Solution structures of nisin A and its two major degradation products determined by n.m.r.

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The conformations of nisin and two major degradation products, nisin-(1–32)-peptide (nisin^{1–32}) and des-ΔAla⁵-nisin^{1–32} (where ΔAla is αβ-didehydroalanine), in aqueous solution have been determined from n.m.r. data. Sequential assignments of the peptides using correlation spectroscopy ('COSY'), homonuclear Hartmann–Hahn spectroscopy ('HOHAHA'), nuclear Overhauser enhancement spectroscopy (NOESY), relayed NOESY and rotating-frame nuclear Overhauser spectroscopy (ROESY) experiments are presented, including stereospecific assignments of β-methylene protons of the lanthionine residues. ROESY experiments are also used to detect flexible regions in the polypeptide chain. A dynamic-stimulated-annealing approach is used for structural determination. It can be concluded that all these peptides are flexible in aqueous solution, with no experimental evidence of preferred overall conformations; the only defined conformational features are imposed by the presence of the lanthionine residues. Low-temperature studies also reveal that des-ΔAla⁵-nisin^{1–32} adopts conformations similar to those when the ring is intact, suggesting that the loss of activity of this degradation product is due to the absence of the ΔAla⁵ residue rather than to the conformational consequences of ring-opening.

INTRODUCTION

The peptide nisin A (hereinafter referred to as nisin; Fig. 1), produced by strains of *Lactococcus lactis*, possesses antimicrobial activity against a spectrum of Gram-positive organisms. Nisin contains several post-translationally modified amino acid residues, namely α,β-didehydroalanine, α,β-didehydrobutyrine, *m*-lanthionine and (2*S*,3*S*,6*R*)-3-methyl-lanthionine; the last two residues introduce thioether bridges at various locations in the molecule, resulting in a series of cyclic units.

The structural gene for the precursor of nisin has recently been cloned and sequenced [1,2]; it encodes a peptide, prenisin, of 57 amino acid residues, comprising a 23-residue leader sequence followed by a 34-residue sequence which corresponds to that of mature nisin, except that it contains serine, threonine and cysteine residues as precursors of the dehydrobutyrine, dehydroalanine, lanthionine and 3-methyl-lanthionine residues. It is believed that the serine and threonine residues undergo enzymic dehydration to dehydroalanine and dehydrobutyrine respectively, followed by the stereospecific addition of the thiol groups of cysteine residues to the α,β-double bonds of the dehydroamino acids to form the lanthionine and 3-methyl-lanthionine residues, and hence the cyclic structures. Either before or, more probably, after these modifications, the leader sequence is cleaved off, and the mature nisin is secreted from the cell.

The importance of the post-translational modifications for biological activity has yet to be established in detail, but some evidence has come from studies of the two major degradation products of nisin formed during isolation and/or on storage. These have been identified [3] as nisin-(1–32)-peptide (nisin^{1–32}), lacking the two C-terminal residues, and des-ΔAla⁵-nisin^{1–32} (where ΔAla is αβ-didehydroalanine). Nisin^{1–32} has similar antimicrobial activity to that of nisin against a number of Gram-positive organisms, but the additional loss of the dehydroalanine

residue at position 5 leads to a decrease in activity of several hundred-fold. In the light of these observations, one obvious possible role of the post-translational modifications is that they serve to constrain the nisin molecule into a conformation which is required for activity.

As part of our programme of work on the biosynthesis and structure–activity relationships of nisin, we have now used high-resolution n.m.r. to study the conformations of nisin and related peptides in aqueous solution; a preliminary report of this work has appeared elsewhere [5].

MATERIALS AND METHODS

Sample preparation

Nisin (37000 units/mg) was obtained from Aplin and Barrett, Beamster, Dorchester, Dorset, U.K., and purified by h.p.l.c. to yield homogeneous nisin A [4]. The purified nisin, after dialysis and freeze-drying, generally contains < 1% triethylammonium acetate from the h.p.l.c. solvent. Nisin^{1–32} and des-ΔAla⁵-nisin^{1–32} were prepared as described in [3]. Samples for n.m.r. spectroscopy were routinely prepared as 3–5 mM solutions of peptide in ²H₂O or in 85% ¹H₂O/15% ²H₂O, each containing 0.1 M-sodium phosphate. Most spectra were recorded at pH 2.25.

N.m.r. spectroscopy

Spectra were obtained at 500 or 600 MHz by using, respectively, Bruker AM500 and Bruker AMX600 spectrometers. All two-dimensional (2D) spectra were acquired and processed in the phase-sensitive mode using the time-proportional phase-incrementation method [6]. Spectra were recorded at temperatures between 278 and 303 K and referenced to sodium 3-(trimethylsilyl)propane-1-sulphonate.

For identification of spin-systems and sequential assignments, correlation spectroscopy (COSY), homonuclear–Hartmann

Abbreviations used: COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; ROESY, rotating-frame nuclear Overhauser spectroscopy; nisin, nisin A; nisin^{1–32}, nisin-(1–32)-peptide; 1D and 2D, one- and two-dimensional; PE-COSY, Primitive Exclusive COSY; n.o.e., nuclear Overhauser enhancement; ΔAla, αβ-didehydroalanine; ΔAbu, αβ-didehydrobutyrine; Ala₂ and Abu are the two 'halves' of lanthionine and/or methyl-lanthionine residues.

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Fig. 1. Structure of nisin

Hahn spectroscopy (HOHAHA), Nuclear Overhauser enhancement spectroscopy (NOESY) and relayed NOESY experiments were used. Accurate measurements of $^3J_{\alpha\beta}$ spin-spin coupling constants were obtained from Primitive Exclusive (PE) COSY experiments, utilizing a 32-step phase-cycling scheme [7]. The water resonance was usually suppressed using low-power pre-irradiation. In the HOHAHA experiments, however, in order to allow detection of the peptide proton resonances 'under' the water resonance, the solvent resonance was suppressed either by using the SCUBA pulse sequence [8] or by presaturation followed by a 1-1 (90° - τ - 90°) observe sequence after the spin-lock period [9]. An MLEV-17 mixing sequence (duration 50 to 120 ms) was used with a spin-locking field in the range 8-15 kHz.

For the NOESY spectra, mixing times of 80, 100, 200, 350 and 550 ms were used. When quantitative nuclear Overhauser enhancement (n.O.e.s) were required, the final 90° observe pulse in the NOESY pulse sequence was replaced by a 1-1 sequence with the carrier placed at the position of the solvent. This approach is taken to avoid attenuation of the amide proton resonance intensities due to amide proton-water exchange. Intra-residue n.O.e. measurements for stereospecific assignments of the β -methylene protons were taken primarily from the 80 ms NOESY spectra recorded in water.

For the rotating-frame nuclear Overhauser spectroscopy (ROESY) spectra, mixing times of 80 ms and 200 ms were used. In these experiments, two transmitter powers were used, with a low-power continuous spin-lock pulse to give a spin-locking field of 2-3 kHz [10]. The relayed-NOESY experiment was composed of a standard NOESY pulse sequence, in which incoherent magnetization was transferred during t_m , followed by a period of coherent magnetization transfer by spin-locking using the MLEV-17 pulse sequence. The mixing period for the incoherent transfer was 350 ms, and that for the coherent transfer was 40 ms (optimized for scalar coupling constants of 6-7 Hz).

For the majority of the 2D spectra, 400-512 t_1 increments were collected, each with 2048 data points over a spectral width of 11 p.p.m. in both dimensions. Most spectra were collected with the receiver phase optimized by using the 'ADC' instead of the 'GO' command for explicit acquisition in order to improve the overall baseline of the 2D spectrum. 2D data were processed using either Bruker software on a Bruker X32 workstation or the FELIX program, provided by Dr. Denis Hare (Hare Research, Woodinville, WA, U.S.A.), on a Silicon Graphics 4D/20

workstation. Data were processed in the phase-sensitive mode using either a Gaussian or a sine-bell squared-window function together with zero-filling in f_1 to give a final 1024×1024 data matrix, except for the PE-COSY spectrum, where a final 4096×4096 data matrix was used. The quality of the spectra was greatly enhanced by the use of baseplane correction in f_1 and f_2 .

Structure calculations

Proton-proton distance constraints were derived from the n.m.r. data as follows. The lower limit in all cases was taken as the sum of the van der Waals' radii, 0.19 nm (1.9 Å). Upper limits were based on the time-course of the development of the n.O.e.s, using 'reference' distances. Thus the maximum intra-residue NH_i - αH_i distance is 0.29 nm (2.9 Å); all NH_i - αH_i n.O.e.s were observed at a mixing time of 100 ms, and hence all the n.O.e.s observed at this mixing time were taken as indicating a maximum inter-proton distance of 0.29 nm (2.9 Å). A similar argument, based on the inter-residue NH_i - NH_{i+1} distance, led to the assignment of an upper limit of 0.4 nm (4.0 Å) to inter-proton distances corresponding to n.O.e.s observed at 200 ms, but not at 100 ms. Finally, additional cross-peaks observed at mixing times greater than 200 ms (up to 550 ms) were taken as indicating an upper limit for the inter-proton distance of 0.55 nm (5.5 Å).

Structure calculations were carried out by using the hybrid metric-matrix-distance-geometry-dynamical-simulated-annealing approach [11]. The distance-geometry part of the calculation was carried out using the program DSPACE (supplied by Dr. Dennis Hare), using a total of 132 inter-proton distance constraints derived from the observed n.O.e.s as described above. The initial distance-geometry structures were obtained by using standard amino acid residues, but covalent constraints for the five lanthionine or methyl-lanthionine bridges were included. The co-ordinate generation (embedding stage) was followed by a randomization stage in an attempt to ensure adequate sampling of conformational space. Conjugate gradient energy minimization (100 steps) was followed by a simulated annealing optimization to overcome local minima, and a final conjugate gradient minimization was applied. In all, 20 distance geometry structures were calculated using this approach.

All 20 structures were then subjected to the dynamical-simulated-annealing process using the program XPLOR [12], using the correct (post-translationally modified) structure of the

Table 1. $^3J_{\alpha\beta}$ coupling constant and n.O.e. data for stereospecific assignment of C_β proton resonances of the lanthionine residues of nisin, together with estimates of χ_1 torsion angles for these residues

Residue	Coupling constant (Hz)* (Chemical shift, p.p.m.)		n.O.e.s†‡				χ_1 (°)
	$^3J_{\alpha\beta^3}$	$^3J_{\alpha\beta^2}$	$d_{\alpha\beta^3}$	$d_{\alpha\beta^2}$	$d_{N\beta^3}$	$d_{N\beta^2}$	
Ala _s ³	4.20 (3.18)	6.50 (3.32)	s	w	vw	m	(+60)§
Ala _s ⁷	9.00 (3.11)	6.80 (2.99)	w	w	m	vw	(180)§
Ala _s ¹¹	3.40 (3.70)	13.7 (3.07)	s	w	w	s	-60
Ala _s ¹⁹	9.40 (3.04)	4.70 (3.01)	Degenerate		s	w	(180)§
Ala _s ²⁶	3.00 (3.48)	13.0 (2.72)	s	w	w	s	-60
Ala _s ²⁸	4.30 (3.73)	12.0 (2.75)	s	w	w	s	-60

* Coupling constants were measured from PE-COSY spectra (see the text).

† n.O.e.s were taken from 80 ms NOESY and ROESY experiments; the following notation is used: $d_{\alpha\beta^3}$, $C_\alpha H-C_\beta H^3$; $d_{\alpha\beta^2}$, $C_\alpha H-C_\beta H^2$; $d_{N\beta^3}$, $NH-C_\beta H^3$; $d_{N\beta^2}$, $NH-C_\beta H^2$.

‡ Abbreviations: s, strong; m, medium; w, weak; vw, very weak.

§ Predominant conformation in a mixture.

Table 2. 1H -n.m.r. (500 MHz) chemical shifts of nisin (5 mM) in aqueous solution (100 mM-sodium phosphate buffer, pH 2.25 ($^1H_2O/^2H_2O$, 17:3, v/v; 303 K))

Residue	δ (p.p.m.)					
	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	$C_\epsilon H$
Ile ¹	8.22	4.19	2.16	1.37, 6.62; 1.16	0.95	
Δ^2 Abu ²	9.93		6.65	1.87		
D-Ala _s ⁴	8.24	4.68	3.18, 3.32			
Ile ⁴	7.89	4.39	2.12	1.22, 1.45; 0.99	0.88	
Δ Ala ⁵	9.85		5.49, 5.61			
Leu ⁶	8.94	4.47	1.78	1.69	0.94, 1.00	
Ala _s ⁷	8.27	4.60	2.99, 3.11			
D-Abu ⁸	8.88	5.16	3.61	1.40		
Pro ⁹		4.48	1.77, 2.50	2.22, 1.90	3.50	
Gly ¹⁰	8.70	3.66, 4.45				
Ala _s ¹¹	7.96	4.07	3.07, 3.70			
Lys ¹²	8.64	4.39	1.86	1.44, 1.54	1.75	3.05
	7.60	($N_\epsilon H_3^+$)				
D-Abu ¹³	8.29	4.60	3.66	1.37		
Gly ¹⁴	8.35	4.12, 4.16				
Ala ¹⁵	8.57	4.30	1.51			
Leu ¹⁶	8.47	4.37	1.78	1.70	0.95, 0.98	
Met ¹⁷	7.86	4.68	2.15, 2.32	2.53, 2.70	2.18	
Gly ¹⁸	8.12	3.90, 4.18				
Ala _s ¹⁹	7.72	4.55	3.01, 3.04			
Asn ²⁰	8.55	4.75	2.86	6.94, 7.60 ($\beta CONH_2$)		
Met ²¹	8.27	4.57	2.04, 2.17	2.56, 2.64	2.17	
Lys ²²	8.43	4.37	1.90	1.48, 1.55	1.77	3.06
	7.60	($N_\epsilon H_3^+$)				
D-Abu ²³	8.80	5.05	3.60	1.40		
Ala ²⁴	8.24	4.72	1.50			
D-Abu ²⁵	9.09	4.85	3.57	1.48		
Ala _s ²⁶	7.87	3.93	2.72, 3.48			
His ²⁷	8.74	4.98	3.13, 3.42	8.65(H_2),	7.35(H_1)	
Ala _s ²⁸	7.90	4.47	2.75, 3.73			
Ser ²⁹	8.47	4.54	3.90			
Ile ³⁰	8.13	4.24	1.87	1.20, 1.38; 0.90	0.90	
His ³¹	8.65	4.85	3.21, 3.31	8.63(H_2)	7.32(H_1)	
Val ³²	8.32	4.24	2.17	1.01		
Δ Ala ³³	9.71		5.74			
Lys ³⁴	8.50	4.51	1.91, 2.01	1.54	1.77	3.07
	7.60	($N_\epsilon H_3^+$)				

molecule. Residues 2, 5 and 33 were modified to didehydroamino acids, and residues 3, 8, 13, 23 and 25 to D-amino acids. In addition, new patches were constructed for the formation of the lanthionine and methyl-lanthionine moieties, as these were not

readily available from the residue topology file library within XPLOR. When a preferred rotamer of the lanthionine side chain could be identified, the torsion angle restraint used was described by a square-well-potential function with a width $\pm 60^\circ$ about the rotamer position; that is, $\chi_1 = 60 \pm 60^\circ$, $180 \pm 60^\circ$ or $-60 \pm 60^\circ$.

The protocol for the dynamic-simulated-annealing calculation was described in [13] and is summarized below.

1. Perform 200 cycles of energy minimization with the following force constants: bond length, 20.9 kJ/nm² per mol (500 kcal/Å² per mol); bond angle, 2090 kJ/rad² per mol (500 kcal/rad² per mol); torsion, 0 kJ/rad² per mol. except peptide-bond torsion, 836 kJ/rad² per mol (200 kcal/rad² per mol); improper, 2090 kJ/radian² per mol (500 kcal/rad² per mol); atom-atom repulsion, 4×10^{-3} J/nm⁴ per mol (0.01 kcal/Å⁴ per mol). The pseudo-force constant corresponding to satisfaction of the n.O.e.-derived distance constraints, $k_{n.o.e.}$, was set to 0 kJ/nm² per mol for this initial minimization.

2. Set $k_{n.o.e.}$ to 2.09 kJ/nm² per mol (50 kcal/Å² per mol), the torsional force constant to 836 kJ/rad² per mol (200 kcal/rad² per mol), the force constant for the dihedral angle about the proline peptide bond to 83.6 kJ/rad² per mol (20 kcal/rad² per mol) and all masses to 10 atomic mass units, and perform 1.5 ps dynamics coupled to a temperature bath at 1000 K.

3. Set $k_{n.o.e.}$ to 0.42 kJ/nm² per mol (10 kcal/Å² per mol), the torsional force constant to 83.6 kJ/rad² per mol (20 kcal/rad² per mol), and the remaining parameters as in the standard XPLOR parameter set PARMALLH3X; perform 40 75 fs stages of dynamics at 1000 K, increasing atom-atom repulsion by a factor of $1000^{1/40}$ after each stage.

4. Cool over 1.4 ps in 25 K stages from 1000 K to 300 K.

5. Energy-minimize for 200 cycles with the proline peptide bond dihedral angle force constant set to 836 kJ/rad² per mol (200 kcal/rad² per mol).

Calculations were performed on a Silicon Graphics 4D/25-GT workstation and an Alliant FX40-3 computer.

RESULTS AND DISCUSSION

Resonance assignments in nisin, nisin¹⁻³² and des- Δ Ala⁵-nisin¹⁻³²

The first stage in the conformational analysis of these peptides

Table 3. ^1H -n.m.r. (500 MHz) data of nisin¹⁻³² (4 mM) in aqueous solution [100 mM sodium phosphate buffer, pH 3.05 ($^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$, 17:3, v/v; 303 K)]

Residue	δ (p.p.m.)					
	NH	C_αH	C_βH	C_γH	C_δH	$\text{C}_\epsilon\text{H}$
Ile ¹		4.19	2.16	1.34, 1.61; 1.16	1.04	
$\Delta^2\text{Abu}^2$	9.86		6.69	1.89		
D-Ala ³	8.25	4.69	3.16, 3.32			
Ile ⁴	7.89	4.39	2.12	1.21, 1.43; 0.99	0.88	
ΔAla^5	9.86		5.52, 5.65			
Leu ⁶	8.95	4.48	1.80	1.71	0.96, 1.01	
Ala ⁷	8.27	4.60	2.97, 3.11			
D-Abu ⁸	8.89	5.15	3.64	1.35		
Pro ⁹		4.48	1.87, 2.50	2.00, 2.21	3.50	
Gly ¹⁰	8.70	4.45, 3.66				
Ala ¹¹	7.99	4.07	3.08, 3.71			
Lys ¹²	8.64	4.39	1.86	1.43, 1.55	1.75	3.05
	7.62	($\text{N}_\epsilon\text{H}_3^+$)				
D-Abu ¹³	8.30	4.70	3.65	1.36		
Gly ¹⁴	8.35	4.13, 4.19				
Ala ¹⁵	8.59	4.30	1.52			
Leu ¹⁶	8.47	4.35	1.80	1.70	0.97, 0.99	
Met ¹⁷	7.86	4.67	2.16, 2.32	2.51, 2.68	2.15	
Gly ¹⁸	8.12	3.90, 4.16				
Ala ¹⁹	7.74	4.54	3.02, 3.07			
Asn ²⁰	8.55	4.73	2.88	6.96, 7.62(βCONH_2)		
Met ²¹	8.27	4.57	2.05, 2.18	2.57, 2.65	2.14	
Lys ²²	8.44	4.36	1.90	1.47, 1.55	1.77	3.06
	7.62	($\text{N}_\epsilon\text{H}_3^+$)				
D-Abu ²³	8.80	5.02	3.60	1.40		
Ala ²⁴	8.24	4.73	1.50			
D-Abu ²⁵	9.10	4.83	3.55	1.49		
Ala ²⁶	7.87	3.91	2.72, 3.48			
His ²⁷	8.75	4.95	3.13, 3.43	8.69(H_β), 7.38(H_α)		
Ala ²⁸	7.92	4.48	2.75, 3.73			
Ser ²⁹	8.48	4.54	3.92			
Ile ³⁰	8.14	4.24	1.88	1.19, 1.40; 0.89	0.89	
His ³¹	8.65	4.83	3.23, 3.31	8.68(H_β), 7.35(H_α)		
Val ³² -NH ₂	8.24	4.15	2.07	1.00		
	7.14, 7.73	(αCONH_2)				

by n.m.r. is the assignment of their ^1H n.m.r. spectra [3,4]. In each case, the first stage of resonance assignment was carried out by determining scalar connectivities within amino acid residues using COSY and HOHAHA spectra. This was followed by sequential assignment, based on a search for short-range NOESY cross-peaks between the C_α , C_β , or amide proton to residue i and the amide protons of residue $i+1$ in the sequence. This procedure has been described in detail for nisin [4]. We have now extended the nisin assignments to a complete stereospecific assignment of all the pro-chiral β -methylene proton resonances. These stereospecific assignments were based on $^3J_{\alpha\beta}$ spin-spin coupling constants obtained from the PE-COSY spectrum in $^2\text{H}_2\text{O}$, and intra-residue $\text{C}_\alpha\text{H}-\text{C}_\beta\text{H}$ and $\text{NH}-\text{C}_\beta\text{H}$ n.O.e.s obtained from both NOESY and ROESY data. These parameters have a characteristic pattern from each of the three energetically preferred staggered rotamer positions ($\chi_1 = 60^\circ$, 180° or -60°) [14]. For nisin, the prochiral β -methylene protons are involved in the 'bridges' of the rings; stereospecific assignments allow much better definition of the local conformations in these ring units. The $^3J_{\alpha\beta}$ spin-spin coupling constants and n.O.e. data for the lanthionine residues of nisin, with the deduced stereospecific assignments, are given in Table 1, which also summarizes the information obtained from these data concerning the χ_1 torsion angles (about the $\text{C}_\alpha-\text{C}_\beta$ bond) of the lanthionine residues. For three sets of lanthionine β -methylene protons, those of Ala¹¹, Ala²⁶ and Ala²⁸ (where Ala_s is one half of the lanthionine and/or methyl-lanthionine residue), the observation that one $^3J_{\alpha\beta}$ coupling constant is greater than 12 Hz and the other is smaller than 5 Hz is consistent with the existence of a single conformation about the $\text{C}_\alpha-\text{C}_\beta$ bond and rules out the $\chi_1 = 60^\circ$ rotamer. For

Table 4. ^1H -n.m.r. (500 MHz) data of des- ΔAla^5 -nisin¹⁻³² (4 mM) in aqueous solution [100 mM-phosphate buffer, pH 3.05 ($^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$, 17:3, v/v; 303 K)]

Residue	δ (p.p.m.)					
	NH	C_αH	C_βH	C_γH	C_δH	$\text{C}_\epsilon\text{H}$
Ile ¹		4.18	2.15	1.38, 1.64; 1.17	1.06	
$\Delta^2\text{Abu}^2$	9.92		6.67	1.89		
D-Ala ³	8.35	4.72	3.08, 3.22			
Ile ⁴ -NH ₂	8.15	4.29	2.01	1.39, 1.56; 1.04	0.96	
	7.19, 7.74(αCONH_2)					
	2.51($\text{PyrC}_\beta\text{H}_2$)					
Pyr-Leu ⁶	8.67	4.50	1.81	1.70	0.97, 1.01	
Ala ⁷	8.66	*	3.06			
D-Abu ⁸	9.10	5.15	3.68	1.40		
Pro ⁹		4.50	1.96, 2.49	1.96, 2.19	3.48, 3.56	
Gly ¹⁰	8.64	3.74, 4.43				
Ala ¹¹	8.05	4.12	3.11, 3.73			
Lys ¹²	8.62	4.42	1.90	1.46, 1.57	1.77	3.08
	7.62	($\text{N}_\epsilon\text{H}_3^+$)				
D-Abu ¹³	8.32	4.71	3.68	1.40		
Gly ¹⁴	8.38	4.14, 4.21				
Ala ¹⁵	8.59	4.32	1.54			
Leu ¹⁶	8.49	4.35	1.83	1.73	0.98	
Met ¹⁷	7.88	4.70	2.20, 2.34	2.54, 2.73		
Gly ¹⁸	8.14	3.92, 4.18				
Ala ¹⁹	7.77	4.58	3.05, 3.09			
Asn ²⁰	8.58	4.77	2.88	6.97, 7.62(βCONH_2)		
Met ²¹	8.30	4.58	2.05, 2.20	2.59, 2.68		
Lys ²²	8.45	4.38	1.92	1.47, 1.57	1.80	3.10
	7.62($\text{N}_\epsilon\text{H}_3^+$)					
D-Abu ²³	8.81	5.03	3.63	1.42		
Ala ²⁴	8.24	4.73	1.52			
D-Abu ²⁵	9.11	4.87	3.60	1.51		
Ala ²⁶	7.88	3.94	2.75, 3.52			
His ²⁷	8.78	4.96	3.15, 3.45	8.69(H_β), 7.40(H_α)		
Ala ²⁸	7.92	4.48	2.80, 3.75			
Ser ²⁹	8.50	4.54	3.92			
Ile ³⁰	8.20	4.25	1.89	1.20, 1.43; 0.90	0.90	
His ³¹	8.70	4.83	3.27, 3.34	8.68(H_β), 7.37(H_α)		
Val ³² -NH ₂	8.27	4.18	2.10	1.01		
	7.15, 7.73(αCONH_2)					

* Bleached out owing to solvent pre-irradiation.

Ala_s³ and Ala_s⁷, on the other hand, the $^3J_{\alpha\beta}$ scalar coupling constants are not consistent with the existence of a single rotameric state about the $\text{C}_\alpha-\text{C}_\beta$ bond, indicating that this lanthionine residue, and hence ring A (residues 3-7) of nisin, has some flexibility.

The temperature-dependence of the amide-proton chemical shifts was determined over the range 286-308 K. The majority of amide protons displayed temperature (T) coefficients ($-\Delta\delta/\Delta T$ values) in the range 8.0-16.5 p.p.b./K. Significantly lower temperature coefficients ($-\Delta\delta/\Delta T = 1.2-3.1$ p.p.b./K) were observed for the backbone amide protons of Ile⁴, Ala_s⁷, Met¹⁷, Ala_s¹⁹ and Ala_s²⁶, suggesting that these amide protons may be involved in intramolecular hydrogen bonds or otherwise shielded from the solvent. It is noteworthy that all these residues are within one of the ring structures of the nisin molecule.

Once the complete assignment of the nisin spectrum was available, the assignments were readily extended to the spectra of the two degradation products, using the same techniques. As shown in Tables 2, 3 and 4, the chemical shifts of most of the resonances were similar, with the exception of those from residues adjacent to the cleavage sites.

N.O.e. measurements

The determination of the solution structure of a peptide or protein from n.m.r. data depends on obtaining as complete a set as possible of distance constraints from n.O.e. measurements. Although NOESY experiments give good n.O.e. cross-peaks for nisin, it remains possible, given the small size of the peptide, that some dipolar coupling cross-peaks are too weak to be observed,

Fig. 2. Portions of the NOESY (a, 300 ms mixing time) and ROESY (b, 200 ms mixing time) spectra of nisin at 303 K, showing n.O.e. connectivities between amide protons and C_α and side-chain protons

Additional n.O.e. connectivities for residues 2, 31 and 33 not observed in the NOESY spectrum are indicated in the ROESY spectrum. Cross-peaks for residues 11, 26 and 28 arising from spin-diffusion are identified in the NOESY spectrum; these cross-peaks were of inverted phase in the ROESY spectrum (see the text for details).

Fig. 3. Spatial distribution of the n.O.e.s observed

□, Sequential C_α -H-NH; ■, sequential NH- C_β -H; ▲, non-sequential side-chain to side-chain.

owing to unfavourable correlation times. This is particularly true if there are flexible regions in the peptide chain. In order to address this question, the rotating-frame n.O.e. (ROESY or CAMELSPIN) experiment was used. In this experiment, the n.O.e. remains positive, irrespective of the value of τ_c (rotational correlation time) and ω_0 (spectrometer frequency): the dipolar cross-peak appears negative with respect to the diagonal. However, the ROESY experiment must be interpreted with care, since artifacts can arise due to the presence of Hartmann-Hahn transfer [15], which leads to a contribution to cross-peak intensity of the same sign as the diagonal. (Cross-peaks can in fact have an inverted phase if the Hartmann-Hahn contribution is greater than the dipolar contribution.) Such artifacts can be detected by

altering the conditions under which the experiment is done, for example, by changing the frequency offset of the carrier, the radiofrequency power or the mixing time of the spin-lock pulse. In the experiments discussed here, particular care was taken to ensure that the ROESY cross-peaks reported represent true proximity between protons and are not the result of Hartmann-Hahn transfer coupled with incoherent magnetization transfer.

Fig. 2 shows portions of the 2D ROESY and NOESY spectra containing connectivities between amide and C_α or side-chain protons. Cross-peaks due to dipolar coupling which are observed in the ROESY as compared with the NOESY spectra are indicated. These additional cross-peaks are observed for a number of protons in residues 1–3 and 29–34, strongly suggesting that the *N*- and *C*-termini of the molecule are flexible; that is, they have shorter correlation times than the bulk of the molecule. With the exception of a few amide-side-chain connectivities, where additional dipolar cross-peaks in the ROESY spectrum probably arise from motion of the side chains, the ROESY and NOESY cross-peak maps for the rest of the molecule were closely similar. Signals in the NOESY spectrum that result from spin-diffusion effects are readily identified by their absence (or inverted phase) in the ROESY spectrum. For example, a spin-diffusion cross-peak between the amide and β -protons of Ala₂₆ is clearly visible in the NOESY spectrum at mixing times greater than 200 ms. The observed n.O.e.s are summarized in Fig. 3.

It was notable that no long-range n.O.e.s (between residues distant in the sequence) were observed in either NOESY or ROESY spectra, even at low temperature (278 K) and with long mixing times. There are thus no experimental constraints which would serve to define the orientation of the rings with respect to one another. A continuous stretch of very strong C_α -H- NH_{i+1}

Fig. 4. Superimposition of 20 structures of ring A of nisin, derived from n.m.r. data as described in the text

Fig. 5. Superimposition of 20 structures of ring B of nisin, derived from n.m.r. data as described in the text

n.O.e.s from Ala_s¹⁹ to Abu²³ is indicative of an extended secondary structure in this part of the molecule, although the observation of a very strong NH_i-NH_{i+1} n.O.e. between Abu²³ and Ala²⁴ suggests some degree of conformational preference at the junction of the 'linker' region and ring D. In the absence of additional scalar coupling information (such as heteronuclear ¹⁵N-¹H and ¹³C-¹H *vicinal* coupling), it is difficult to define the ϕ and ψ torsion angles of the residues at the 'junction' of the rings; this makes it impossible to draw any definite conclusions regarding the orientation of the rings with respect to one another.

All the n.m.r. evidence suggests that, under the solution conditions used for our experiments, nisin does not have a single

overall conformation. We have therefore concentrated our efforts on defining the conformations of the individual rings.

Strong NH_i-NH_{i+1} n.O.e.s at short mixing times (less than 100 ms) suggest the presence of turns for all the ring structures of nisin, either within the ring unit or at the junction where the ring begins (e.g. Abu²³ NH-Ala²⁴ NH). Turns in peptides are identified by using three n.m.r. parameters: n.O.e., ³J_{NH-C α H} and hydrogen bonding (as indicated by the temperature-dependence of the chemical shift of amide protons or by their rate of exchange with the solvent). Since in the solution state only averaged n.m.r. parameters are observed, unambiguous identification of a particular *type* of turn is only possible when its population is very high; in most cases, including nisin, the presence of a turn can be identified, but its precise type is more difficult to specify.

Structure calculations

The distance constraints derived from the observed n.O.e.s were used, as outlined in the Materials and methods section, to derive sets of possible conformations for each of the ring structures in nisin. A set of 20 structures was generated by distance geometry and refined by dynamical simulated annealing. This procedure was carried out both for the individual rings and for the whole nisin molecule; both sets of calculations gave essentially identical results. The structures obtained for rings A (residues 3-7), B (residues 8-11) and D/E (residues 23-28) are shown in Figs. 4-6. In these Figures the 20 calculated structures consistent with the n.m.r. data have been superimposed so as to superimpose (by the least-squares criterion) the *backbone* atoms of each ring. It is clear that ring B, the smallest ring, which also contains a proline residue, is conformationally relatively well defined. By contrast, there appears to be a great deal of conformational flexibility in the largest ring, C (residues 13-19; results not shown). In residues 16-19 within this ring, the NH_i-NH_{i+1} n.O.e. connectivities are generally more intense than the C α H_i-NH_{i+1} connectivities, this difference being greater at lower temperatures (278 K). It can be concluded that there is a tendency for this region of the polypeptide chain to exist as a helical turn, but that this is only one of a number of conformations adopted.

Ring A (Fig. 4) is reasonably well-defined, but shows the flexibility of the lanthionine bridge discussed above; in addition, the Ile and Leu side chains can be seen to have considerable

Fig. 6. Superimposition of 20 structures of rings D and E of nisin, derived from n.m.r. data as described in the text

flexibility. The linked rings D and E also have quite limited conformational freedom, but do seem to be able to undergo a 'butterfly' motion about the linkage between residues 25 and 26 (not apparent in the view of Fig. 4). The very strong $\text{NH}_i\text{-NH}_{i+1}$ n.O.e. connectivity between Abu²³ and Ala²⁴ suggests a rotational restriction around the N-C_α bond of Abu²³ and therefore the orientation of the ring with respect to the 'linker' region.

We conclude that nisin is, in aqueous solution, a very flexible molecule, essentially the only defined conformational features being those imposed by the presence of the lanthionine residues. In particular, we have obtained no experimental evidence for a preferred *overall* conformation for the nisin molecule under these conditions. The conformation of nisin in a lipid bilayer, which is likely to be much more relevant to its mechanism of action, remains to be established.

Comparison of the structures of nisin and its degradation products

We have shown [3] that the two C-terminal residues of nisin, ΔAla^{33} and Lys^{34} , are not required for antimicrobial activity. The 1D and 2D n.m.r. spectra of nisin¹⁻³² were essentially identical with those of nisin, except for the absence of the resonances of ΔAla^{33} and Lys^{34} and some small chemical-shift changes for residues 23 and 32. We conclude that the loss of these two residues, which we have shown to be highly flexible in solution (see above), has no effect on the conformation of nisin.

The additional loss of ΔAla^5 , to give des- ΔAla^5 -nisin¹⁻³², leads to a much more substantial loss of activity [3]. It has not been established whether this is due to the loss of ΔAla^5 *per se*, or to changes in conformation as a result of the opening of ring A. As shown in Fig. 7, the n.O.e. patterns of nisin and des- ΔAla^5 -nisin¹⁻³² were very similar, *except* for the N-terminal region from residues Ile¹ to Abu⁸. An increased flexibility and a lack of well-defined structure in this region of des- ΔAla^5 -nisin¹⁻³² is evident from the absence of n.O.e. connectivities and also from an increased amide proton-water exchange rate in this region of the

Fig. 7. Comparison of the n.O.e. connectivities observed for nisin and for des- ΔAla^5 -nisin¹⁻³²

The connectivity indicated between Abu⁸ and Pro⁹ is between Abu⁸ C _{α} H and Pro⁹ C _{δ} H₂.

molecule. It is important to note, however, that it was possible to observe in des- Δ Ala⁵-nisin¹⁻³² qualitatively the *same* n.O.e.s as in nisin by decreasing the temperature by 20° and using longer mixing times (400 ms).

There is clearly an increase in conformational flexibility as a result of the removal of Δ Ala⁵ and the opening of ring A. However, the re-appearance of the n.O.e.s for residues 1-8 at low temperature suggests that a conformation or group of conformations similar to those adopted when the ring is intact still has a low energy for the modified molecule, perhaps stabilized by hydrogen-bonding between the amide on Ile⁴ and the pyruvoyl group on Leu⁶ (cf. [3]). This might suggest that the lower biological activity of des- Δ Ala⁵-nisin¹⁻³² is more likely to be due to the absence of Δ Ala⁵ *per se* than to the conformational consequences of ring-opening.

We are most grateful to Dr. M. J. Sutcliffe for his help with the distance-geometry and molecular-dynamics calculations. We acknowledge the Science and Engineering Research Council for financial support.

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Received 19 August 1991; accepted 10 October 1991