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Sequence-specific ^{1}H assignment and secondary structure of the bacteriocin AS-48 cyclic peptide

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Abstract

The bacteriocin AS-48 is a cationic peptide (7149 Da) having a broad antimicrobial spectrum, encoded by the 68 kb conjugative plasmid pMB2 from *Enterococcus faecalis* S-48. It is a unique peptide since it has a cyclic structure, which is achieved by the formation of a tail–head peptide bond after ribosomal synthesis (Gálvez et al., 1989; Martínez-Bueno et al., 1994; Samyn et al., 1994). Preliminary CD and calorimetric studies (data not shown) pointed towards a highly helical and very stable three dimensional structure.

All the information gathered until now indicates that the target of AS-48 is the cytoplasmic membrane in which it opens channels or pores, leading to dissipation of the proton motive force and cell death, which in some cases is also followed by bacterial lysis (Gálvez et al., 1991). This peptide is a suitable tool for studying protein–membrane interactions, and it also offers promising perspectives for biotechnological applications.

Knowledge of the 3D structure of AS-48 is a first step in the conduct of further structure–function studies. Here we report the complete ^{1}H NMR assignment of its proton resonances together with the resulting secondary structure pattern as prerequisites for the determination of a high-resolution 3D solution structure.

Methods and Results

NMR samples were prepared by dissolving the cyclic peptide antibiotic AS-48 at a concentration of ca. 1.5 mM, 0.1 M NaCl, in 0.5 ml H_2O:D_2O (9:1 by vol.) or D_2O. Sedimentation equilibrium experiments indicate that the protein is entirely monomeric in these conditions (data not shown). Data were collected at 298 K and the pH (not corrected for isotope effects) adjusted to 3.0 in all cases. Sodium 3-trimethylsilyl(2,2,3,3-$^2H_4$)propionate was used as internal reference. NMR experiments were performed on a Bruker AMX-600 spectrometer equipped with z-axis pulsed field gradient capabilities. All 2D spectra were acquired in the phase sensitive mode using the time proportional phase incrementation mode (Marion and Wüthrich, 1983). Water suppression was achieved by selective presaturation of the water signal or by including the WATERGATE module (Piotto et al., 1992) in the original pulse sequences. Conventional 2D pulse sequences and phase-cycling procedures were used for COSY (Aue et al., 1976); TOCSY (Bax and Davies, 1985) and NOESY (Kumar et al., 1980). Mixing times of 80 ms and 150 ms in the NOESY and 60 ms in the TOCSY sequences were used. The size of the acquisition data matrix was 2048 $\times$ 512 words in f_2 and f_1, respectively. All spectra were processed using the standard Bruker software. Before Fourier transformation, the 2D data matrix was multiplied by a phase-shifted sine-bell or square-sine-bell window function in both dimensions. The corresponding shift was optimised in every experiment. Baseline correction was applied in both dimensions.

Qualitative measurements of amide hydrogen exchange were carried out by dissolving the lyophilized, fully protonated protein in D_2O and observing the intensity of peptide NH resonances in TOCSY experiments over a period of 50 days at 25 °C. A total

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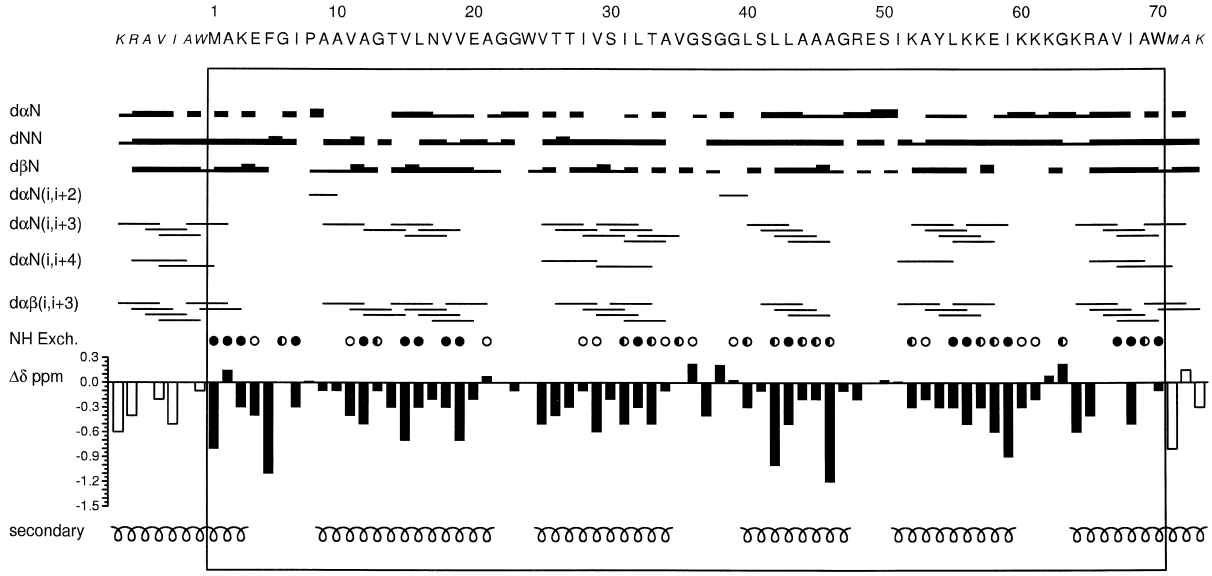

Figure 1. Summary of observed sequential and short range NOEs, conformational chemical shifts ($\Delta\delta$ ppm = δ observed $-\delta$ random coil) and exchange data of bacteriocin AS-48 at 298 K and pH 3.0. In the box, data of the cyclic protein are included and the sequence is indicated in the top by normal case letters. Flanking residues are indicated in *italics* and their data are also shown. The thickness of the sequential NOEs is a qualitative indication of their intensity. In the exchange plot, a circle indicates that the amide proton of that residue remains in D_2O solution at least for 15 min, a half-filled circle that the corresponding HN exchange between 15 min and 50 days and a filled circle that the corresponding NH is observed for more than 50 days.

of 46 NHs could be detected after 15 minutes of H–D exchange, 17 of which were still present at the end of the experiment. The assignment process was conducted by using the standard sequence-specific strategy (Wüthrich, 1986).

On the basis of the sequence, 70 independent spin systems were detected and classified from the 2D correlation spectra. Sequential cross correlations were established on the basis of H_α–HN (H_α–H_δ for Pro), H_β–HN and/or HN–HN NOEs observed between adjacent residues of the cyclic protein. For all residues at least one sequential NOE could be assigned with the exception of A34–V35 due to HN chemical shift overlap. The relative intensities of the sequential NOEs as well as the presence of short-range cross-correlations constitute an important source for the characterization of the secondary structural elements. These observations are in general supported by other independent parameters, such as the conformational chemical shifts and HN-exchange data. Resonances in loop regions, mainly those in the segments G36–S37–G38–G39 and I59–K60–K61–K62, were the last ones to be assigned. All data determined from the NMR spectra of the bacteriocin AS-48 are schematically presented for the protein sequence in Figure 1. Due to the cyclic structure of the protein, data corresponding to the primary sequence are included in the box and, for clarity, flanking residues are also shown. On this basis, most of the residues in bacteriocin AS-48 are found in helical structures connected by short turn regions. Five helical regions have been characterized spanning residues 9–21 (α_1), 24–34 (α_2), 40–47 (α_3), 51–59 (α_4) and 64–3 (α_5). These regions are characterized by intense HN-NH sequential NOEs and the presence of a great number of non-sequential H_α–HN (i, $i+3$), H_α–H_β (i, $i+3$), H_α–HN (i, $i+4$) cross correlations. Further, the location of the protected protons as well as the signs of the conformational chemical shifts , $\Delta\delta$ ppm = δ observed $-$ δ random coil (Wishart et al., 1995), show a broad agreement with the limits determined by the NOE data.

Extent of assignment and data deposition

The assignment extends to all protons in the peptide, included those in long-chain residues. The only ambiguity correspond to protons QD and QE of Lys 60 and Lys 61, which could be exchanged. Stereospecific assignments have been performed for the methyl groups of all nine valines, and for the HB2 and HB3 of 4 out of five AMX residues.

In summary, the assignment of the proton resonances of the cyclic peptide bacteriocin AS-48 has been accomplished and its secondary structure delineated, thus paving the way for the NMR determination of the three-dimensional structure. ^{1}H chemical shift assignments have been deposited at BioMagResBank (http://www.bmrb.wisc.edu/) under the accession number 4112.

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