Solution structure of plantaricin C, a novel lantibiotic

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Plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strain of dairy origin, is a lantibiotic. One dehydroalanine, one lanthionine and three β-methyl-lanthionine residues were found in its 27 amino acid sequence. The plantaricin C structure has two parts: the first comprises the six NH$_2$-terminal residues, four of which are lysines, which confer a strong positive charge to this stretch. The amino acids in positions 7 and 27 form the lanthionine bridge, giving a globular conformation to the rest of the molecule. The β-methyl-lanthionine bridges are established between residues 12–15, 13–18 and 23–26. This central region has a charge distribution compatible with an amphipathic α-helix, through which plantaricin C would become inserted into the membrane matrix of sensitive organisms, provoking the opening of pores and leakage of the cytoplasmic content.

Keywords: bacteriocin; lantibiotic; NMR; plantaricin C; solution structure.

Bacteriocins are ribosomally synthesized polypeptides, produced by bacteria that inhibit the growth of other bacteria. Production of bacteriocins is widespread among lactic acid bacteria, a group of Gram-positive organisms used as starters in food fermentation. These compounds potentially fulfil the requirements for optimal food preservatives: first, some of them have a wide spectrum of sensitive spoilage and pathogenic bacteria; second, they are presumed to be degraded by intestinal proteases, being of no harm to consumers and, last but not least, they are produced by the starters in the fermenting mass, which means that no further costs are associated with their use [1–3]. Bacteriocins have been grouped into three classes based on their structure. Class I comprises the lantibiotics, a series of small heat-resistant peptides that undergo post-translational modifications leading to the formation of rings through reaction between dehydrated serine and threonine residues with the sulfhydryl group of cysteine [4,5]. Class II is represented by heat-resistant unmodified peptides and, finally, class III is made of heat-labile proteins with sizes in excess of 15 kDa [3].

In a survey of lactobacilli isolated from traditional fermented products, one strain of *Lactobacillus plantarum* produced a 3.5-kDa bacteriocin of wide inhibitory spectrum against Gram-positive bacteria. This compound, named plantaricin C, is quite resistant to environmental/storage conditions and presents a high specific activity [6]. It inserts into the cytoplasmic membrane of sensitive organisms in a voltage-independent manner without the need for protein receptors. Presumably, it polymerizes there, creating a barrel structure that results in formation of pores through which the plasma content is released [7]. As a consequence the cell dies and, in some cases, lysis is induced.

In this paper we report the amino acid sequence and solution structure of plantaricin C, which is shown to be a lantibiotic, and relate its structure with the bactericidal action described previously.

MATERIALS AND METHODS

Bacterial strains, culture conditions and purification of plantaricin C

The bacteriocin was produced by *L. plantarum* LL441, and *L. sake* CECT 906 was used as the indicator strain. Cultures were grown in MRS medium (Biokar) containing 1% glucose at 30 °C without aeration. Plantaricin C was purified as described previously [6] and stored at 4 °C.

N-terminal protein determinations

Pure plantaricin C was subjected to automated Edman degradation [8] in an Applied Biosystems 477A protein sequenator. Tryptic peptides were obtained after dissolving a 50 µg aliquot of lyophilized plantaricin C in 1 mL of 0.1 M NH$_4$HCO$_3$, pH 8.5, through addition of 1 µg trypsin (Sigma) and incubation at 37 °C for 24 h. The resulting peptides were separated by HPLC on a C-18 reversed phase column and detected by their absorbance at 220 nm. Chemical derivatization of plantaricin C to open the lanthionine bridges, prior to sequencing, was performed as follows [9]: 500 pmol aliquots of plantaricin C were treated with either ethanthiol or 1-propanthiol to split the lanthionines and methyllanthionines to S-ethyl-cysteines and S-ethyl-methylcysteines (using ethanthiol) or S-propyl-cysteines and S-propyl-methyl-cysteines (using 1-propanthiol), respectively. These modified amino acids were identified during Edman degradation as their phenylthiohydantoin derivatives.

To test the position of the only dehydroalanine, 500 pmol unmodified plantaricin C was sequenced. After performing 20 steps of Edman degradation, the filter disk was removed from the sequencer cartridge and treated with pertrifluoroacetic acid

at 4 °C for 30 min (in the gas phase), and then dried under high vacuum to remove the excess pertrifluoroacetic acid. The filter disk was then replaced in the sequencer cartridge and the Edman degradation resumed for another 11 cycles.

Matrix-assisted laser desorption mass spectrometry (MALDI-MS)

The MALDI mass spectrum was recorded in linear mode using a Finnigan Lasermat 2000 (Finnigan MAT Ltd, Hemel Hempstead, UK) equipped with a nitrogen laser (337 nm, pulse duration: 3 ns). The analyser operated in positive ion mode with an accelerating voltage of 20 kV. The spectrum was obtained by summing 10 scans.

A solution of 10 mg·mL^{-1} α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid was used as matrix. A 3-μL aliquot of the matrix was then mixed with 1 μL sample (dissolved in methanol/water 1 : 1, 5% acetic acid) and 1 μL of this solution was applied to the target, air dried and analysed. For internal calibration insulin B with a molecular mass of $(M + H)^+ = 3496.9$ Da was used.

Electrospray mass spectrometry (ESI-MS)

Plantaricin C was dissolved in methanol/water 1 : 1, 5% acetic acid (10 pmol·μL^{-1}). The sample was on-line injected to the electrospray ion source of a Finnigan TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT Ltd, Bremen, Germany) with a flow of 3 μL·min^{-1}. The spectra were recorded under unit-mass resolution in the positive ion mode. The total ion current chromatogram was obtained by scanning the mass range corresponding to *m/z*-values of 200–2500 every 4 s. The spectra were accumulated and deconvoluted to obtain the calculated mass of plantaricin C.

High resolution mass measurements with a resolution of ≈ 7000 were performed on a double-focusing sector field mass spectrometer MAT 90° (Finnigan) equipped with an ESI II electrospray interface. The mass accuracy was increased using mellitin as an internal standard. The mellitin solution was added to the plantaricin C sample to obtain final concentrations of 5 pmol·μL^{-1}. This solution was infused into the electrospray ion source with a flow rate of 1 μL·min^{-1}. In addition, a sheath liquid of 2-propanol was used with the same flow rate. Mass spectra were acquired in the positive ion mode in the mass range *m/z* 710–725 in an electric scan. The scan rate was 500 s per decade and 35 spectra were accumulated. Peak matching experiments were performed using the first isotopic peak of plantaricin C and mellitin.

NMR spectroscopy

The NMR experiments were carried out using a Varian VXR500 spectrometer operating at 499.8 MHz with a 0.5 mM sample of plantaricin C dissolved in a 65 : 35 mixture of C^{2}H$_3$CN and H$_2$O. Two-dimensional spectra were recorded with 4096 point free induction decays and 1024 increments with time proportional phase incrementation [10] to give spectral widths of 6.5 kHz in F$_2$ and 13 kHz in F$_1$. The H$_2$O peak was saturated for 1.8 s before each pulse sequence.

NOESY [11] and TOCSY [12] spectra were recorded at 55, 45, 35 and 25 °C. A WALTZ-modulated, 5.6-kHz spin lock field was applied during the 40-ms mixing periods of the TOCSY experiments. Rotating frame NOE (ROESY) spectra [13] were recorded at 45 °C with a continuous spin lock field of 3.5 kHz applied during the 150 ms mixing periods.

Structure calculations

Cross-peaks in the ROESY spectrum were integrated together with blanks on each side in the F$_1$ dimension. The average value of each pair of blanks was used to correct the intensities for baseline distortion and their difference was used to derive upper and lower limits for the cross-peak volumes [14]. The upper and lower limits of the volumes were used as input to a version of the program DYANA [15], which has been modified for automatic calibration of the conversion from volumes to distance constraints with the approximation of proportionality to the inverse sixth root. Separate scaling factors for proton–proton, proton–methyl, and methyl–methyl interactions were optimized iteratively for each structure together with the torsion angle parameters. Automatic calibration avoids the subjectivity of manual calibration and the possible introduction of bias caused by the use of a single set of scaling factors for all structures. The use of maximum volumes to provide lower limit distance constraints facilitates calibration, and also provides a convenient way of incorporating information from overlapping cross-peaks and cross-peaks of negligible intensity. The functions of the modified program, INDYANA, will be described fully elsewhere (S. G. Chamberlin, L. Brennan, S. M. Puddicombe, D. E. Davies & D. L. Turner, unpublished results).

Three modified amino acid residues were added to the standard AMBER library: dehydroalanine, D-Abu$_s$ and D-Ala$_s$. The sulfide bridge in lanthionine was constructed by superimposing the C$_\beta$ and S$_\beta$ atoms of L-cysteine on corresponding pseudoatoms of D-Ala$_s$ with fixed upper distance constraints of 0.01 nm. Methyllanthionine was constructed in a similar way using D-Abu$_s$ and L-cysteine. For each bridge, the two upper fixed distances result in a distance of 0.18 nm between the C$_\beta$ and the S and a C$_\beta$–S–C$_\beta$_ angle of about 100°. These fixed distances were treated with the softer target function type 2 of the program DYANA, whereas the volumes were treated as target function type 1 [17]. To avoid steric repulsion, the bridges were defined as covalent links in the sequence file.

The computed structures were processed using a separate program to find any interproton distances under 0.25 nm for which there were no constraints. The volumes at the expected positions of such cross-peaks were measured wherever possible to provide additional constraints, most of which gave only lower limit distances. This procedure was particularly important because of the flexibility of the molecule and the low concentration of the sample, but it is recommended generally as a means to ensure that the final structures are fully compatible with the experimental data with regard both to the presence and absence of visible cross-peaks. Stereospecific assignments were made with the aid of the program GLOMSA [15] and corrections were applied automatically to the distance constraints in the calculation of the target function whenever stereospecific assignments were not available. The structures were annealed with 20 000 steps, using the standard annealing procedure from DYANA.

The major disadvantage of the ROESY experiment is the reduction in cross-peak intensity between scalar-coupled protons, which may be caused by coherent magnetization transfer (the TOCSY effect): possible systematic errors were avoided by using only lower limit volumes (upper limit distances) for vicinal protons. Conversely, only maximum volumes were used for protons involved in overlapping cross-peaks, except in the case of degeneracy between methylene protons for which half the maximum intensity of the cross-peak was ascribed to one of the protons, but without stereospecific assignment, such that lower distance limits were generated for both protons.

RESULTS

The first 11 amino acids of plantaricin C, which were obtained through automated Edman degradation, have been reported previously [6]. The sequence, KKTKKNXSGDI, had a gap in position 7 and was blocked after residue 11. In order to determine the complete sequence of plantaricin C, several methods were applied. First, tryptic digestion of the peptide was tried, but the two peaks resulting from HPLC separation rendered sequences that were part of the known sequence, namely KNXSGDI and NXSGDI, confirming the position of the blockage. The compound was then analysed independently by NMR and by a combination of chemical modification and degradation.

Sequence and resonance assignment

The spin systems of the amino acid residues were identified from TOCSY spectra and placed in sequence with the aid of $d\alpha N_{(i,i+1)}$ connectivities in NOESY and ROESY experiments [18], with confirmation from NH_i-NH_{i+1} cross-peaks. The compound was found to comprise 27 residues, with a dehydroalanine at position 16, which was identified by its singlet NH proton and its two downfield β protons. The Ala_s and Abu_s residues were identified by their side chain AMBX and A_3MPX spin systems, respectively (excluding the backbone NH), and their characteristic chemical shifts. The side chains of residues 6, 10 and 19 were identified as AMX spin systems which could arise from Asn or Asp and, similarly, the AM(PT)X spin system at position 17 could arise from Glu or Gln but remained ambiguous because of the difficulty of assigning NOE cross-peaks to the terminal NH_2 protons. Overlapping Lys spin systems were positioned by a combination of the integrated signal intensities and the absence of a signal from the N-terminal amide protons, which exchange rapidly with water. Two unassigned peaks remain at 7.47 p.p.m. and 8.66 p.p.m. (at 45 °C). These peaks show no cross peaks in NOESY or ROESY spectra at various temperatures. The resonance assignments and the summary of the data obtained

concerning the primary sequence of plantaricin C are given in Tables 1 and 2, respectively.

Chemical derivatization

To solve the ambiguities left after NMR sequence analysis plantaricin C was subjected to chemical derivatization [9] to open the β-methyl-lanthionine bridges and the resulting linear peptide was subjected to Edman degradation (Table 2). In this way the three AMX residues (Asp or Asn) located at positions 6, 10 and 19 were determined to be Asn, Asp and Asp, respectively, confirming previous data on the nature of residue number 6. Similarly, the residue placed at position 17 was found to be glutamic acid. The last residue of the peptide was ambiguous in the NMR determination, but was assigned as Ala_s using the chemical derivatization method. Blockage of the sequence at position 16 was resolved through previous treatment of the sample with pertrifluoroacetic acid.

The theoretical monoisotopic mass of plantaricin C is 2880.3 Da, which is in excellent agreement with the value of 2880.4 Da found by mass spectroscopy. The high resolution mass spectrum is shown in Fig. 1.

Bridge positions

The sequence analysis positively identified nine modified amino acid residues: one dehydroalanine, five Ala_s residues and three Abu_s residues. The plantaricin C structure is therefore expected to contain four rings formed by sulfide bridges. The positions of bridges in previous studies of lantibiotics have been confirmed by NOE cross-peaks between β protons in the L-alanine and the D-alanine fragments of (2S, 6R) lanthionine or between the β protons of the L-alanine moiety and the β proton or the methyl protons of the Abu_s moiety in methyllanthionine [19,20]. In the present case the following connectivities were observed: $12Ala_sH\beta-15Abu_sH\beta$, $12Ala_sH\beta-15Abu_sH\beta$ and $13Abu_sH\beta-18Ala_sH\beta$. The known biosynthetic pathway for the formation of the bridges [4,21,22] permits two patterns for the remaining four residues: (a) 7–26, 23–27 or (b) 7–27, 23–26, in which the lanthionine bridges may be L–D or D–L.
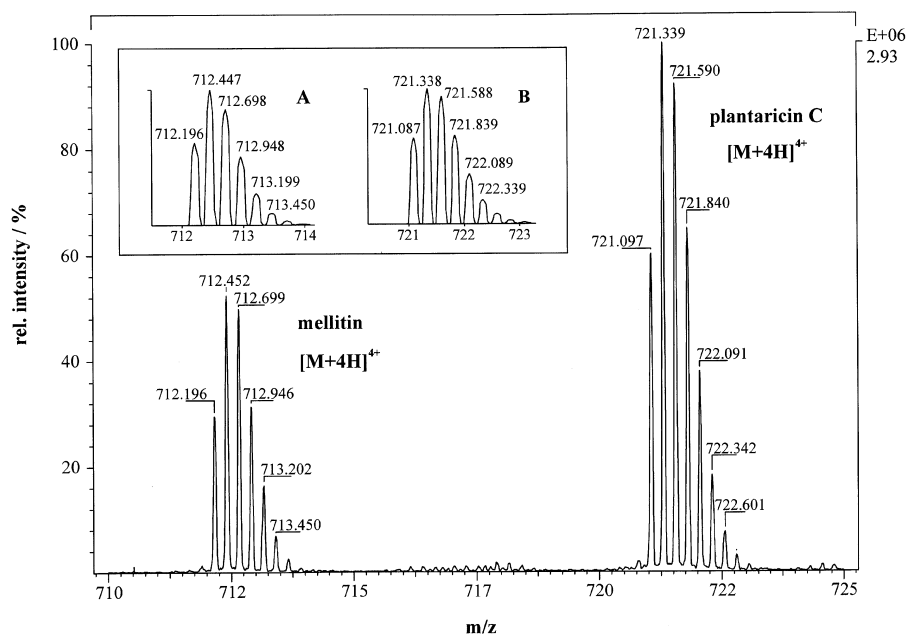

Fig. 1. Electrospray mass spectrum of a solution of 5 pmol·µL^{-1} mellitin (*m* = 2844.75 Da) and plantaricin C (*m* = 2880.31 Da). The resolution was adjusted to ≈ 7000. The first isotopic peak of mellitin was used as internal mass standard. Inset: theoretically calculated isotopic pattern of mellitin (A) and plantaricin C (B).

Table 1. Chemical shifts (p.p.m.) for plantaricin C at 45°C in C^2H_3CN/H_2O. Stereospecific assignments are indicated by an asterisk.

Lys	1		4.4	1.8	1.8	γCH_2 1.4 δCH_2 1.7 εCH_2 3.0
Lys	2	8.4	4.4	1.8	1.8	γCH_2 1.4 δCH_2 1.7 εCH_2 3.0
Thr	3	7.93	4.43	4.19		γCH_3 1.15
Lys	4	8.0	4.3	1.8	1.7	γCH_2 1.4 δCH_2 1.7 εCH_2 3.0
Lys	5	8.0	4.3	1.8	1.7	γCH_2 1.4 δCH_2 1.7 εCH_2 3.0
Asn	6	8.21	4.66	2.75	2.75	γNH_2 7.40, 6.71
Ala$_S$	7	8.29	4.56	3.06	3.21	
Ser	8	8.00	4.43	3.82	3.82	
Gly	9	8.23	3.97			
Asp	10	7.99	4.58	2.85	2.85	
Ile	11	7.71	4.23	1.91		γCH_3 0.86 γH 1.47, 1.15 δCH_3 0.86
Ala$_S$	12	7.91	4.18	2.99*	2.83*	
D-Abu	13	7.67	4.68	3.71		γCH_3 1.26
Leu	14	7.51	4.75	1.56	1.50	δCH_3 0.85, 0.85
D-Abu	15	7.77	4.72	3.61		γCH_3 1.36
Dha	16	10.03	5.34	5.47		
Glu	17	8.31	4.30	2.05*	2.14*	γCH_2 2.50
Ala$_S$	18	8.14	4.59	3.13*	3.01*	
Asp	19	7.58	4.73	3.06	2.85	
His	20	8.33	4.97	3.39	3.32	1H 7.31 2H 8.69
Leu	21	8.90	3.90	1.93	1.93	γCH 1.70 δCH_3 0.92, 0.92
Ala	22	8.22	3.97	1.38		
D-Abu	23	6.77	4.78	3.31		γCH_3 1.20
Trp	24	7.97	4.45	3.36	3.31	1H 9.79,2H 7.35,4H 7.63,5H 7.08,6H 7.16,7H 7.40
Val	25	7.36	4.14	1.55		γCH_3 0.84* 0.77*
Ala$_s$	26	7.60	4.62	3.29	2.57	
Ala$_s$	27	7.93	4.32	3.05		

Table 2. Sequence information for plantaricin C obtained from (A) automated sequencing (B) NMR and (C) combined chemical methods; (D) represents the bacteriocin consensus sequence. AMX: Asp or Asn; Glx: Gln or Glu

Residue	A	B	C	D
1	Lys	Lys	Lys	Lys
2	Lys	Lys	Lys	Lys
3	Thr	Thr	Thr	Thr
4	Lys	Lys	Lys	Lys
5	Lys	Lys	Lys	Lys
6	Asn	AMX	Asn	Asn
7	–	Ala$_s$	Ala$_s$	Ala$_s$
8	Ser	Ser	Ser	Ser
9	Gly	Gly	Gly	Gly
10	Asp	AMX	Asp	Asp
11	–	Leu?	Ile	Ile
12	–	Ala$_s$	Ala$_s$	Ala$_s$
13	–	Abu$_s$	Abu$_s$	Abu$_s$
14	–	Leu	Leu	Leu
15	–	Abu$_s$	Abu$_s$	Abu$_s$
16	–	Dha	Dha	Dha
17	–	Glx	Glu	Glu
18	–	Ala$_s$	Ala$_s$	Ala$_s$
19	–	AMX	Asp	Asp
20	–	His	His	His
21	–	Leu	Leu	Leu
22	–	Ala	Ala	Ala
23	–	Abu$_s$	Dhb/Abu$_s$	Abu$_s$
24	–	Trp	Trp	Trp
25	–	Val	Val	Val
26	–	Ala$_s$	Ala$_s$	Ala$_s$
27	–	Ala?	Ala$_s$	Ala$_s$

Table 3. The possible bridging patterns in plantaricin C.

Type	Bridge A	Bridge B	Bridge C	Bridge D
1	7 (D) – 26	12–15	13–18	23–27
2	7 (D) – 27	12–15	13–18	23–26
3	7–26 (D)	12–15	13–18	23–27
4	7–27 (D)	12–15	13–18	23–26

The four possibilities are listed in Table 3 and a schematic representation of pattern (b) is shown in Fig. 2. No cross-peaks were observed which could confirm the bridging pattern. In view of the weak signals obtained with the low sample concentration and the possibility of misleading NOE cross-peaks arising from adjacent methyllanthionine groups, we sought to establish the position of the bridges by determining the complete three-dimensional structure of the molecule.

Fig. 2. Schematic representation of plantaricin C showing the most probable pattern of linkages. Lanthionines are shown as linked open circles and the position of the methyl group in methyl lanthionines is marked 'm'. The dehydroalanine is marked 'dha'.

Table 4. Constraints used in the structure calculation.

Type of constraint	Lower volume limit	Upper volume limit
Intraresidue ($\Delta i = 0$)	98	76
Sequential ($\Delta i = 1$)	60	103
Medium range ($2 \leq \Delta i \leq 4$)	15	149
Long range ($\Delta i \geq 5$)	5	260

Table 5. The distances (Å) between β-carbons obtained in a family of structures calculated without constrained bridges. The standard deviations are given in parenthesis. The bridge positions are defined in Table 3.

Pattern	Bridge A	Bridge B	Bridge C	Bridge D
1	8.98 (1.77)	4.06 (0.19)	3.13 (0.47)	8.75 (0.63)
2	12.47 (1.90)	4.06 (0.19)	3.13 (0.47)	4.35 (0.76)
3	8.98 (1.77)	4.06 (0.19)	3.13 (0.47)	8.75 (0.63)
4	12.47 (1.90)	4.06 (0.19)	3.13 (0.47)	4.35 (0.76)

Table 6. Summary of results obtained for structures calculated with each of the four possible bridging patterns. The rmsd values are given for the backbones of the 10 best structures in each case with respect to their mean.

Pattern	Target function ($Å^2$) and range	Rmsd (Å) and range	Maximum violation	
			upl (Å)	lol (Å)
1	23.3 ± 2.6%	0.05 ± 0.05	1.38	1.29
2	19.6 ± 3.4%	0.43 ± 0.12	1.43	1.28
3	20.7 ± 3.0%	0.43 ± 0.24	1.46	1.25
4	19.6 ± 2.4%	0.23 ± 0.10	1.39	1.27

Structure determination

Integration of the ROESY spectrum yielded 506 maximum volumes and 149 minimum volumes (only the maximum volume is used where cross-peaks overlap or the error bar crosses zero), which were used as input for the modified DYANA program, as described in Materials and methods. These were then modified for lack of stereospecific assignment to give 588 maximum volumes and 178 minimum volumes. A summary of the constraints used is given in Table 4. Structures were generated initially without constrained thio–ether linkages in order to determine whether the peptide fold would position the bridged residues unequivocally, with all Ala$_s$ residues in the L configuration. A family of 10 structures with target functions
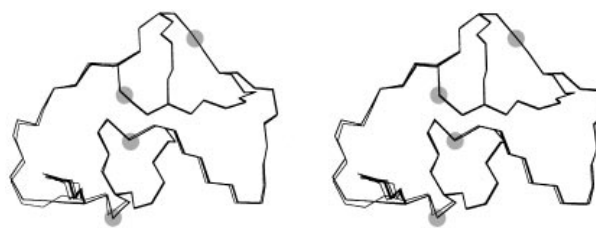
Fig. 3. Backbone structure of plantaricin C. The most favoured family of structures obtained with the linkage pattern 4 [7–27(D), 12–15, 13–18, 23–26] is shown. The ill-defined N-terminal region (residues 1–5) was omitted in this representation. The full circles represent sulfur atoms in the bridges.

up to 9.6% higher than the lowest value was analysed. The results given in Table 5 show that the 13Cβ–18Cβ distance is well-defined and close to the expected value of ≈ 0.3 nm, and the 12Cβ–15Cβ distance is also comparatively short. This confirms both of these ring positions. The 23Cβ–26Cβ distance is also short enough to indicate a link but, as these two residues are close in the sequence, it is not sufficient to confirm the pattern.

As a further test, structures were calculated with bridges corresponding to each of the four possible patterns, constrained by means of eight upper limit distances. In each case, 10 structures with similar target function ranges were used for the analysis. The structures for patterns 2 and 4 were each obtained from 200 random starting structures, those of pattern 3 were obtained from 400 random starting structures and, because pattern 1 gave poor convergence, 1800 random starting structures were needed to obtain a comparable set of solutions. The results are summarized in Table 6, from which it is clear that patterns 2 and 4 are the most favoured. These patterns differ only in the position of the D amino acid in the lanthionine formed by residues 7 and 27, and the analysis cannot distinguish between them. The family of structures obtained for pattern 4 is shown in Fig. 3.

The superimposition of residues 6–27 from the best structure of each of the four bridging patterns is shown in Fig. 4; the N-terminus was not included as it is not well defined. The rmsd for the backbone of residues 6–27 is 1.95 ± 0.67 Å. It is clear from the figure that the core of the structure is very well defined and the residual uncertainty in the pattern of bridges is a consequence of the similarity in the positions of the Ala$_s$ and Abu$_s$ residues found in each case.

DISCUSSION

In a previous report we postulated that plantaricin C might be a new member of the lantibiotic family of bacteriocins because

Fig. 4. The superimposition of backbone structures calculated for plantaricin C (residues 6–27) for each of the four possible bridging patterns shows that the overall fold of the polypeptide chain is similar in all bridging possibilities. The best structure in each of the four families is shown.
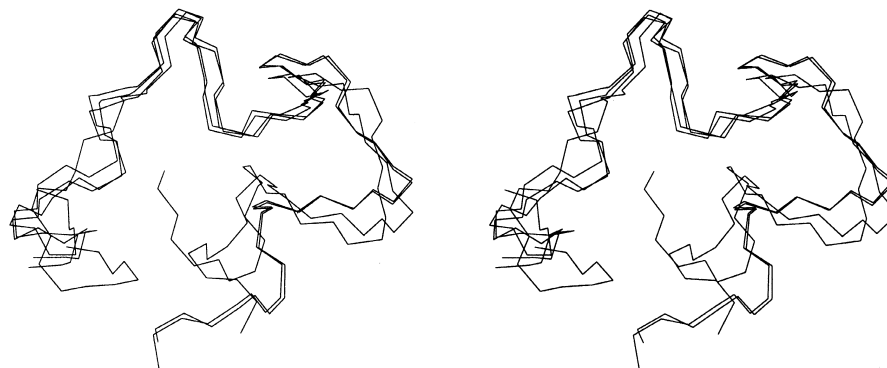
its complete sequence was not accessible to automated Edman degradation and also because a blank was found in the accessible portion [6]. Lanthionine (ala-*S*-ala) and β-methyl-lanthionine (ala-*S*-abu) [23] result in gaps during Edman degradation and could represent the amino acid in position 7 of plantaricin C, while dehydrated amino acids produce blockage, as was found at position 12 of the bacteriocin [6,24].

In order to obtain further insight into the nature of plantaricin C, a series of methods was used to determine its complete sequence as well as its secondary and tertiary structures. First, it was found that plantaricin C is a polypeptide made of 27 amino acids and that it is indeed a lantibiotic since one dehydroalanine, one lanthionine and three β-methyl-lanthionine residues were found in the molecule.

The complete sequence of plantaricin C helps to explain previously observed data. First, although the presence of a lanthionine at position 7 is confirmed, the apparent blockage of the sequencing reaction at position 12 is not caused by a dehydrated amino acid. This discrepancy could be explained because the residues at positions 12, 13 and 15 are part of β-methyl-lanthionines, which would render blank cycles, and then the sequencing reaction is effectively interrupted by a dehydroalanine at position 16. Careful inspection of the old chromatograms reveals a leucine residue at position 14 that was initially ignored. Knowledge of the sequence also explains the data obtained after tryptic digestion of the polypeptide. Trypsin hydrolyses peptide bonds after lysine or arginine residues, and the only ones present in plantaricin C are located at positions 1–5, hence all of the tryptic peptides obtained simply lack the N-terminal end of the polypeptide.

The deduced structure of plantaricin C comprises two distinct regions: a linear N-terminal end composed of six residues and a second, globular domain, created by the formation of a thio–ether bond between residues 7 and 27. This structure predicts that the N-terminal section will be mobile, whereas the rest of the molecule will be rigid and compact because of the 7–27 bridge and the presence of three internal loops between residues 12–15, 13–18 and 23–26, respectively. The overall structure of plantaricin C has some similarity to that of nisin in aqueous solvents [25], although both the N-terminus and C-terminus of the nisin molecule are flexible. The central part of nisin is also very rigid, containing five thio–ether bridges. However, there is an internal hinge at around amino acid 21 that allows the movement of the two halves of the central region with respect to each other.

Finally, the distribution of charges in the molecule would help to explain the mode of action of plantaricin C [7]. It was found that the bacteriocin inserts into the plasma membrane, where it generates pores through which cytoplasmic solutes are leaked. This effect was even observed in liposomes, which excludes the necessity of a protein cell receptor. Two hypotheses have been proposed to explain the action of nisin in the plasma membrane. The insertion model predicts that it inserts into the membrane after adhesion of the lantibiotic to the lipid surface, forming clusters that surround a central pore [26]. The wedge model predicts that adhesion of an amphipathic molecule to the lipid bilayer external surface will provoke destabilization of its structure, promoting the formation of pores [27]. The results of Driessen *et al.* [28] favour the wedge model for nisin, partly because of the high potential across the membrane, $\Delta\psi$, that is necessary to promote pore formation. However, it was shown recently that nisin will recognize lipid II, the last monomeric peptidoglycan precursor, *in vivo*, which relieves the necessity for a potential across the membrane [29]. Thus, it appears that the insertion model best explains the effect of lantibiotics under natural conditions, as postulated for plantaricin C and molecules such as gallidermin and epidermin, which promote solute leakage even from de-energized membranes and liposomes [7,30].

Hence, it may be postulated that the first interaction of plantaricin C with the membrane depends on the positive charge of its N-terminal end (out of the first five amino acids four are lysines), which would be attracted by the negative charge of the phospholipids. This is supported by the fact that the peptides generated by tryptic digestion of plantaricin C (see above) are devoid of any inhibitory activity (unpublished data). Once the contact is established, the globular domain would integrate into the membrane matrix due to its essentially uncharged nature. Out of the 21 residues that form this domain, 15 are nonpolar, two are polar without charge, three are negatively charged and one has a positive charge. The insertion of the hydrophobic domain of the bacteriocin into the membrane could be accomplished while its N-terminal part remains attached to the heads of the phospholipids because of the flexibility that the molecule has around amino acid 6.

As postulated for nisin [25,31], the portion of plantaricin C that extends through the membrane (potentially amino acids 6–27) might form an amphiphilic helix that, following the insertion model, would mediate the progressive combination of new molecules to form a barrel structure with a central pore. The diameter of the pore would increase by the addition of new 'staves' which, in turn, would allow the leakage of progressively larger solutes from the cell. This is exemplified by the almost immediate loss of uridine upon addition of the bacteriocin to sensitive cultures, but not of ATP, which started to be released 40 min after bacteriocin addition [7]. The function of the negatively charged amino acids in the hydrophobic domain is unclear, but it may be significant that plantaricin C is only produced by cultures growing at pH 5.5 or lower [32] and its activity becomes maximized at these low pHs. Under these conditions the net charge of the dicarboxylic amino acids would be much reduced. This has two consequences: first it allows the coexistence of several bacteriocin molecules surrounding the pore, in spite of having their dicarboxylic acids juxtaposed in the channel and second, it explains how negatively charged molecules, such as ATP, can pass through the pores.

CONCLUSION

The structural data reported here help us understand some of the properties shown by plantaricin C, such as the lack of the necessity for a receptor in the cell surface, which probably accounts for its wide spectrum of sensitive bacteria, and its action on membranes. Furthermore, these data will contribute to the design of future fundamental and applied studies with the aim of using the bacteriocin as a safe food preservative of natural origin.

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