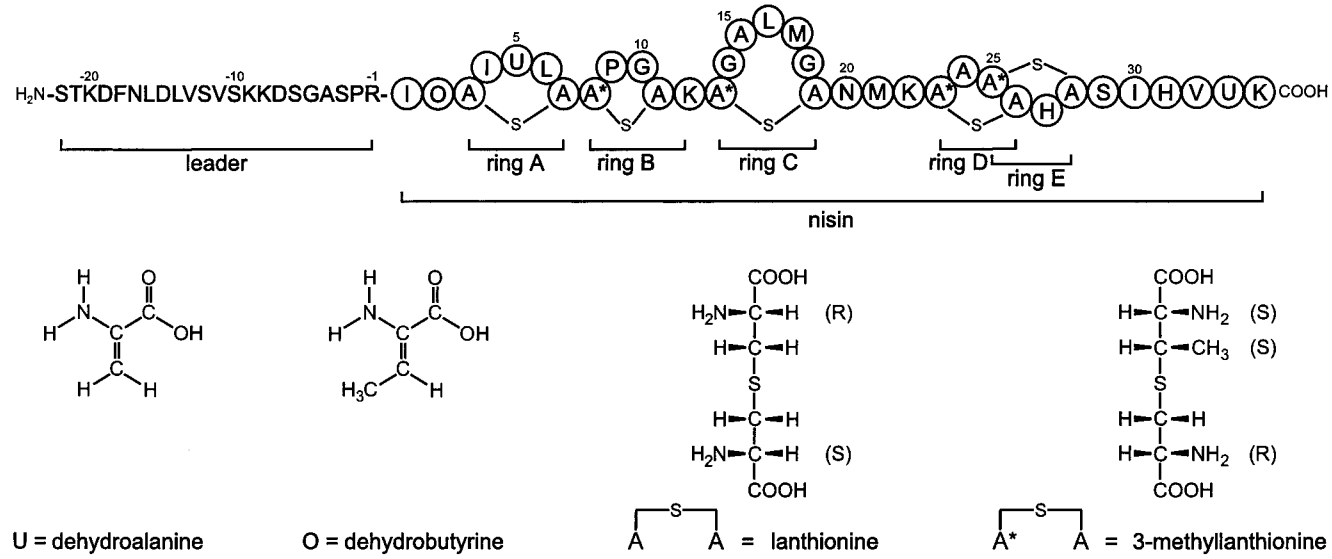

FIGURE 1: Representation of the structure of the final intermediate in biosynthesis of nisin and structures of the unusual residues.

Biosynthesis of nisin starts with transcription of the *nisA* gene (Buchman et al., 1988), which encodes an N-terminal 23-residue leader peptide and a C-terminal 34-residue part corresponding to the unmodified nisin sequence. The next steps are post-translational modification reactions, transport across the membrane, and cleavage of the leader peptide. A nisin precursor polypeptide has been isolated, which was secreted by a producing cell in which the gene encoding the leader peptidase was inactivated (Van der Meer et al., 1993). Preliminary studies indicated that this molecule (referred to as precursor nisin) is fully modified nisin with its unmodified leader peptide (without the initial methionine) still attached (Figure 1). Evidence has been obtained that cleavage of the leader peptide is the last step in the biosynthesis of nisin (Van der Meer et al., 1993, 1994). This process most likely occurs outside the lactococcal cells. The precursor nisin molecule has an antimicrobial activity that is at least 200-fold lower than that of wild-type nisin (O.P.K., unpublished results).

Also, for the type A lantibiotic Pep5, biosynthetic intermediates have been isolated (Weil et al., 1990). These intermediates, found in a cytoplasmic protein fraction, contained dehydrated residues but no lanthionines, while the leader peptide was unmodified. This suggested that maturation is initiated by selective dehydration and that thioether-ring formation is not closely linked to the dehydration process (Weil et al., 1990). It has recently been suggested that the Pep5 leader peptide is cleaved off inside the staphylococcal cells (Meyer et al., 1995). The order of the biosynthetic events, processing and transport across the membrane, apparently can vary between the different lantibiotic producing bacteria.

Of the few currently described lantibiotic precursor molecules, only the final intermediate in nisin biosynthesis, precursor nisin, can be isolated in amounts sufficient for conformational analysis by high-resolution NMR spectroscopy. Such studies are necessary to obtain insight into the complex biosynthesis of lantibiotics. The role of the leader peptide in lantibiotic precursors is currently not known, but several functions have been proposed (Jung, 1991), namely keeping the precursor molecule inactive, assisting in the modifications, or being involved in secretion. These leader peptides were predicted to be α-helical (Jung, 1991). Now for the first time the spatial structure of the leader peptide in a biosynthetic intermediate can be elucidated, together with the interactions between the leader peptide and the lantibiotic part of the precursor. Also, an NMR analysis is presented of the synthetic leader peptide of nisin, which includes an N-terminal methionine residue (Beck-Sickinger & Jung, 1993), so that the nisin precursor can be compared to its two individual components, i.e. the leader peptide and mature nisin (Van de Ven et al., 1991; Van den Hooven et al., 1993, 1996a,b). In this way the influence of the presence of the leader peptide on the nisin part of the precursor can be examined. This will be discussed in terms of the low activity of the precursor molecule and of related molecules.

MATERIALS AND METHODS

Production and Purification of the Final Intermediate in Nisin Biosynthesis. The isolation procedure for this precursor nisin (Van der Meer et al., 1993) is similar to that of nisin mutants (Kuipers et al., 1993a). Essentially, *L. lactis* strain MG1614 (Gasson, 1984) harboring plasmid pNZ9111 (containing *nisABTCIR*) (Van der Meer et al., 1993) was grown without aeration at 30 °C in a medium containing 1% sucrose, 1% peptone (Difco), 1% yeast extract (Difco), 1% KH_2PO_4, 0.2% NaCl, and 0.002% $MgSO_4 \cdot 7H_2O$, supplemented with 10 μg/mL erythromycin. During 20-L fermentation the pH was maintained at 6.1. After growth for 8.5 h, precursor nisin was purified from the culture supernatant by hydrophobic interaction chromatography on Fractogel TSK butyl 650-S (Merck) (Mulders et al., 1991; Kuipers et al., 1992), followed by RP-HPLC on a Hi-Pore RP318 column of 250 × 21.5 mm (Bio-Rad) (Rollema et al., 1995). The HPLC elution buffers consisted of 10% aqueous acetonitrile with 0.1% trifluoroacetic acid (buffer A) and 90% aqueous acetonitrile with 0.08% trifluoroacetic acid (buffer B). The gradient used in preparative runs was from 22% to 26% buffer B in buffer A, linear in 50 min at a flow rate of 10 mL/min.

NMR Spectroscopy. The synthetic leader peptide of nisin (Beck-Sickinger & Jung, 1993) was a kind gift of Prof. Dr. G. Jung and Dr. A. G. Beck-Sickinger (University of Tübingen, Germany). Samples contained 2.6 mg of the synthetic leader peptide of nisin or 7−9 mg of precursor

nisin in 0.5 mL of $H_2O/^2H_2O$ (9:1) or in 0.5 mL of ^2H_2O. To a sample containing 5.4 mg of precursor nisin in 0.5 mL of $H_2O/^2H_2O$ (9:1) was added 26.9 mg of perdeuterated DPC (Campro Scientific) (molecular ratio precursor nisin/DPC = 1:73). The pH of all samples was adjusted at 20 °C to 3.5 (pH meter reading). NMR experiments were conducted at 5 and 25 °C for the synthetic leader peptide, at 5, 25, and 40 °C for precursor nisin in aqueous solution, and at 40 °C for precursor nisin complexed to DPC micelles.

1D-NMR spectra, NOESY spectra (Jeener et al., 1979; Bodenhausen et al., 1984) with mixing times of 300−450 ms, 2D-MLEV17-TOCSY spectra (Bax & Davis, 1985; Griesinger et al., 1988) with mixing times of 40−50 ms, and P.COSY spectra (Marion & Bax, 1988) were recorded at 400 MHz on a Bruker AM400 spectrometer, interfaced to an Aspect3000 computer. For the synthetic leader peptide also a ROESY spectrum (Bothner-By et al., 1984) with a mixing time of 100 ms was recorded at 400 MHz. NOESY spectra were also recorded at 600 MHz on a Bruker AMX2 600, interfaced to an Aspect station, on a sample of precursor nisin in aqueous solution at 5 °C (mixing times of 150 and 450 ms) and on a sample of precursor nisin complexed to DPC micelles at 40 °C (mixing time of 450 ms). Acquisition and processing parameters were essentially the same as those described previously (Van den Hooven et al., 1996a,b). The spectra were referenced to sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS).

$^3J_{NH-H^\alpha}$ coupling constants were determined by inverse Fourier transformation of in-phase multiplets (Szyperski et al., 1992) from NOESY spectra with mixing times of 400−450 ms and 4096 data points in t_2. For this purpose the ETH automated spectroscopy program package for X window systems (XEASY, T.-H. Xia and C. Bartels, Zürich, Switzerland) together with the program INFIT (Szyperski et al., 1992) were used.

The spin-label experiment involved a sample containing 2.7 mg of precursor nisin and 13.4 mg of DPC-d_{38} in 0.5 mL of ^2H_2O at pH 3.5 (pH meter reading). The spin-label 5-doxylstearic acid (Aldrich) was added as previously described (Van den Hooven et al., 1996b) to a concentration of 0.2 mM. 2D-MLEV17-TOCSY spectra with mixing times of 32 ms were recorded at 40 °C on a Bruker AM400 spectrometer. The effect of the micelle-inserted 5-doxylstearic acid on the ^{1}H-NMR spectrum of precursor nisin complexed to DPC micelles was analyzed by subtracting Fourier-transformed TOCSY spectra recorded for the sample with and without spin-label as described for nisin (Van den Hooven et al., 1996b). The computed relative-difference spectrum was analyzed as previously reported (Van den Hooven et al., 1996b). A line-broadening effect is attributed to a single residue of precursor nisin, if a majority of the cross-peaks arising from that residue show up in the relative-difference spectrum.

RESULTS AND DISCUSSION

General Remarks. A part of the data presented in this paper are interpreted in relation to previous studies of nisin in aqueous solution (Van de Ven et al., 1991) and when complexed to membrane-mimicking micelles of anionic sodium dodecyl sulfate (SDS) and of zwitterionic dodecylphosphocholine (DPC) (Van de Hooven et al., 1993, 1996a,b). In all three systems the spatial structure of nisin consists of a structured N-terminal domain encompassing the first three lanthionine rings A, B, and C, connected (via a hinge region) to a structured C-terminal domain encompassing the residues Lys22 to $_S$Ala28 including the two intertwined lanthionine rings D and E (Van de Ven et al., 1991; Van den Hooven et al., 1996a). Despite the different polar head-groups of the detergents, the spatial structures of nisin complexed to DPC or to SDS micelles appear to be similar. However, the structure of the first lanthionine ring of nisin differs from that in aqueous solution (Van den Hooven et al., 1996a). The molecule is amphipathic in water, a property that is retained when the molecule complexes to micelles. Nisin is located on the surface of DPC and SDS micelles with the hydrophobic residues immersed into the micelles and the hydrophilic residues oriented outward (Van den Hooven et al., 1996b). The interaction of nisin with the micelle systems is conceived to model the first step in the mechanism underlying its antimicrobial activity, namely the binding of nisin to the cytoplasmic membrane of target bacteria. This step is proposed to be followed by aggregation and membrane-potential-dependent insertion.

Precursor Nisin and the Synthetic Leader Peptide in Aqueous Solution. To study the conformations of precursor nisin and of the synthetic leader peptide, NMR spectra were recorded for each molecule in an aqueous solution of pH 3.5 (see parts A and B of Figure 2, respectively), under the same conditions at which NMR spectra had been collected for nisin (Figure 2C) (Slijper et al., 1989; Van de Ven et al., 1991). In COSY and TOCSY spectra of precursor nisin and of the synthetic leader peptide the cross-peak patterns of the individual residues were identified. The same unusual residues found in nisin were also encountered in precursor nisin, as established by the observation of their characteristic signals in the NMR spectrum [e.g. Chan et al. (1989) and Slijper et al. (1989)]. A sequential assignment was performed through the observation of NOEs between $H_i^\alpha-NH_i$ and NH_i-NH_{i+1} protons (Wüthrich, 1986). For the synthetic leader peptide also ROEs were used for this purpose. The combined analysis of all NMR spectra resulted in complete sets of resonance assignments for precursor nisin in water at 5 (available as Supporting Information), 25, and 40 °C and for the synthetic leader peptide in water at 5 and 25 °C. These results demonstrate that the tentatively described covalent structure of precursor nisin as fully matured nisin attached to its unmodified leader peptide lacking the initial methionine residue (Figure 1) (Van der Meer et al., 1993) is correct.

NH-proton temperature coefficients were calculated from the chemical shift (δ) data obtained from NMR experiments taken at 5 and 25 °C [as $-1000 \times (\Delta\delta/\Delta T)$]. In general, low NH-proton temperature coefficients are indicative of hydrogen bonding and/or shielding from the solvent. These coefficients, together with the observed chemical shifts, give qualitative information on the conformation of a molecule. Thus, using these results in combination with $^3J_{NH-H^\alpha}$ coupling constants (related to the backbone torsion angle ϕ), the influence of the presence of the leader peptide part of the precursor on the conformation of the nisin part and vice versa was examined (Figures 3, 4, and 5, respectively). From Figures 3−5 it is apparent that the NMR data of the residues in the synthetic leader peptide and in nisin are highly similar to the data of the corresponding residues in precursor nisin.

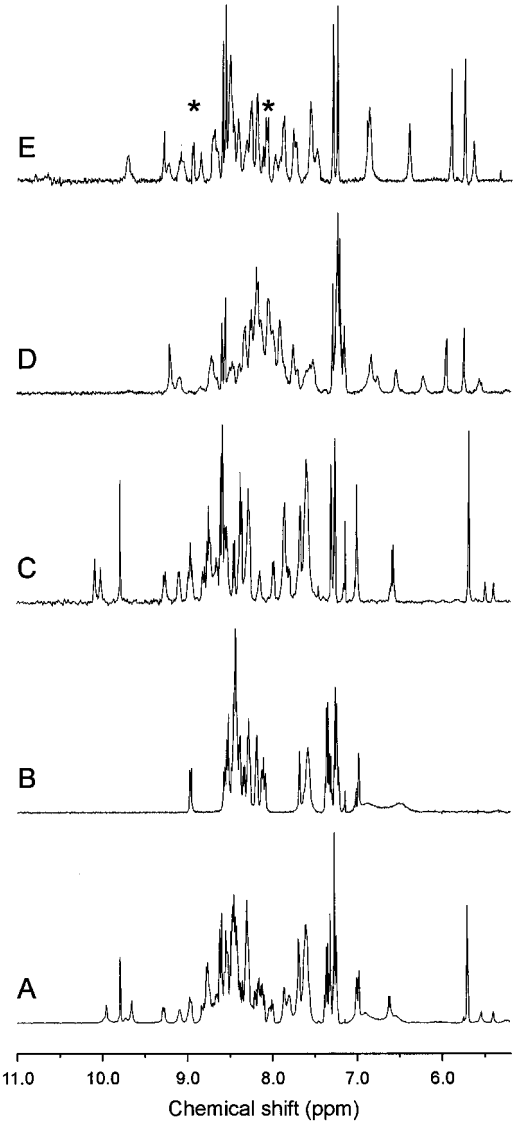

FIGURE 2: Amide, aromatic, and vinyl proton region of the ^{1}H-NMR spectra of (A) precursor nisin in aqueous solution at 5 °C, (B) the synthetic leader peptide of nisin in aqueous solution at 5 °C, (C) nisin in aqueous solution at 5 °C, (D) precursor nisin complexed to DPC micelles (precursor nisin/DPC = 1:73) at 40 °C, and (E) nisin complexed to DPC micelles (nisin/DPC = 1:45) at 40 °C. The pH of all samples was adjusted at 20 °C to 3.5 (pH meter reading). Pyridine was present as an impurity in the DPC used for nisin/DPC, and two of its resonances at 8.95 and 8.12 ppm are indicated by asterisks; the third resonance at 8.58 ppm overlaps with resonances of nisin and is not indicated [see also Van den Hooven et al. (1993)].

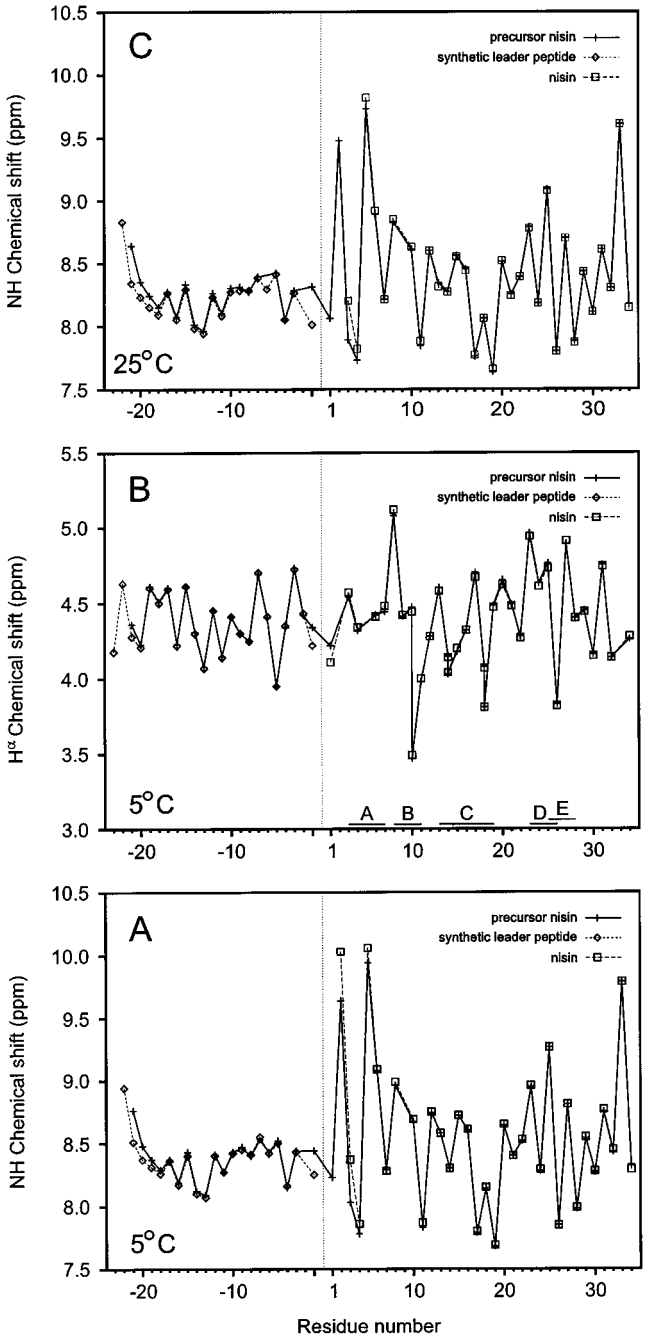

FIGURE 3: Chemical shifts (in ppm) of backbone protons of precursor nisin (+, solid line), of the synthetic leader peptide (◇, dotted line), and of nisin (□, dashed line) versus the residue number. Chemical shifts were obtained from NMR spectra of all three molecules in an aqueous solution of pH 3.5. (A) Chemical shifts of NH protons derived from NMR spectra recorded at 5 °C, (B) chemical shifts of H^α protons from measurements at 5 °C, and (C) chemical shifts of NH protons at 25 °C. The positions of the lanthionine rings A−E in nisin and in precursor nisin are indicated in (B).

Significant differences are only observed at the N terminus of the leader peptide and near the processing site (Arg−1, Ile1). The first difference can be ascribed to the missing methionine residue in precursor nisin, which is present in the synthetic leader peptide, while the second difference is due to the covalent linking of the leader peptide to the nisin part in precursor nisin.

The NOEs observed for the central part of the leader peptide part of precursor nisin are similar to the NOEs observed for the central part of the (isolated) synthetic leader peptide. This confirms that the conformation of the leader peptide part of precursor nisin is similar to that of the synthetic leader peptide as discussed above. Furthermore, it can be concluded that in aqueous solution both the leader peptide part of precursor nisin and the synthetic leader peptide predominantly adopt a random coil conformation: First, the backbone NH- and H^α-proton chemical shifts of the leader peptide cluster around values typically expected for residues in random coil conformations (Figure 3). Second, the chemical shifts of the side-chain protons of residues of the leader peptide are very close to the corresponding values for residues in random coils (Wüthrich, 1986). In addition, all $^3J_{NH-H^\alpha}$ coupling constants determined for residues of the leader peptide are in the 6−8 Hz range (Figure 5), typically expected for flexible peptides. The NH-

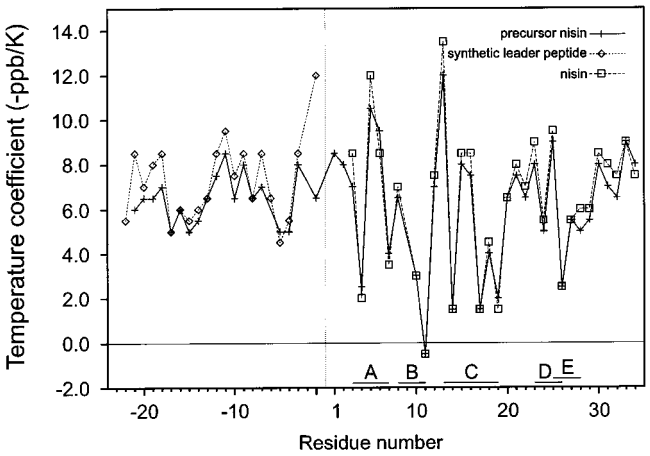

FIGURE 4: Backbone amide proton temperature coefficients (in −ppb/K) versus the residue number for precursor nisin (+, solid line), the synthetic leader peptide (◇, dotted line), and nisin (□, dashed line). These coefficients were calculated using the resonance assignments of NMR spectra recorded at 5 and 25 °C for all three molecules in an aqueous solution of pH 3.5. The positions of the lanthionine rings A−E in nisin and in precursor nisin are indicated.

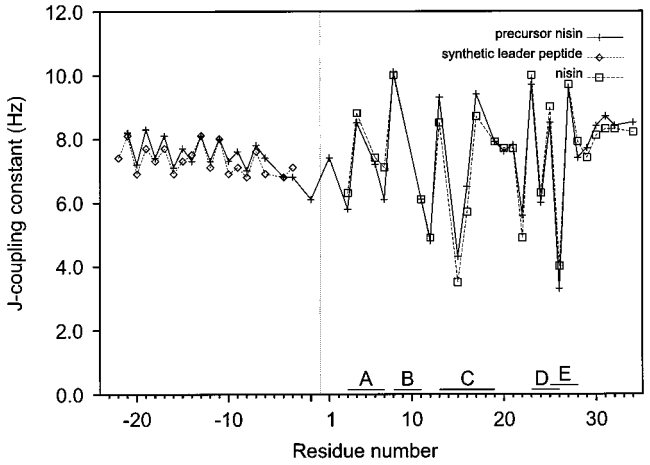

FIGURE 5: $^3J_{NH-H^\alpha}$ coupling constants (in Hz) versus the residue number for precursor nisin (+, solid line), the synthetic leader peptide (◇, dotted line), and nisin (□, dashed line). These constants were obtained from spectra recorded for the three molecules in an aqueous solution of pH 3.5 at 5 °C. The positions of the lanthionine rings A−E in nisin and in precursor nisin are indicated.

proton temperature coefficients obtained for the leader peptide are in line with these observations (Figure 4); no indications are found for hydrogen bonding or solvent shielding. Also, long-range NOEs between protons of the leader peptide are absent and no typical "secondary structure NOEs" could be identified. It has, however, been observed that in a mixture of trifluoroethanol and water the synthetic leader peptide shows helical content (Lian et al., 1991; Beck-Sickinger & Jung, 1993).

At this point, the conformation of the nisin part of the precursor can be compared to the spatial structure of nisin in aqueous solution (Van de Ven et al., 1991). On the basis of chemical shifts, NH-proton temperature coefficients, $^3J_{NH-H^\alpha}$ coupling constants (Figures 3−5), and the observation that the NOEs for the residues Ala^*_S8 to Lys34 of precursor nisin and nisin are comparable, it can be concluded that the conformation of this part of precursor nisin is identical to that of the corresponding part of mature nisin. For the residues Ile1−$_S$Ala7, including lanthionine ring A, of precursor nisin and mature nisin, differences in chemical shift are observed for the NH protons of the residues Dhb2−Dha5 and for the H^α proton of Ile1 (Figure 3). These chemical shift differences may be caused by the presence of the link to the leader peptide in precursor nisin, which is absent in nisin, by a difference in spatial structure of the residues Ile1−$_S$Ala7 of both molecules, or by both. We have the following reasons to believe it is the first effect: first, the observed differences in chemical shift are much smaller than the differences between the chemical shifts of the N-terminal NH and H^α protons of nisin in aqueous solution and of nisin complexed to DPC micelles (Van den Hooven et al., 1993), where two (trans) peptide bonds flanking residue Dha5 were almost completely inverted when nisin was brought from one environment into the other (Van den Hooven et al, 1996a). Second, no significant differences in NH-proton temperature coefficients and $^3J_{NH-H^\alpha}$ coupling constants for the residues Ile1−$_S$Ala7 of both precursor nisin and nisin were observed (Figures 4 and 5). Third, the NOEs observed for the residues Ile1−$_S$Ala7 of precursor nisin did not indicate conformational changes when compared to those of mature nisin.

The interactions between the leader peptide part and the nisin part of the precursor have also been investigated by analyzing NOESY spectra recorded at 5 °C with mixing times of 150 and 450 ms. Almost all NOEs were observed within the leader peptide part or within the nisin part. Only a few trivial NOEs were detected between protons from different parts, which involved protons at the processing site, namely contacts between the neighboring residues Arg−1 and Ile1 and between the residues Arg−1 and Dhb2. Hence, we conclude that the leader peptide part is apparently not folded onto the nisin part of precursor nisin. This is corroborated by the aforementioned similarity in chemical shifts, temperature coefficients, and $^3J_{NH-H^\alpha}$ coupling constant data of the residues of precursor nisin when compared to the data obtained for the corresponding residues of the synthetic leader peptide and of nisin (Figures 3−5). All of these data together indicate that the presence of the leader peptide in precursor nisin has little influence on the conformation of the nisin part and vice versa. The spatial structure of precursor nisin in aqueous solution can thus be described as follows: the leader peptide part is unstructured and the nisin part adopts the same 3D structure as nisin (Van de Ven et al., 1991).

Precursor Nisin in the Presence of Membrane-Mimicking Micelles of Dodecylphosphocholine. Precursor nisin has also been investigated in the presence of DPC micelles to mimic the interaction with its target site, i.e. the cytoplasmic membrane. This study was initiated by examining the effect of the detergent on the ^{1}H-NMR spectrum of the precursor by starting with the molecule in an aqueous solution of pH 3.5 at room temperature and gradually increasing the amount of DPC. At detergent concentrations below the critical micelle concentration [cmc, which is 1.1 mM at 22 °C (Lauterwein et al., 1979)], no spectral changes were observed, indicating that there is no interaction between precursor nisin and monomeric DPC. Just above the cmc of DPC shifting and broadening of resonances of precursor nisin were observed. The resonances shifted continuously until a precursor nisin/DPC ratio of about 1:40 was reached. Further addition of DPC no longer affected the NMR spectrum of precursor nisin. These results are similar to those observed for the nisin−DPC system (hereafter referred to as

nisin/DPC) (Van de Hooven et al., 1993) and are interpreted as evidence for complexation between precursor nisin and DPC micelles.

Subsequently, 2D-NMR spectra of precursor nisin complexed to DPC micelles (precursor/DPC) were recorded at 40 °C and pH 3.5, the same conditions at which nisin/DPC (Figure 2E) was studied. The NMR spectrum of precursor/DPC (Figure 2D) is more involved than that of precursor nisin in aqueous solution (precursor/H_2O), the main reason being a significant line broadening of the resonances of precursor/DPC compared to those of precursor/H_2O, resulting in a decrease in the signal-to-noise ratio. The positions of cross-peaks could, however, still be determined with good accuracy, so that almost all of the resonances of precursor/DPC at 40 °C could be assigned (resonance assignments available as Supporting Information). Those that could not be identified are mostly from the N-terminal half of the nisin part of the precursor. The large line widths and the low signal-to-noise ratio hamper an accurate determination of cross-peak volumes and of $^3J_{NH-H^\alpha}$ coupling constants and, thus, a calculation of the 3D structure. Unfortunately, a direct comparison of the NOEs of precursor/DPC and precursor/H_2O did not yield sufficiently reliable information; thus, the conformation of precursor/DPC relative to that of precursor/H_2O could not be investigated in this way. At temperatures below 40 °C the line widths are significantly larger and attempted assignments of resonances gave poor results, so no NH-proton temperature coefficients could be obtained in this way. For nisin NH-protons, temperature coefficients had been obtained for the molecule in water and complexed to micelles. Also in this case the coefficients of the individual NH resonances were not interpreted; instead, differences in the coefficients between the two systems were examined. Only significant differences were observed for NH protons of the N and C termini, which could be explained by a conformational change and solvent shielding, respectively (Van den Hooven et al., 1996a,b).

The presence of DPC micelles affects the chemical shifts of the majority of the backbone protons of precursor nisin upon going from water to the membrane-mimicking environment (Figure 6). The leader peptide part of the precursor shows moderate differences in chemical shift of NH and H^α protons with maxima of 0.16 and 0.14 ppm, respectively. This suggests that there is no gross effect on the conformation of the leader peptide part when going from the one to the other system. To analyze the nisin part of precursor/DPC the differences between the chemical shifts of the backbone protons (amide-NH and H^α) of nisin/DPC and the corresponding ones of precursor/DPC are examined (Figure 7), together with these differences between nisin/H_2O and precursor/DPC and between precursor/DPC and precursor/H_2O. Significant differences between precursor/DPC and nisin/DPC are only observed for the residues Ile1−$_S$Ala19 (Figure 7). The largest differences in chemical shift between precursor/DPC and nisin/DPC are observed for the residues Ile1, Dhb2, Ile4, Dha5, Leu6, and Ala$^*_S$8 (Figure 7), all part of or close to lanthionine ring A. No chemical shift data are available for the backbone protons of the lanthionine residues Ala$_S$3 and $_S$Ala7 of precursor/DPC. From our previous studies on nisin it was concluded that the differences in chemical shift of the residues Ile1−$_S$Ala19 between nisin/H_2O and nisin/DPC were caused by an interaction with

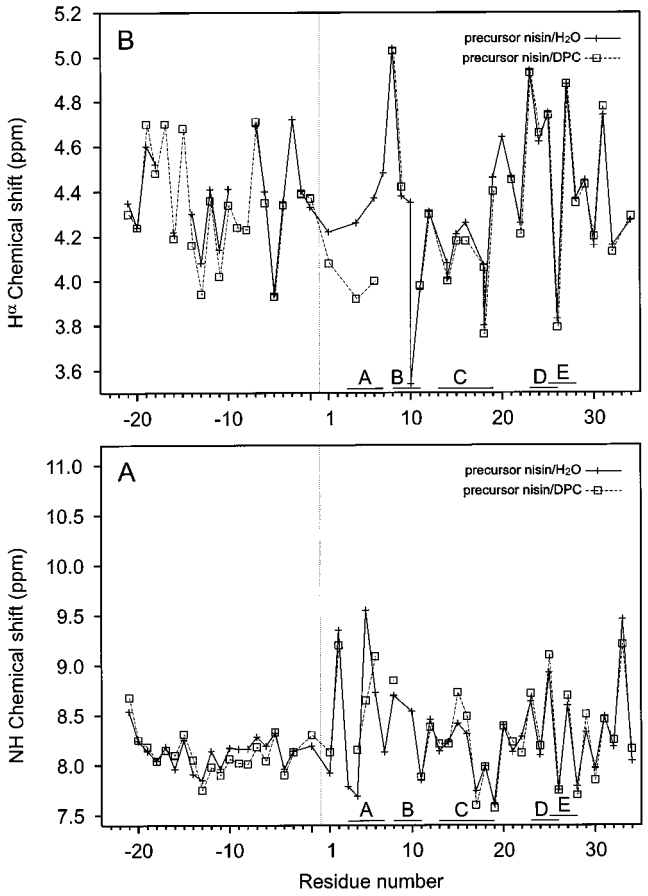

FIGURE 6: Chemical shifts (in ppm) of backbone protons versus the residue number for precursor nisin in aqueous solution (+, solid line) and for precursor nisin complexed to DPC micelles (□, dashed line). (A) Chemical shifts of NH protons and (B) of H^α protons. The data were derived from NMR spectra taken at 40 °C. For glycines the chemical shifts are given for both H^α protons. A break in the line indicates missing data for residues. Lines are connected between data points of residues where the connecting residue lacks a backbone proton, i.e. Pro, Dha, and Dhb. The positions of the lanthionine rings A−E in nisin and in precursor nisin are indicated.

the micelles and, for the residues Ile4-Dha5-Leu6, also by a change in conformation upon binding to micelles (Van de Ven et al., 1991; Van den Hooven et al., 1996a,b). For precursor/DPC the NH-proton chemical shifts of the residues Ile4-Dha5-Leu6 and Ala$^*_S$8 have intermediate values between those of nisin/DPC and those of nisin/H_2O (Figure 7). This is also observed for the H^α protons of the residues Ile1 and Leu6. The conformation of this part of the molecule may be different for precursor/DPC and nisin/DPC. The differences in chemical shift of the residues Asn20−Lys34 between nisin/H_2O and nisin/DPC were mainly caused by the presence of DPC micelles and not by conformational changes (Van den Ven et al., 1991; Van den Hooven et al., 1996a,b). This is probably also the case for these residues of the precursor, since the chemical shifts of the residues Asn20−Lys34 are similar for precursor/H_2O and nisin/H_2O and also for precursor/DPC and nisin/DPC.

Incorporation of spin-labeled 5-doxylstearic acid into DPC micelles allows investigation of the location of precursor nisin relative to the micelles as recently described for nisin (Van den Hooven et al., 1996b). The chemical shifts of precursor/DPC did not change upon the addition of 5-doxylstearic acid, indicating that the spin-label has little or no effect on the conformation of precursor nisin. Those protons

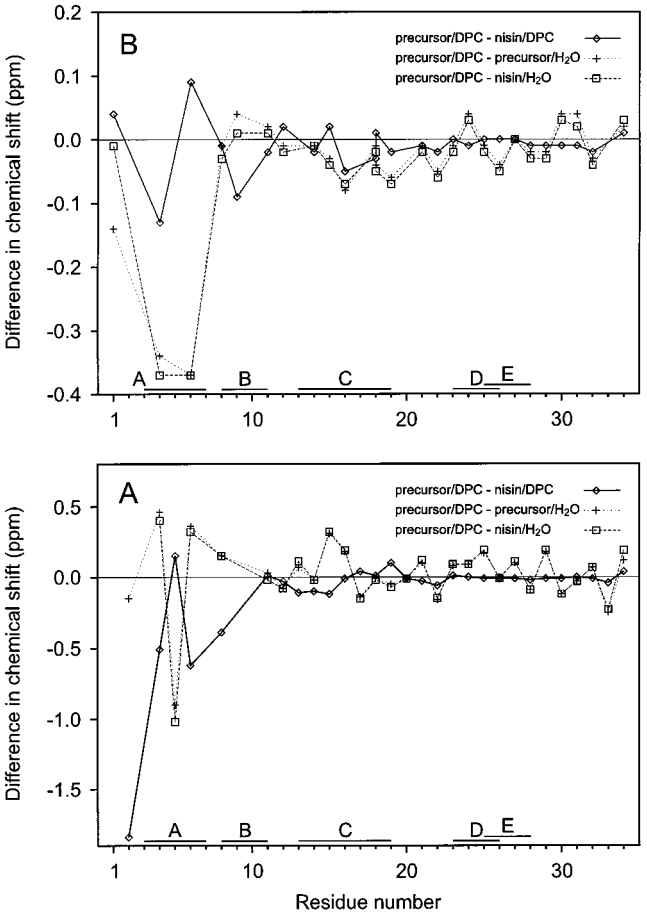

FIGURE 7: Differences in chemical shift (in ppm) of (A) NH protons and (B) H^{α} protons versus the residue number for, on the one hand, the nisin part of precursor nisin complexed to DPC micelles and, on the other hand, the nisin part of precursor nisin in aqueous solution (+, dotted line), nisin in aqueous solution (□, dashed line), and nisin complexed to DPC micelles (◇, solid line). The data were derived from NMR spectra recorded at 40 °C. The positions of the lanthionine rings A−E in nisin and in precursor nisin are indicated.

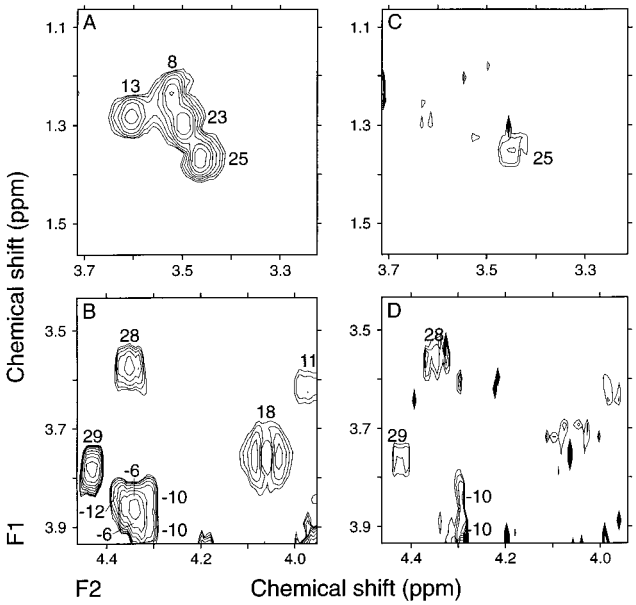

FIGURE 8: Effect of the micelle-inserted spin-labeled molecule 5-doxylstearic acid on the ^{1}H-NMR spectrum of precursor nisin complexed to DPC micelles. (A, B) Regions of a TOCSY spectrum, recorded with a mixing time of 32 ms, at 313 K and pH 3.5, and (C, D) corresponding regions of the relative-difference spectrum, respectively (see Materials and Methods). The numbers in the subfigures refer to the residues to which the cross-peaks have been assigned (see Figure 1). The contouring in the relative-difference spectrum starts at 20% with increments of 10%. Intensities in the difference spectrum at the edges of peaks in the unperturbed TOCSY spectrum are not interpreted as spin-label-induced effects (e.g. for Gly18). Spurious peaks resulting from noise can be seen in the difference spectrum as intense diamond-shaped cross-peaks at positions where no precursor nisin cross-peaks are expected. In (A) and (C) cross-peaks characteristic of 3-methyllanthionines are shown; in (B) and (D) several cross-peaks from both the leader peptide part and the nisin part of the precursor are shown.

of the precursor in the proximity of the micelle-inserted spin-label show line broadening, resulting in a decrease in peak height, which can be observed in difference spectra (Figure 8) obtained by subtracting Fourier-transformed TOCSY spectra recorded for samples with and without spin-label (Van den Hooven et al., 1996b). The effect induced by the spin-label is specific. However, for a number of residues overlap of cross-peaks precludes the determination of the broadening effect.

A number of residues of the leader peptide part of precursor nisin were observed to be in the proximity of the micelle-inserted spin-label, namely the residues Ser−10, Val−11, Val−13, and Phe−18. Resonances of the residues Ser−3, Ala−4, Ser−6, and Ser−12 were not affected by the spin-label. The leader peptide part is clearly in contact with the micelles and is not fully exposed to the water phase.

For the majority of the residues of the nisin part of the precursor, the qualitative line-broadening effect of micelle-inserted spin-label was comparable to that observed for nisin/DPC. Only for the residues Ile1 and Ile4 were somewhat different results obtained for precursor/DPC and nisin/DPC; the effects on the residues Ile1 and Ile4 of precursor/DPC were weaker and stronger, respectively. Spin-label and chemical shift data together show that the residues Asn20−Lys34 of precursor/DPC and nisin/DPC adopt similar conformations and interact with DPC micelles in a similar way. The described differences for the residues Ile1−$_S$Ala19 of precursor/DPC and nisin/DPC can be ascribed to the presence of the leader peptide in precursor nisin. The region in which these differences were observed happens to coincide with the first structured domain in nisin/DPC, just N-terminal of a stretch of residues showing structural variability (Van den Hooven et al., 1996a). This variable region makes it conceivable that the effect of the leader peptide stops just at these residues (Figure 7).

Possible Functions for the Leader Peptide. Leader peptides of type A lantibiotics are hydrophilic, highly charged, devoid of cysteine residues and do not show the characteristics of *sec*-dependent signal sequences (De Vos et al., 1995; Sahl et al., 1995). These leader peptides are not post-translationally modified as shown for nisin and Pep5 (Weil et al., 1990; Kuipers et al., 1993b, Van der Meer et al., 1993, 1994). It has already been demonstrated that the leader peptide of nisin is involved in biosynthesis (Rintala et al., 1993; Van der Meer et al., 1994; Chakicherla & Hansen, 1995). The conserved residues Ser6 and Phe18 to Asp15 (De Vos et al., 1995) appeared essential for modification, secretion, or both (Van der Meer et al., 1994). The leader peptide part of precursor nisin, the synthetic leader peptide, and probably also the leader peptide in the primary translation product (Van den Hooven, 1995) are predominantly random coiled in aqueous solution. Thus, the conserved sequence Phe-Asn-Leu-Asp of the nisin leader peptide does not adopt

a well-defined structure that could act as a rigid recognition site. However, conformational adjustment is possible to binding sites of the modifying enzyme(s), of proteins involved in transport, or at a membrane. In this respect, it is noted that in a mixture of trifluoroethanol and water the synthetic leader peptide of nisin shows helical content (Lian et al., 1991; Beck-Sickinger & Jung, 1993).

Four possible functions have been proposed for type A lantibiotic leader peptides (Jung, 1991). First, the leader peptide may keep precursor molecules biologically inactive. Indeed, the final intermediate in biosynthesis of nisin is almost inactive. Second, assistance of the precursor prepeptide in transport across the cytoplasmic membrane may be envisioned. In this respect, it is noted that the leader peptide part of precursor nisin was observed to be in contact with, but not traversing, the membrane-mimicking micelles; the biological relevance of this observation is not clear. Third, it may act as a recognition or binding site for biosynthetic enzymes. Fourth, an interaction between the leader peptide part and the rest of the molecule may favor a conformation essential for correct modification, preventing nonspecific reactions. In precursor nisin, which is already modified, the leader peptide part and the nisin part do not interact, so that at this point in the maturation there are no indications for such a function.

Low Biological Activity of Precursor Nisin and Related Molecules. The low biological activity of precursor nisin is not caused by a shielding of the membrane interaction surface of the nisin part of the precursor by the leader peptide. Neither does the spatial structure of precursor/H_2O give a clue to this low activity, because the 3D structure of the nisin part is almost identical to that of nisin/H_2O. It was, however, observed that the N-terminal half of the nisin part of precursor nisin interacts in a different way with DPC micelles than does the corresponding part of nisin, whereas the C-terminal half of the nisin part of the precursor binds to the micelles in a similar way as the corresponding part of nisin (Van den Hooven et al., 1996a,b). This difference can be ascribed to the presence of the hydrophilic leader peptide, which may alter the penetration of at least the first nisin residue, the hydrophobic micelle-inserted Ile1, into the micelles. By extrapolating these data to membranes, it is suggested that nisin and precursor nisin interact in a different way with membranes. This difference is likely to contribute to the low biological activity of precursor nisin. Since in the formation of pores binding to the cytoplasmic membrane is thought to be followed by insertion and aggregation, it is conceivable that a different membrane interaction can have a large effect on the activity, i.e. pore formation. Other, not yet investigated, factors may be of relevance for the low biological activity of precursor nisin as well, such as the accessibility of the cytoplasmic membrane via the cell wall or the behavior of the molecules in the actual pore.

The results obtained in this study can be used to rationalize results of previous studies on related molecules. A chimera has been constructed containing the unmodified leader peptide of the closely related lantibiotic subtilin (~65% sequence identity between the leader peptides of nisin and subtilin) and fully modified nisin (Kuipers et al., 1993b). This chimera and its main tryptic fragment, i.e. [ITPQ]-nisin (Kuipers et al., 1993b), showed in comparison with nisin a decrease in interaction with anionic and with zwitterionic lipids in a monolayer study (Demel et al., 1996) and also an at least 200-fold lower antimicrobial activity (Kuipers et al., 1993b), similar to that of precursor nisin. The presence of the residues in positions −1 to −4 in the leader peptide is likely to be the main cause for the low activity of these extended nisin molecules. These residues in the nisin and the subtilin leader peptide constitute the sequences ASPR and ITPQ, respectively, which show similar hydropathy profiles. From our data the comparable low activities of the extended nisin molecules can be understood, because the random coil character of the leader peptide of precursor nisin and the lack of interaction between the leader peptide and nisin part strongly suggest that only one or a few residues of the leader peptide just next to the Ile1 can influence the nisin part and the activity of the molecule. The presence of a hydrophilic residue, Arg−1, just before the first hydrophobic residue of nisin, Ile1, known to insert into micelles, is likely to make the effect on activity quite substantial.

Conclusion. The final intermediate in the biosynthesis of nisin consists of a fully matured nisin part and an unmodified leader peptide part without the initial methionine residue. This molecule is the first intermediate in the biosynthesis of lantibiotics for which the spatial structure has been characterized. In aqueous solution the leader peptide part adopts a random coil conformation; the conserved sequence does not adopt a well-defined structure that could act as a rigid recognition site. The spatial structure of the nisin part of the precursor closely resembles that of wild-type nisin. There is almost no interaction between these parts. The interaction of the precursor residues Ile1−$_S$Ala19 with DPC micelles differed from that observed for wild-type nisin, whereas similarities were observed for the residues Asn20−Lys34. This different interaction is likely to contribute to the strong decrease in biological activity when compared to wild-type nisin. This low activity is not caused by a shielding of the membrane-interacting side of the nisin part of precursor nisin by an interaction with the leader peptide, or by a different conformation of the nisin part relative to nisin in aqueous solution. The low activities of the nisin molecules with the subtilin leader peptide or the sequence ITPQ linked to the N terminus can now be rationalized.

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SUPPORTING INFORMATION AVAILABLE

^{1}H-NMR resonance assignments for precursor nisin in aqueous solution at 5 °C and pH 3.5 and for precursor nisin complexed to DPC micelles at 40 °C and pH 3.5 (6 pages). Ordering information is given on any current masthead page.

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