Three-dimensional structure of the lantibiotic nisin in the presence of membrane-mimetic micelles of dodecylphosphocholine and of sodium dodecylsulphate

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(Received 26 July 1995) – EJB 95 1240/3

The lantibiotic nisin is a cationic, polycyclic bacteriocin of 34 residues, including several unusual dehydro residues and thioether-bridged lanthionines. The primary target of its antimicrobial action is the cytoplasmic membrane. Therefore the conformation of nisin when bound to membrane-mimicking micelles of zwitterionic dodecylphosphocholine and of anionic sodium dodecylsulphate was determined with high-resolution NMR spectroscopy. Structures were calculated on the basis of NMR-derived constraints with the distance-geometry program DIANA and were further refined by restrained energy minimization using X-PLOR. The conformation of nisin complexed to both types of micelles is the same, irrespective of the different polar head-groups of the detergents. The structure consists of two structured domains: an N-terminal domain (residues 3–19) containing three lanthionine rings, A, B and C; and a C-terminal domain (residues 22–28) containing two intertwined lanthionine rings numbered D and E. These domains are flanked by regions showing structural variability. Both domains are clearly amphipathic, a property characteristic for membrane-interacting polypeptides. The structures of the ring systems are better defined than those of the linear segments. The four-residue rings B, D and E of nisin all show a β turn structure, which is closed by the thioether linkage. The backbones of the rings B and D form type II β-turns. Ring E resembles a type I β-turn. Preceding the intertwined rings D (residues 23–26) and E (25–28) another type-II β-turn is found formed by the residues 21–24, so that the C-terminal domain consists of three consecutive β-turns. The structures of nisin in the micellar systems differ significantly from the previously determined (and now partially recalculated) structure in aqueous solution [van de Ven, F. J. M., van den Hooven, H. W., Konings, R. N. H. & Hilbers, C. W. (1991) Eur. J. Biochem. 202, 1181–1188] in the first lanthionine ring around dehydroalanine 5.

Keywords: bacteriocin; lanthionine-containing polypeptide; membrane-interaction; NMR; distance geometry.

Nisin is a bacteriocin produced by Lactococcus lactis subsp. lactis and is active against a broad range of Gram-positive bacteria. It contains a number of unusual amino acids, namely lanthionine, 3-methyllanthionine, dehydroalanine and dehydrobutyrine (Fig. 1). Because of the presence of lanthionines, it is a member of the group of related polypeptides termed lantibiotics (Schnell et al., 1988); for reviews see (Jung, 1991; Bierbaum and Sahl, 1993; Entian and Klein, 1993; Hansen, 1993; Sahl et al., 1995). More specifically, nisin belongs to the class of type-A lantibiotics.

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Abbreviations. Ala, 3-methylallyl moiety of (2S, 3S, 6R)-3-methyllanthionine; Alaγ, d-allyl moiety of meso-lanthionine; γ-Ala, l-allyl moiety of meso-lanthionine or of (2S, 3S, 6R)-3-methyllanthionine; COSY, correlated spectroscopy; 2/3D, two/three-dimensional; Dha, dehydroalanine; Dhb, dehydrobutyrylpeptide (3-methyldehydroalanine); DopPCho, dodecylphosphocholine; DQF-COSY, double-quantum-filtered COSY; ROE, rotating-frame nuclear Overhauser enhancement; ROESY, ROE spectroscopy; TOCSY, total correlated spectroscopy; TPPI, time-proportional phase incrementation.

The primary target of the antimicrobial nisin molecule is the membrane of sensitive cells (Ruhr and Sahl, 1985; Kordel and Sahl, 1986; Henning et al., 1986; Sahl et al., 1987; Bruno et al., 1992; Okereke and Montville, 1992; Abee et al., 1994; Winland et al., 1994). Its antimicrobial activity is based on an interference with the membrane function, which is caused by its interaction with the phospholipid components of the cytoplasmic membrane (Henning et al., 1986). Nisin does not seem to require a specific membrane receptor. The activity of nisin was found to be influenced by the phospholipid composition of the liposomal membrane (Gao et al., 1991; García García et al., 1993; Diessens et al., 1995) and depends on a trans-negative membrane potential of sufficient height (Sahl et al., 1987; Diressen et al., 1995). Type-A lantibiotics seem in general to act by membrane perturbation.

Lantibiotics are ribosomally synthesized as prepeptides, after which the unusual amino acids are introduced via post-translational modifications (Schnell et al., 1988). Dehydroalanine and dehydrobutyrylpeptide are formed by dehydration of serine and threonine residues, respectively. Subsequently, thiol groups of cysteine residues can undergo an addition reaction with these α,β-unsaturated amino acids, resulting in, respectively, lanthionine or 3-methyl-2-lanthionine (Ingram, 1970). The part of the lanthio-
nine or of the 3-methylanthionine which is derived from serine or threonine is always in the D configuration, whereas the other half, which originates from a cysteine residue, retains its L configuration (Gross and Morell, 1971). The Cα of 3-methylanthionine is in the S configuration (Morell and Gross, 1973). The dehydrobutyryne is in the Z configuration (Chan et al., 1989).

The antimicrobial activity of nisin against certain food-spoilage bacteria such as *Clostridia* and its non-toxic nature has led to its application as a preservative in a variety of food products around the world, such as processed cheese, dairy desserts, milk, fermented beverages, meat and canned foods (Hurst, 1981; Delves-Broughton, 1990; Vandenbergh, 1993).

NMR studies have been performed on nisin in aqueous solution in order to contribute to the elucidation of its structure/function relationship. As a result the complete assignments of the 'H-NMR spectrum (Chan et al., 1989; Slijper et al., 1989) and determination of the three-dimensional (3D) structure (van de Ven et al., 1991b; Lian et al., 1992) have been reported. Recently, the conformation of nisin has been studied by NMR and CD spectroscopy in membrane-mimicking environments, simulating its target site (van den Hooven et al., 1993). The results indicated that nisin undergoes a local structural transition when brought into the membrane-like environments. Here we report on the 3D structures of nisin complexed to membrandic micelles of zwiterionic dodecylphosphocholine (DodPCho) and of anionic sodium dodecyl sulphate. These structures are compared to the previously determined structure of nisin in aqueous solution (van de Ven et al., 1991b). The structure of nisin in water was determined via a different protocol and has now partially been recalculated. The newly calculated structures are somewhat better determined than the previous one. The structures of the nisin molecules when complexed to DodPCho and to SDS micelles are similar to each other and differ significantly from that in water in ring A, particularly around residue Dha5 (Dha, dehydroalanine).

**Fig. 1. The primary structure of nisin and structures of the unusual amino acids.** (a) U, dehydroalanine; (b) O, dehydrobutyryne; (c) L-3-s-3, 3-methylanthionine.

**MATERIALS AND METHODS**

**Chemicals.** Nisin was obtained as a gift of Aplin & Barrett. Prior to use the material was further purified by reverse-phase HPLC (Rollema et al., 1991). [H3]SDS and [H30]DodPCho were purchased from MSD Isotopes.

**NMR spectroscopy.** 2D NMR measurements were performed at 40°C. NOESY spectra (Jeener et al., 1979; Bodenhausen et al., 1984) with mixing times of 50, 150 and 450 ms were recorded for a sample containing 3 mM nisin and 100 mM [H30]SDS and for a sample with 3.6 mM nisin and 160 mM [H30]DodPCho. The pH was adjusted to 3.5 (pH meter reading). The NOESY spectra were obtained at 600 MHz on a Bruker AM600 spectrometer, interfaced to an Aspect3000 computer. The carrier was placed at the water resonance. The solvent signal was suppressed by continuous irradiation during the relaxation delay (1.5 s) and also during the mixing time. The spectra were acquired in the phase-sensitive mode using the time-proportional-phase-incrementation (TPPI) method (Redfield and Bax, 1988) and double-quantum-filtered (DQF)-COSY spectra were recorded with samples similar to those used for the NOESY experiments. The ROESY spectra were measured at 600 MHz on a Bruker AMX2 600 spectrometer, interfaced to an Aspect station equipped with a RS3000 processor. In the ROESY experiment the spin-lock field was 3.5 kHz and the mixing time was 80 ms. Two π/2 pulses were flanking the spin-lock pulse to eliminate offset effects (Griesinger and Ernst, 1987). Data processing involved multiplication of the free induction decays (FID) by a π/3 shifted sine bell and zero-filling once in t2 and twice in t1.

Rotating-frame NOE spectroscopy (ROESY; Bothner-By et al., 1984), purged correlated spectroscopy (P. COSY; Marion and Bax, 1988) and double-quantum-filtered (DQF)-COSY spectra were recorded with samples similar to those used for the NOESY experiments. The ROESY spectra were measured at 600 MHz on a Bruker AMX2 600 spectrometer, interfaced to an Aspect station equipped with a RS3000 processor. In the ROESY experiment the spin-lock field was 3.5 kHz and the mixing time was 80 ms. Two π/2 pulses were flanking the spin-lock pulse to eliminate offset effects (Griesinger and Ernst, 1987). Data processing involved multiplication of the free induction decays (FID) by a π/3 shifted sine bell and zero-filling once in t2. The COSY type spectra were recorded on a Bruker AM400 spectrometer operating at 400 MHz and interfaced to an Aspect3000 computer. The number of data points in the t2 direction was 8192 and 512 in t1. The FIDs were multiplied by shifted sine bells with phase shifts π/6 and π/4 along t1 and t2, respectively. The final resolution in the F2 direction of the COSY spectra was 1.2 Hz/point.

Temperature coefficients (van den Hooven et al., 1995) were calculated from the resonance assignments at 5–7°C, 25°C and 40°C (van den Hooven et al., 1993). Amide proton exchange was measured by dissolving lyophilized H2O samples in H2O.
and following the time dependence of the disappearance of amide proton signals.

Angle constraints. For both micellar systems, due to the large NH line width, only the larger $\tilde{J}_{\text{cis},\text{cis}}$ coupling constants (>8 Hz) could be determined reliably from the COSY cross-peaks. The large coupling constants $\tilde{J}_{\text{cis},\text{trans}}$ were used to restrict the torsion angle $\phi$ in the structure calculations to $[-160^\circ, -80^\circ]$ (Kline et al., 1988) or to $[80^\circ, 160^\circ]$ in the case of L-amino acids or D-amino acids, respectively.

Distance constraints. The majority of the constraints that were used in the structure calculations were derived from the NOESY spectra. The cross-peak volumes were determined using the NMRi software package. The conversion to distances was carried out with the program NO2DI, which takes spin diffusion into account (van de Ven et al., 1991a). The NOESY spectra with mixing times of 50 ms and 150 ms yielded similar distances and the average distance was used. The distances derived from the NOESY spectrum with a mixing time of 450 ms often deviated from the distances derived from the 50-ms and 150-ms NOESY. Only those NOE cross-peaks in the 450-ms NOESY spectrum that were not observed in the 50-ms and 150-ms NOESY spectra were taken into account. These long-mixing-time NOEs were carefully checked in the spectrum and the corresponding distances, which were in the $0.5-0.7$ nm range, were added to the list derived from the 50-ms and 150-ms NOESY spectra. The thus-obtained distances $(d_i)$ were converted to upper-distance bounds $(d_u) = 0.95 \times d_i + 0.05 \times d_i^2$. Lower-distance bounds $(d_l)$ were calculated only for $\text{H}^\alpha-\text{NH}_2$, $\text{H}^\beta-\text{NH}_2$, and $\text{NH}_2-\text{NH}_2$ distances according to $d_l = 1.03 \times d - 0.04 \times d^2$. These formulae were chosen to obtain an allowed deviation of $5\%$ for a distance of 0.2 nm and $15\%$ for a distance of 0.45 nm. Lower-distance bounds that are close to the sum of the van der Waals' radii of two protons are not informative and were therefore omitted.

Structure calculations. The 3D structures were calculated by the program DIANA 2.1 (Güntert et al., 1991a) using the aforementioned angle and distance constraints as input. Residue topologies for dehydroalanine and dehydrobutyryne were added. Standard X-PLOR patches for the N-terminus and C-terminus and for the conversion of L-amino acids to D-amino acids were used. Patches were constructed to build lanthionines from Ser, Thr and Cys. The following energy constants were used: $k_{\text{distance}} = 10.5 \text{ MJ} \cdot \text{mol}^{-1} \cdot \text{nm}^{-2}$, $k_{\text{torsion-angletension}} = 84 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$ and $k_{\text{gausian}}$, $k_{\text{torque angle}}$ and $k_{\text{compperp}}$ had default values (from the X-PLOR manual). A planar energy term was introduced to maintain the constituents of the double bond of Dha and Db (dehydrobutyryne) within a given plane. A torsion-angle energy term with an energy constant of $8.4 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$ was included for the side chains to preclude eclipsed conformations. However for lanthionines, where the side chain is part of the backbone of a ring, this term was excluded. The van der Waals' function was a simple repulsive potential; the energy constant for this repulsion function was $168 \text{ MJ} \cdot \text{mol}^{-1} \cdot \text{nm}^{-2}$. The van der Waals' radii were multiplied by 0.8 to obtain radii which are similar to those used in the DIANA program (Brünger, 1992). A square-well function was used as the restraining function for NOE-derived distances. The minimization consisted of two parts. First 1000 steps of unrestrained energy minimization were performed with only covalent-bond, bond-angle and improper-dihedral-angle energy terms to convert the covalent geometry of the structures calculated by DIANA to the one used by X-PLOR. Then 4000 steps of restrained energy

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Table 1. Number of distance bounds used for the structure calculations.

<table>
<thead>
<tr>
<th>Micelle</th>
<th>Type of bound</th>
<th>Number of distance bounds</th>
<th>other sequential*</th>
<th>medium-range and long-range*</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>upper</td>
<td>83</td>
<td>33</td>
<td>148 (33)</td>
<td>326</td>
</tr>
<tr>
<td>SDS</td>
<td>lower</td>
<td>80</td>
<td>13</td>
<td>152 (11)</td>
<td>2053</td>
</tr>
<tr>
<td>DodPClo</td>
<td>upper</td>
<td>105</td>
<td>40</td>
<td>221 (50)</td>
<td>484</td>
</tr>
<tr>
<td>DodPClo</td>
<td>lower</td>
<td>78</td>
<td>31</td>
<td>184 (21)</td>
<td>916</td>
</tr>
</tbody>
</table>

* The number of constraints involving lanthionines, which is included in the first number in this column, is indicated within parentheses.
* The number of inter-ring constraints, which is included in the number of medium-range and long-range distance bounds, is indicated within parentheses.

with the GLOMSA program (Güntert et al., 1991a and b). The criteria for acceptance of a stereospecific assignment were essentially the same as those published for the Antp(C39=S) homeodomain (Güntert et al., 1991b). The obtained structures satisfied all constraints.

In the next step of the protocol a further refinement of the structures was carried out in which the back-calculated NOESY spectrum was fitted to the experimental NOESY spectrum recorded with a mixing time of 150 ms. For this purpose the program CORMA 2.25 was used (Borgias et al., 1990). A single effective isotropic correlation time of 2.5 ns for all interactions was assumed. A three-site jump model for all methyl relaxation was used. In cases where a NOE was back-calculated and the corresponding cross-peak was clearly absent in the NOESY and in the ROESY spectrum lower-distance bounds, with a maximum of 0.4 nm, were introduced in subsequent DIANA calculations. These lower-distance bounds were introduced for protons for which NOEs to other nisin resonances had been observed. Lower-distance bounds were also introduced when the calculated NOE intensity was much larger than the measured intensity. Subsequently, another DIANA calculation was carried out with the new set of lower-distance bounds and the CORMA/DIANA cycle was repeated.

The structures were further refined by restrained energy minimization in vacuo with X-PLOR 3.1 (Brünger, 1992). Residue topologies for dehydroalanine and dehydrobutyryne were added. Standard X-PLOR patches for the N-terminus and C-terminus and for the conversion of L-amino acids to D-amino acids were used. Patches were constructed to build lanthionines from Ser, Thr and Cys. The following energy constants were used:

- $k_{\text{distance}} = 10.5 \text{ MJ} \cdot \text{mol}^{-1} \cdot \text{nm}^{-2}$
- $k_{\text{torsion-angletension}} = 84 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$
- $k_{\text{gausian}}$, $k_{\text{torque angle}}$ and $k_{\text{compperp}}$ had default values (from the X-PLOR manual).

A planar energy term was introduced to maintain the constituents of the double bond of Dha and Db (dehydrobutyryne) within a given plane. A torsion-angle energy term with an energy constant of $8.4 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{rad}^{-2}$ was included for the side chains to preclude eclipsed conformations. However for lanthionines, where the side chain is part of the backbone of a ring, this term was excluded. The van der Waals' function was a simple repulsive potential; the energy constant for this repulsion function was $168 \text{ MJ} \cdot \text{mol}^{-1} \cdot \text{nm}^{-2}$. The van der Waals' radii were multiplied by 0.8 to obtain radii which are similar to those used in the DIANA program (Brünger, 1992). A square-well function was used as the restraining function for NOE-derived distances. The minimization consisted of two parts. First 1000 steps of unrestrained energy minimization were performed with only covalent-bond, bond-angle and improper-dihedral-angle energy terms to convert the covalent geometry of the structures calculated by DIANA to the one used by X-PLOR. Then 4000 steps of restrained energy
minimization were carried out with covalent-bond, bond-angle, improper-dihedral-angle, torsion-angle, van der Waals', torsion-angle-restraints and distance-restraints energy. In both steps no electrostatic energy term was included. The structures were visualized using the QUANTA 3.3 program from Polygen, MSI.

RESULTS

Previously, the structure of nisin has been studied in aqueous solution by NMR spectroscopy (Chan et al., 1989; Slijper et al., 1989; van de Ven et al., 1991b; Lian et al., 1992). However, since the active conformation of nisin is presumably that at its target site, i.e. at the cytoplasmic membrane, we considered it worthwhile to study the molecule by high-resolution NMR spectroscopy in a membrane-like environment. For this purpose membrane-mimetic micelles of anionic SDS and of zwitterionic DodPCho were used. The complete resonance assignments of the ^H-NMR spectrum of nisin complexed to DodPCho and to SDS micelles have been published previously (van den Hooven et al., 1993) and have been deposited in the BioMagResBank, a NMR structure database (Seavey et al., 1991), under ref ID 2521. The three systems will henceforth be referred to as nisin/ H,O, nisin/SDS and nisin/DodPCho.

Structure calculations for nisin complexed to DodPCho and to SDS micelles. For the first round of DIANA calculations, distance constraints that were directly obtained from the observations of NOEs and angle constraints were used. Especially for nisin/SDS but also for nisin/DodPCho this procedure yielded many structures, that were rather compact, i.e. with contacts between residues far apart in the sequence. Since there was no evidence for such contacts in the NOESY and ROESY spectra, these structures had to have incorrect overall folds. Therefore, based on NOE extraction, lower-distance bounds were added to cure the structures of these spurious contacts. For the nisin/DodPCho system the combined distance-geometry/NOE-back-calculation procedure converged to yield extended structures or structures with an obtuse angle between N-terminal and C-terminal halves of the molecule (vide infra). For the nisin/SDS system the procedure did not converge and was stopped after more than 1000 'push-apart' constraints had been added. It is important to note that these non-NOE constraints had little or no effect on the structures of the individual lanthionine rings.

The final DIANA calculations were carried out with 500 and 300 randomly generated start structures for nisin complexed to SDS and DodPCho micelles, respectively. The numbers of distance constraints is given in Table 1. The numbers of intra-residue and inter-residue upper-bound-distance constraints/residue are presented in Fig. 2. The number of intra-residue constraints/residue is the same for nisin/SDS and nisin/DodPCho. The variation in the number of inter-residue constraints/residue is similar in both micellar systems, but the absolute numbers are higher for nisin/DodPCho than for nisin/SDS. The residues located in linear peptide segments have in general the lowest number of inter-residue constraints. Five φ torsion-angle constraints were obtained, namely for the residues Ala^8, Ala^23, Ala^25 | Ala^g, Fig. 2. Number of upper-distance constraints. Number of intra-residue (△) and inter-residue (■) upper-bound-distance constraints for nisin/ SDS and the number of intra-residue (□) and inter-residue (■) upper-bound-distance constraints for nisin/DodPCho. An intra-residue constraint is counted once for the corresponding residue, an inter-residue constraint is counted twice, namely for both. The ring systems are formed by the residues 3–7, 8–11, 13–19 and 23–28 and their positions are indicated by bars at the top of the figure.

Table 2. Analysis of 15 structures of nisin complexed to SDS and to DodPCho micelles and of 15 structures of the recalculated fragment of nisin-(1–11) in water.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value for SDS</th>
<th>DodPCho</th>
<th>fragment in H_{2}O</th>
</tr>
</thead>
</table>

| DIANA target function* (Å²) | 1.10 ± 0.22 | 0.94 ± 0.20 | 0.20 ± 0.02 |
| range (Å²) | 0.49 – 1.37 | 0.48 – 1.21 | 0.17 – 0.24 |
| X-PLOR energy (kJ/mol)* | 510 ± 60 | 530 ± 60 | 250 ± 30 |
| range (kJ/mol) | 450 – 647 | 462 – 626 | 227 – 290 |
| NOE violations | | | |
| number >20 pm | 5 – 12 | 2 – 10 | 0 – 4 |
| maximum (pm) | 28 – 60 | 26 – 60 | 40 |
| Torsion-angle violations | | | |
| number >5° | 0 – 1 | 0 | 0 |
| maximum (deg) | 7 | | |

* The distance geometry program DIANA gives its target function in Å². 1 Å² = 0.01 nm².
* The X-PLOR energy was calculated as the sum of covalent bond energy, bond angle energy, improper dihedral angle energy, torsion-angle energy and intramolecular van der Waals' energy.
Fig. 3. Ramachandran plots. (a) L-amino acids and (b) D-amino acids of nisin/DodPCho, (c) L-amino acids and (d) D-amino acids of nisin/SDS and (e) L-amino acids and (f) D-amino acids of the fragment 1–11 of nisin in aqueous solution. The allowed regions of L-amino acids (Brünger, 1992) were converted in the case of D-amino acids by changing the sign of both torsion angles. In all cases $\phi$ and $\psi$ angles were taken of 15 structures.

3-methylalanyl moiety of (2S, 3S, 6R)-3-methyllanthionine], His27 and in the case of SDS also for Ile4 and in the case of DodPCho micelles also for $\delta$Ala7 [Ala, L-alanyl moiety of meso-lanthionine or of (2S, 3S, 6R)-3-methyllanthionine]. In the majority of the preliminary structures obtained after the first round of DIANA calculations a hydrogen bond between the NH proton of $\delta$Ala11 and the carbonyl oxygen atom of Ala$^8$ was found, in agreement with the low temperature coefficient and the slow exchange observed for this NH proton; subsequently, a hydrogen-bond distance bound of 0.24 nm was introduced for this atom pair.

For nisin/DodPCho, the $H^o$ protons of the residues Ala$^3$ (Ala$\alpha$, d-alanyl moiety of meso-lanthionine), $\delta$Ala7, Pro9, $\delta$Ala11, Met17, $\delta$Ala26, His27 and $\delta$Ala28, the $H^o$ protons of Gly10 and Gly18 and the $H^o$ protons of Pro9 were assigned stereospecifically using the program GLOMSA (Güntert et al., 1991b). In the same manner, for nisin/SDS, the $H^o$ protons of the residues Pro9, $\delta$Ala11, Asn20, $\delta$Ala26 and His27 and the $H^o$ protons of Gly10 were assigned stereospecifically. For both systems the $H^o$ protons of dehydroalanine were assigned stereospecifically using intraresidue $H^o\cdot\cdot\cdotNH^\alpha$ and sequential $H^o\cdot\cdot\cdotNH^\alpha$, NOEs.

The 15 structures which gave the best results both in distance-geometry and NOE back-calculations were selected for both micellar systems. They were subjected to further refinement by restrained energy minimization using the program
Recalculation of the structure of a part of the nisin molecule in aqueous solution. The protocol for the structure calculation described above was somewhat different from the approach used previously to calculate nisin in aqueous solution (DISMAN/CHARMm versus DIANA/X-PLOR; van de Ven et al., 1991 b). Previous studies (van den Hooven et al., 1993) already suggested that the most important conformational differences between nisin in aqueous solution and in micellar systems occur in the N-terminus. To eliminate the possibility that these differences are caused by the different protocols of structure calculation, the structure of the segment of nisin in aqueous solution comprising the residues Ile1 to ωAla11, including the rings A and B, was recalculated with exactly the same protocol as used for nisin/DodPCho and for nisin/SDS.

DIANA and X-PLOR calculations were carried out with 101 upper-distance bounds, of which 27 were intra-residual, 52 sequential (including 11 intra-lanthionine) and 22 medium-range or long-range (including 11 interring), and a total of 169 lower-distance bounds. The torsion-angle constraints were the same as those used in the previous structure calculation for nisin in aqueous solution (van de Ven et al., 1991 b). DIANA calculations were performed with 200 start structures. The 15 structures with lowest target function were selected. The $H^\beta$ protons of the amino acids Ala3, Dha5, ωAla7, Pro9 and ωAla11 were stereo-specifically assigned using GLOMSA. The results of the DIANA/X-PLOR calculations are presented in Table 2.

The recalculated conformation of ring B is identical to the previously reported one (van de Ven et al., 1991 b). The structure of ring A was determined with greater precision compared to the previously used protocol. The DIANA/X-PLOR approach yielded structures, which are a subset of the previously determined ones.

Analysis of the quality of the structures. The results of the DIANA and X-PLOR calculations are summarized in Table 2. The calculated structures satisfy all the NMR constraints and have a correct covalent geometry. Furthermore, in a Ramachandran plot (Fig. 3) the $\phi$ and $\psi$ angles of the majority of the L-amino acids and D-amino acids of all 15 structures fall within or near the sterically allowed regions.

Local backbone root-mean-square deviations (RMSD), calculated using X-PLOR for the 15 structures of nisin complexed to SDS (X) and DodPCho (■) micelles and the fragment 1–11 of nisin in aqueous solution (△). The ring systems are formed by the residues 3–7, 8–11, 13–19 and 23–28 and their positions are indicated by bars at the top of the figure.

![Fig. 4](image_url) Local RMSD for backbone N, C$^\alpha$ and C$^\gamma$ atoms of segments of three residues versus the position of the central residue of the segment in the sequence of nisin. Averaged pairwise RMSDs were calculated using X-PLOR for the 15 structures of nisin complexed to SDS (X) and DodPCho (■) micelles and the fragment 1–11 of nisin in aqueous solution (△). The ring systems are formed by the residues 3–7, 8–11, 13–19 and 23–28 and their positions are indicated by bars at the top of the figure.

Table 3. Averaged pairwise RMSD of nisin and nisin fragments. RMSD for nisin fragments were only calculated for structures with distance bound violations within the fragments smaller than 40 pm. Backbone atoms are N, C$^\alpha$ and C$^\gamma$ atoms. Values are means ± range.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>RMSD of</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nisin/SDS</td>
</tr>
<tr>
<td></td>
<td>backbone</td>
</tr>
<tr>
<td>Ring A</td>
<td>52 ± 22</td>
</tr>
<tr>
<td>Ring B</td>
<td>8 ± 8</td>
</tr>
<tr>
<td>Ring C</td>
<td>186 ± 66</td>
</tr>
<tr>
<td>Ring D, E</td>
<td>11 ± 8</td>
</tr>
<tr>
<td>Residues 21–24</td>
<td>41 ± 20</td>
</tr>
<tr>
<td>Ring A, B</td>
<td>94 ± 46</td>
</tr>
<tr>
<td>Ring A, B, C</td>
<td>350 ± 79</td>
</tr>
<tr>
<td>Whole nisin</td>
<td>739 ± 146</td>
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</tbody>
</table>
Fig. 5. Representation of 15 superimposed structures of nisin complexed to micelles. (a, b) Nisin in the presence of DodPCho and (c, d) of SDS micelles. Only non-hydrogen atoms are shown. Optimal superposition of backbone N, NH, C', O' and C atoms of (a) the residues 3–19 and (b) 23–28 of the nisin/DodPCho and of (c) the residues 3–11 and (d) 23–28 of the nisin/SDS system. The N-terminus and C-terminus are at the left and right side, respectively. The position of ring C relative to the rings A and B is less determined for nisin/SDS than for nisin/DodPCho. (a–d) are taken from different view points.

Overall fold of nisin in micellar systems. The structure of nisin cannot be described in terms of regular secondary-structure elements, due to the presence of the ring systems in which 65% of the residues are incorporated. As can be gleaned from Table 3 and Fig. 4, only local structured elements can be determined with reasonable precision. For the nisin/DodPCho system, the overall fold of the fragment 3–19 containing the first three rings, A, B and C, as well as the structure of the fragment 22–28 consisting of the rings D and E could be determined. This is illustrated in Fig. 5a and b, respectively. For the nisin/SDS system, data that would allow the orientation of ring C to be constrained relative to ring B were lacking, mainly due to spectral overlap. Therefore in this system only the overall fold of the fragment of the residues Ala3–11 could be determined in addition to the structure of the fragment of the residues Lys22–28 (Fig. 5c and d).

Structured elements of nisin in micellar systems. Most of the rings constitute structurally well-defined elements in nisin (Fig. 4 and Table 3). The structures of the individual ring systems are shown in Fig. 6. The smallest rings B, D and E have the best defined conformations; they contain four residues and adopt a β-turn structure, which is closed by a methyllanthionine.

Ring A. When complexed to micelles no hydrogen bonds and no typical β-turns or γ-turns were found in ring A, residues Ala3–7. The φ, ψ angles of Dha5 are about −130° and 130°, respectively, both for nisin/DodPCho and nisin/SDS (Table 4). The conformation around the dehydroalanine is not fully planar, in contrast to the crystal structure of N-acetylatedehydroalanine (Ajo et al., 1979). Most probably the ring-closure causes the deviation from planarity. For nisin in aqueous solution a γ-turn (Smith and Pease, 1980) around Dha5 is observed (in the recalculated structure). In this ring the single essential structural difference between nisin/DodPCho and nisin/SDS on the one hand and nisin/H2O on the other hand is observed as described in Discussion.

Ring B. The four residues Ala8–11 adopt a type-II β-turn structure, with the Pro9 and the Gly10 in positions 2 and 3 of this turn as is often found for a type-II β-turn (Richardson, 1981). A bifurcated hydrogen bond between the NH protons of Gly10 and Ala11 and the carbonyl oxygen atom of Ala8 is observed (Fig. 7). The amide proton of Ala11 shows a low temperature coefficient and slow exchange, in agreement with its participation in a hydrogen bond. The amide proton of Gly10 is probably only transiently or weakly bonded in the bifurcated hydrogen bond, as can be concluded from the medium sized temperature coefficient and the absence of slowed exchange for nisin/DodPCho.
Table 4. Average backbone torsion angles for the sequence Ile4-Dha5-Leu6.

<table>
<thead>
<tr>
<th>System</th>
<th>Average for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\phi$ of Ile4</td>
</tr>
<tr>
<td>Nisin/DodPCho</td>
<td>-90</td>
</tr>
<tr>
<td>Nisin/SDS</td>
<td>-90</td>
</tr>
<tr>
<td>Nisin/H$_2$O</td>
<td>-130</td>
</tr>
<tr>
<td>Difference*</td>
<td>40</td>
</tr>
</tbody>
</table>

* For a few structures this $\phi$ torsion angle was 150°.

** Differences in torsion angle for nisin in the micellar system and in aqueous solution.

Fig. 7. Hydrogen bonding in parts of a representative structure of nisin/DodPCho. Hydrogen bonds are indicated by dashed lines. Ring B is shown at the left and the fragment of the residues Met21 to Ala28 including three consecutive $\beta$-turns at the right. For nisin/SDS, in the majority of the structures a hydrogen bond between the NH proton of Ala23 and the carbonyl oxygen atom of Met21 was observed, for a few structures a hydrogen bond between Ala24 and Met21 was found. The NH proton of Ala24 in both micellar systems exhibits slowed exchange. The other hydrogen bonds are similar for nisin/DodPCho and nisin/SDS. The hydrogen bonds were present in the structures after the distance-bound violations smaller than 40 pm are shown. The number of structures is 12 and 13 for nisin/DodPCho and nisin/SDS, respectively.

Ring C. Of all the ring systems, ring C, residues Ala13–Ala19, has the least defined structure, although this is more pronounced in the nisin/SDS than in the nisin/DodPCho system. Overlap of resonances was observed for this cyclic element for both micellar systems. The ring adopts an approximately flat structure for nisin/SDS. For nisin/DodPCho, the side chain of Met17 is folded over the ring in the direction of the residue Ala13. Because of the determined structural variability of ring C it is impossible to decide if there are conformational differences between the two micellar systems, though the NOEs between the side chain of Met17 and the N-terminal amino acids of ring C were only observed for nisin/DodPCho.

Intertwined rings D and E. Ring D, residues Ala23–Ala26, adopts a type-II $\beta$-turn conformation. In ring D a bifurcated hydrogen bond, similar to that in ring B, is found between the amide protons of Ala25 and Ala26 and the carbonyl oxygen atom of Ala23 (Fig. 7). The amide proton of Ala26 shows a low temperature coefficient together with slowed exchange, indicative of an involvement in hydrogen bonding. This was not observed for the amide proton of Ala25, which is probably not strongly bonded. $\beta$-Turns with a D-amino acid in the third position tend to be of type II (Smith and Pease, 1980); this type of $\beta$-turn is observed for ring D of nisin.

In the structure of ring D of nisin/DodPCho, the orientation of the peptide bond between Ala23 and Ala24 could not be uniquely defined, due to overlap of resonances. The NOE intensities, $J_{\text{H-H}}$ coupling constants, chemical shifts and temperature coefficients observed for protons in the double ring are comparable for both micellar systems. It is therefore reasonable to assume that the peptide bond between Ala23 and Ala24 of nisin/DodPCho is oriented with the carbonyl pointing to the center of the ring, as is the case for nisin/SDS.

The residues Ala25–Ala28 constitute ring E. The structure of this ring looks like a somewhat distorted type-I $\beta$-turn but shows no hydrogen bonds. Different from ring D, the central amino acids in ring E, Ala26 and His27, have a normal $\alpha$-conformation.

Due to the intertwining of rings D and E, the peptide bond between Ala25 and Ala26 is part of both rings. The orientation of this peptide bond is as expected for the type-II $\beta$-turn of ring D. For ring E, however, this peptide bond deviates from the orientation expected for a type I-like $\beta$-turn. Because of this unusual orientation no hydrogen bonds are found in ring E. The angle between the planes through the $\beta$-turns of both rings is about 120°, so that the peptide bond between Ala25 and Ala26 can only have the correct orientation of a regular $\beta$-turn for one of the rings, which is ring D.

Structured element outside the ring systems. The backbone torsion angles of the amino acids Lys22 and Ala23 are indicative of a type-II $\beta$-turn for the residues Met21–Ala24, of which the last two residues are part of ring D (Figs 7 and 8). The third residue in this turn, Ala23, has a $\gamma$-configuration. Characteristic NMR markers for this type of turn (Wüthrich, 1986) are observed, i.e. $J_{\text{H-H}}$ coupling constants of the two central residues, Lys22 and Ala23, are small and large, respectively, and the NOE between the H-proton of Lys22 and the NH proton of Ala23 is strong. It is noted that ring D was also of type II and also had a $\gamma$-amino acid in the third position.

The three $\beta$-turns observed for the residues Met21–Ala24, Ala23–Ala26 and Ala25–Ala28 have several residues in common. The C-terminal structured domain consists of these three consecutive $\beta$-turns of types II, II and I, respectively.

Evidence for flexibility. Structural variability for nisin/DodPCho and for nisin/SDS, as observed in ring C, is also found in the N-terminus and C-terminus and for the residues Asn20 and Met21, which connect the rings C and D (Fig. 4). This variability can in principle be caused by flexibility or by a lack of NMR-
derived constraints, e.g. due to overlap of resonances. The first option is more likely for several reasons: first, the resonances of the residues in the flexible regions have a somewhat smaller line width compared to those of the ring systems; second, most of the $J_{\text{NH-NH}}$ coupling constants for these residues are in the 7–8 Hz range, typical of motional averaging and, third, lack of NMR data is unlikely because there were almost no overlapping resonances or resonances that were bleached by irradiation of the solvent resonance for the residues involved.

In the distance-geometry approach all time-averaged NMR constraints are required to be simultaneously satisfied in a single molecular structure. However, it is possible that some of the NOEs represent only transient dipolar interactions between protons. If this is the case the weak NOEs between the rings A, B and C are the first to be suspected. In general, if the time-dependence of NOEs was taken into account, this would lead to an increase in the structural variability observed. However, it should be noted that even if some of the NOEs represent only transient dipolar interactions between protons the structure as presented here is a preferred structure which will be present for a significant amount of time.

DISCUSSION

The nisin/micelle complexes are estimated to consist of 30 to 50 detergent molecules and one nisin molecule (van den Hooven et al., 1993, 1995), leading to an estimated mass of about 15 kDa for these complexes. Well resolved spectra, which could be used for the structure elucidation of nisin in the micellar systems, were obtained at 40°C. It was, however, possible to record NOESY spectra at 5–7°C, which were suitable for the assignment of the NH proton resonances, but not for structure elucidation. This allowed a comparison with the NOESY spectra used for the structure calculation for the nisin/H$_2$O system that were recorded at 7°C because of a more favourable $\omega\tau$, at that low temperature (van de Ven et al., 1991b). However, other conditions, such as pH, spectrometer frequency, etc. were the same for nisin/DodPCho, nisin/SDS and nisin/H$_2$O. The pattern of chemical-shift differences between on the one hand nisin/H$_2$O and on the other hand nisin/DodPCho or nisin/SDS is the same for both the 7° and 40°C spectra. This strongly suggests that differences in conformation between nisin in the H$_2$O and micellar systems are indeed due to the binding of nisin to the micelles (van den Hooven et al., 1995) and not to the difference in temperature.

The global structures of nisin in the micellar systems and in aqueous solution are essentially the same; the structures of the ring systems are well or reasonably well defined while the structure of the linear peptide segments is less defined. The rings in the N-terminal half of the molecule are part of a structured domain and the residues Lys22 to Ala28 form a structured domain in the C-terminal half. Overall, the structures in the nisin/H$_2$O and nisin/DodPCho systems are somewhat better defined in that the rings A, B and C have similar orientations with respect to each other. For nisin/SDS, only rings A and B have a well-defined relative orientation whereas the position of ring C relative to ring B is structurally ill-characterized. However, the relative orientation of the rings A and B resemble that of nisin/H$_2$O and nisin/DodPCho. Moreover the (weak) NOEs observed between the rings B and C for nisin/H$_2$O and nisin/DodPCho may be present in the nisin/SDS system, but could not be established due to overlap. Therefore, we assume that within the limits of the present structure determination the structures of nisin are similar in both micellar systems. In all three systems the regions flanking the structured domains are quite flexible, giving the whole molecule a flexible appearance.

The mentioned orientation of the rings A, B and C renders the N-terminal half of the molecule amphipathic. The lantionines and the side chain of Lys12 are located on one side and the hydrophobic residues Ile4, Dha5, Leu6, Ala15, Leu16 and Met17 on the other side (Fig. 5a). The C-terminal structured domain, consisting of three consecutive $\beta$-turns, is also amphipathic; the side chains of the positively charged residues Lys22 and His27 and of the hydrophobic residues Met21 and Ala24 are found at opposite sides. The poorly defined and probably flexible C-terminus, consisting of the residues Ser29–Lys34, does not show a distinct spatial distribution of hydrophobic and hydrophilic charged residues. The molecule is also amphipathic from another point of view; the hydrophilic and charged residues of nisin are mainly located in the C-terminal half of the molecule, whereas the majority of the residues in the N-terminal half is hydrophobic and only a single charged residue, Lys12, is present.

An interesting aspect, not noticed in the nisin/H$_2$O system, is that the residues Met21–Ala24 just preceding and overlapping with the intertwined rings D (which starts at Ala23) and E form a type-II $\beta$-turn (Fig. 8). This is the only structured element in nisin complexed to micelles outside the ring systems. Three other $\beta$-turns have been observed in the rings B, D and E. The backbone conformations of two of these rings, namely B and D, are remarkably similar (Fig. 9).

Apart from the strong similarities between the nisin structures in the different systems there are also some interesting differences, the most outstanding being the difference of the conformation of ring A in aqueous solution and in the micellar systems (Fig. 10). This difference is most pronounced for the $\phi$ angles of the residues Dha5 and Leu6 and the $\psi$ angles of Ile4 and Dha5 (Table 4). The two (trans) peptide bonds flanking Dha5 are almost completely inverted. This is in excellent agreement with our previous observation that the resonances of Ile4 to Leu6 exhibit the largest differences in chemical shift between nisin in water and complexed to micelles (van den Hooven et al., 1993), even up to 1.2 ppm for the NH proton of Dha5. The observed difference in temperature coefficient of the NH proton of Dha5 is also in agreement with calculated structures. Since the amide proton of Dha5 is not involved in hydrogen bonding, the temperature coefficient is indicative of solvent shielding. For nisin/H$_2$O, the NH proton of Dha5 of ring A protrudes from the ring (Fig. 10) and is solvent exposed, which corresponds well with the high temperature coefficient of $-10$ ppm/K/n. In the micellar systems this amide proton points towards the inside of the ring and exhibits a low temperature...
Fig. 10. Conformation of ring A of nisin in water and with DodPCho micelles. (a) 12 Structures of ring A obtained for nisin complexed to DodPCho micelles (identical to that in Fig. 6a) and (b) 13 for nisin in aqueous solution. Optimal superposition of backbone N, NH, C', H', C and O' atoms. The structure of ring A for nisin/SDS is similar to that of nisin/DodPCho (see Fig. 6). The conformational differences observed are due to a flipping of both (trans) peptide bonds flanking residue Dha5 (see text and Table 4).

coefficient, i.e. \(-1\) ppb/K and \(-2\) ppb/K for nisin/DodPCho and nisin/SDS, respectively. For both nisin in water and in the micellar systems the conformation around Dha5 is not fully planar, but deviates by about 60° from planarity (note that in these two situations the orientation of the peptide bond differs by about 180°). This non-planarity is imposed by the NMR-derived constraints and not by a force field; after the distance-geometry approach the restrained energy minimization had only a small effect on the structures. The structural difference observed for ring A of nisin in H2O and the micellar systems is best expressed in the distances 4 NH-5 NH, 5 NH-5 H'\(\text{\textsuperscript{\textprime}}\) and 6 NH-6 H', which of course correspond to differences in NOEs intensities. These NOEs can be considered as indicators of the structure of ring A.

A likely reason for the conformational change in ring A is an interaction of the residues Dha5 and Leu6 with the micelles. This is indeed borne out by the experiments presented in the accompanying report (van den Hooven et al., 1995). Evidence was obtained that also other regions of the nisin molecule are in contact with the micelles, but apparently this has no effect on the structure. Nisin must both be water-soluble and be able to bind to a membrane to exert its antimicrobial activity. The energetically most favoured conformations in these environments appear to differ in ring A. NMR and activity studies on both a nisin Dha5→Dhb mutant (Kuipers et al., 1992) and a degradation product with an α-OH-Ala instead of Dha5 (Rollema, H. S., unpublished results) suggest that this difference in conformation is related to the activity of nisin (van den Hooven, H. W., Rollema, H. S. and Kuiipers, O. P., unpublished results). The importance of the results described here is discussed in the accompanying report in terms of the mode of action of nisin.

It is noted in passing that the chemical shifts and the observed NOEs of nisin in a mixture of trifluoroethanol and water (van den Hooven et al., 1993) indicate that in this system the molecule adopts a conformation which is similar to the one determined for the micellar systems.

Currently about 25 lantibiotics are known, which can be subdivided into two classes: type A, including e.g. nisin, subtilin, Pep5, epilancin K7, epidermin and gallidermin, and type B, including, e.g. duramycin, cinnamicyn, ancovinen, meraci-din and actagardine (Jung, 1991; Bierbaum and Sahl, 1993; Sahl et al., 1995). Lantibiotics of the first type exert their antimicrobial activity primarily by membrane perturbation, while those of the other type are inhibitors of specific enzymes. Only for a few of the lantibiotics the 3D structure has been elucidated (type-B lantibiotics: cinnamicyn (Kessler et al., 1988, 1992), duramycins B and C (Zimmermann et al., 1993) and actagardine (Zimmermann et al., 1995)). The conformation of the type-A lantibiotic subtilin, which is structurally related to nisin, has also been determined and it has been suggested that its conformation in aqueous solution is similar to that of nisin (Chan et al., 1992). The structure of the type-A lantibiotic gallidermin in trifluoroethanol/water has been elucidated (Freund et al., 1991); the molecule adopts an amphiphilic conformation. The amphipathicity seems to be a common feature among type-A lantibiotics. As noticed for nisin, structural variability was also observed in the middle part of the gallidermin molecule. The second ring has the same amino acid sequence and conformation as ring B of nisin and subtilin. So far, type I and II β-turns have been found for four-residue lantionine rings. Nisin is the first type-A lantibiotic for which the structure has been elucidated, both in aqueous solution and in a membrane-mimicking environment. Local structural elements of nisin are affected by bringing the molecule from aqueous solution to a membrane-like environment, whereas the global structure remains more or less the same. This is not a general property of type-A lantibiotics, since preliminary structural studies of a novel type-A lantibiotic epilancin K7 (van de Kamp et al., 1995a,b) indicate that it adopts a completely different structure when complexed to DodPCho micelles compared to the structure in aqueous solution (Monga, S. and van de Kamp, M., unpublished results).

We thank Dr Harry Rollema (NIZO, Dutch Institute for Dairy Research) for expert help with the HPLC purification of nisin and a gift of a nisin degradation product and Dr Oscar Kuipers (NIZO) for a nisin mutant, and Dr Lu-Yun Lian (University of Leicester) for advice in handling uncommon amino acids in X-PLOR. All NMR experiments have been performed at the SON National NMR facility, Nijmegen. Financial support was obtained from the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Technology Foundation (STW). MvdK is supported by the EC-BRIDGE program (contract BIOT-CT91-0265).

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