SECONDARY STRUCTURE OF THE CECROPINS: ANTIBACTERIAL PEPTIDES FROM THE MOTH *HYALOPHORA CECROPIA*

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Received 14 December 1981

1. Introduction

The existence of an inducible humoral immune system in insects has been long known (review [1]). Apart from the presence of lysozyme activity the molecular basis for the killing of bacteria was unknown until cecropins A and B from *Hyalophora cecropia* were isolated [2]. They were shown to be 2 small, basic peptides that could kill and lyse a variety of Gram-positive and Gram-negative bacteria. The amino acid sequences of the cecropins contained a basic N-terminal and a more hydrophobic central part [3]. A synthetic peptide with a sequence similar to residues 1–33 in cecropin A has been shown to have almost full anti-bacterial activity.

In order to obtain some information on the 3-dimensional structure of the cecropins and possibly, their mode of action, this study of their secondary structure was undertaken. It is shown that in dilute buffer solution cecropins A and B exist largely as random coil structures whereas in a hydrophobic environment they fold into more helical conformations. Prediction of secondary structure from sequence data according to Chou and Fasman [4,5] and model building strongly suggest that residues 1–11 form what has been named an amphipathic helix [6] (Merrifield, Vizioli and Boman in preparation).

2. Materials and methods

The cecropins A and B were purified by the method in [2] with the addition of a gel filtration step [3]. The synthetic cecropin $A_{1\text{-}33}$ was kindly supplied by KabiGen AB, Stockholm. Reagent grade chemicals were used without further purification. Liposomes were prepared by sonicating a thin-film of phospholipids with 2.5 mM Na-phosphate buffer (8.5 mg lipid/ml) until the solution became almost clear.

^{1}H NMR spectra were recorded on a Brucker WH270 Fourier Transform Spectrometer.

Circular dichroism measurements were made on a Carry 60 spectropolarimeter. d-10-camphor-sulphonic acid was used for calibration with $\Delta\epsilon$ taken as +2.37 at 290 nm [7]. All spectra were recorded at 25°C in a 0.5 cm cell. Peptide concentrations were ~0.08 mg/ml and always estimated spectrofotometrically at 280 nm in 2.5 mM Na-phosphate (pH 6.4) taking absorptivities of 1.36 and 1.31 cm/mg for the A and B forms, respectively [3]. The mean residue ellipticities expressed in deg . cm^2 . $dmol^{-1}$ were calculated at every 2.5 nm and are the average of 2–3 spectra. The mean residue weight of the cecropins was taken as 108.5. The helical content was either estimated from the mean residue ellipticity at 222 nm according to [8] or by simulating spectra with linear combinations of the α, β and random coil spectra obtained from [9].

3. Results

3.1. *Circular dichroism*

The CD spectra of cecropin A, B and $A_{1\text{-}33}$ are almost identical. The spectrum of cecropin A in 2.5 mM Na-phosphate (pH 6.4) is shown in fig.1. It can be interpreted as reflecting largely random coil structures. The negative Cotton effect around 222 nm indicates some helical content. From the mean residue ellipticity at this wavelength the helical content was found to be 17%, 12% and 15% for cecropin A, B and $A_{1\text{-}33}$, respectively.

To estimate the secondary structure in a more

Published by Elsevier Biomedical Press
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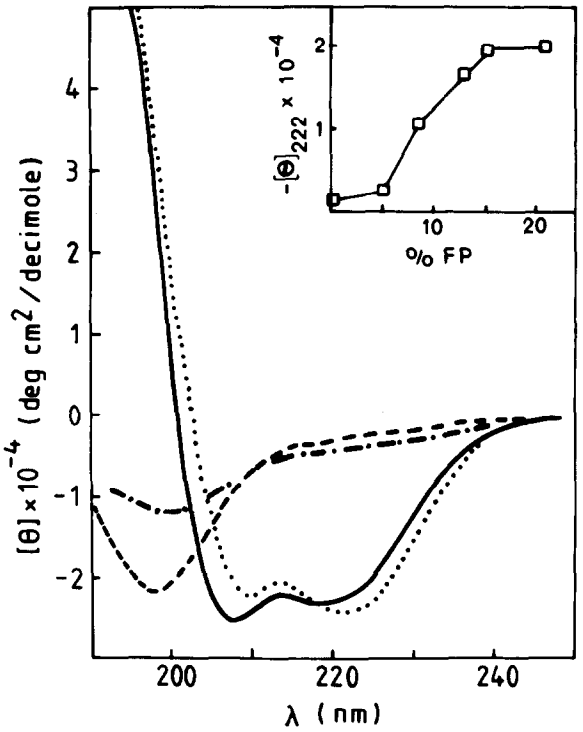

Fig.1. Circular dichroism spectra of cecropins in the far UV: (---) 1.9×10^{-5} M cecropin A in 2.5 mM Na-phosphate buffer (pH 6.4); (——) with 20% 1,1,1,3,3,3-hexafluoro-2-propanol; (–·–·) 2.2×10^{-5} M cecropin B with liposomes containing phosphatidylcholine (150 μg/ml) and dicetyl phosphate (15 μg/ml); (···) a simulated spectrum containing 81% α-helix, 7% β-sheet and 12% random coil. The insert is showing the mean residue ellipticity at 222 nm as a function of added 1,1,1,3,3,3-hexafluoro-2-propanol (FP) for 2.2×10^{-5} M cecropin B in 2.5 mM phosphate buffer (pH 6.4).

hydrophobic, membrane-like, environment CD-spectra were recorded at different concentrations of 1,1,1,3,3,3-hexafluoro-2-propanol. As shown for cecropin A in fig.1, the spectrum changes to a form that reflects large regions of helix. A simulated spectrum with 81% helix, 7% β-strand and 12% random coil is shown for comparison.

In an attempt to actually record CD-spectra of cecropins in membranes, phosphatidylcholine–dicetyl liposomes were prepared. Large unilamellar liposomes prepared by the reverse phase method [10] have been shown to become leaky when cecropins are added (unpublished). These large liposomes were, however, not suitable for CD-measurements due to their extensive light-scattering. Liposomes of the same composition but formed by sonication and thus of a smaller size were instead used and from the CD spectrum shown in fig.1 an increase to ~20% helix content could be estimated for cecropin B.

3.2. ^{1}*H-NMR spectrum*

The 270 MHz proton NMR spectrum of cecropin $A_{1\text{-}33}$ is shown in fig.2. Indicated are also the shifts obtained assuming the peptide to be in random coil conformation [11]. The experimental data seem to be compatible with such a random coil spectrum.

3.3. *Prediction of secondary structures from the amino acid sequences*

I have applied the empirical rules developed by Chou and Fasman [4,5] in the modified form suggested by Dufton and Hider [12] (fig.3). Both cecropins are predicted to possess a long helix from residue 1–11 and another helix at the C-terminal end. The central parts of the molecules are more irregular with regions of turns and β-structures. An attempt to assign secondary structures to the entire molecules is shown in fig.4. The predicted percentages of α-helix, β-sheet and β-turn are for cecropin A 51, 14, 22 and for cecropin B 49, 38, 11. From model building it became apparent that in the N-terminal α-helix the charged residues were located on one side and that the hydrophobic residues constituted the opposite side. This is visualized in fig.4 by a projection along the helical axis. The ε-amino group on the N-terminal lysine residue could easily reach the hydrophilic side as its α-carbon is free to rotate.

4. Discussion

It has been estimated that the minimum length for a peptide chain to fold into a globular structure is between 30 and 50 residues [13]. The CD and NMR results make it very likely that the cecropins, 37 residues long, in dilute buffer exist in a random coil conformation.

The empirical prediction method of Chou and Fasman has been derived from a set of globular proteins. Though it only takes short range interactions into account, one has to remember that the remainder of the protein constitutes the over all milieu wherein the folding takes place. For a peptide like cecropin the predicted secondary structure thus must be considered a potential folding pattern for the peptide in a more hydrophobic environment. The lack of correla-

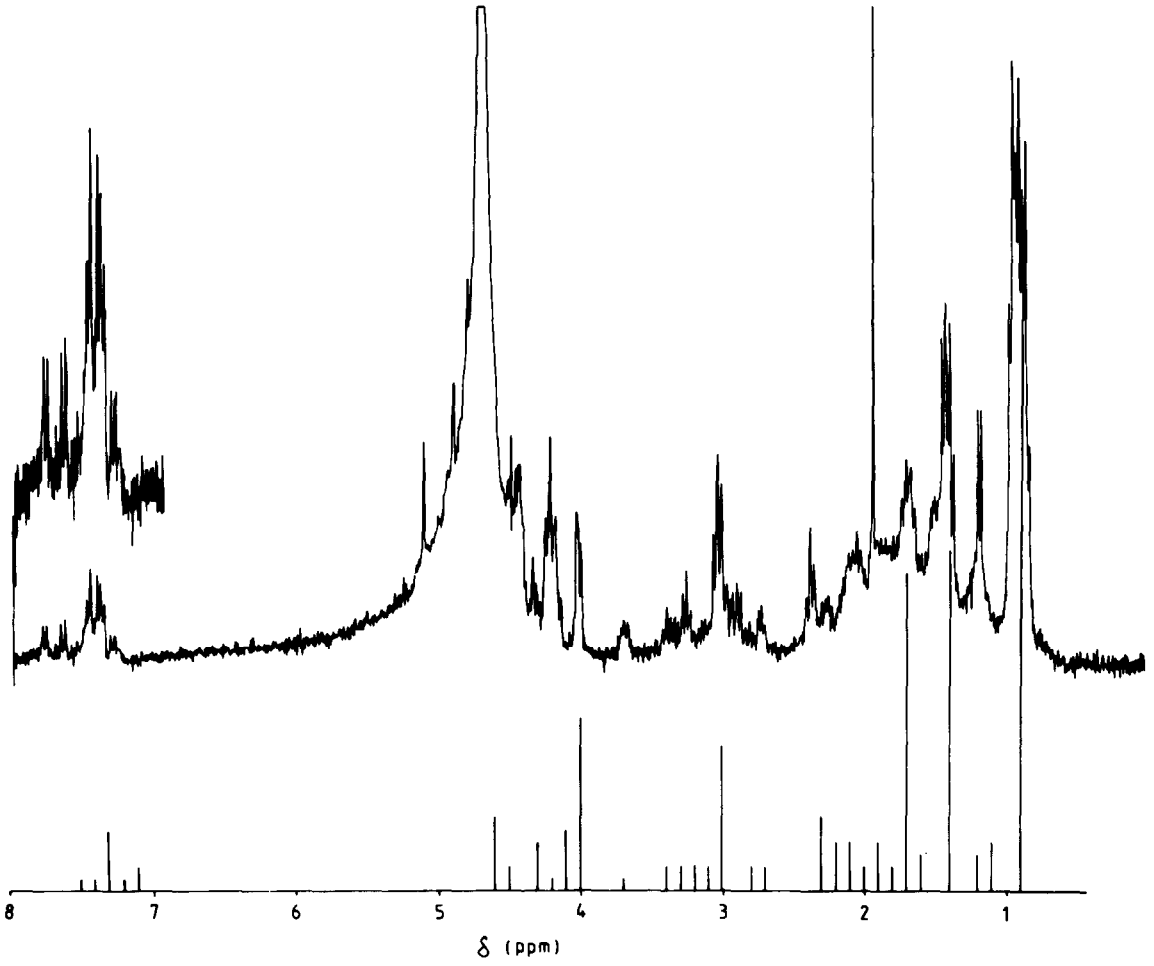

Fig.2. ^{1}H-NMR spectrum of the synthetic cecropin A_{1-33}. Peptide concentration was 1 mM, temperature 309 K and 1000 scans were collected. The expected chemical shifts of cecropin A_{1-33} in a random coil structure are indicated for comparison [11].

tion of the CD results in dilute buffer and the predicted structure is therefore not surprising.

Unfortunately, liposomes that are susceptible to the cecropins could not be used in the CD study. The sonicated liposomes here used induce a moderate increase in the helical content of the cecropins. However, these liposomes are resistant to the lytic action of the cecropins (unpublished). Whether the CD spectrum reflects the conformation of the cecropin bound to the liposome or if it merely reflects a partial binding has not been investigated.

The addition of 20% 1,1,1,3,3,3-hexafluoro-2-propanol caused a dramatic change to an almost totally helical conformation. Organic solvents are known to induce helices because this conformation with its maximal hydrogen bonded backbone is energetically favourable. At some organic solvent concentration a fortuitous correlation between measured and predicted secondary structure may be at hand. However, the low content of organic solvent and a high 80% of helix indicate that the cecropins have a strong potential for a more folded structure. By applying the empirical rules one finds that these helices are most likely to be located at both ends of the peptide. Between these domains there could be bends and some β-strands. The region around Pro-24 has a tendency for β-turn but it is not strong enough to be predicted as such. The rules may be inadequate when it comes to prediction of turns located in a hydrophobic core region as the turns in the original data set are on the hydrophilic

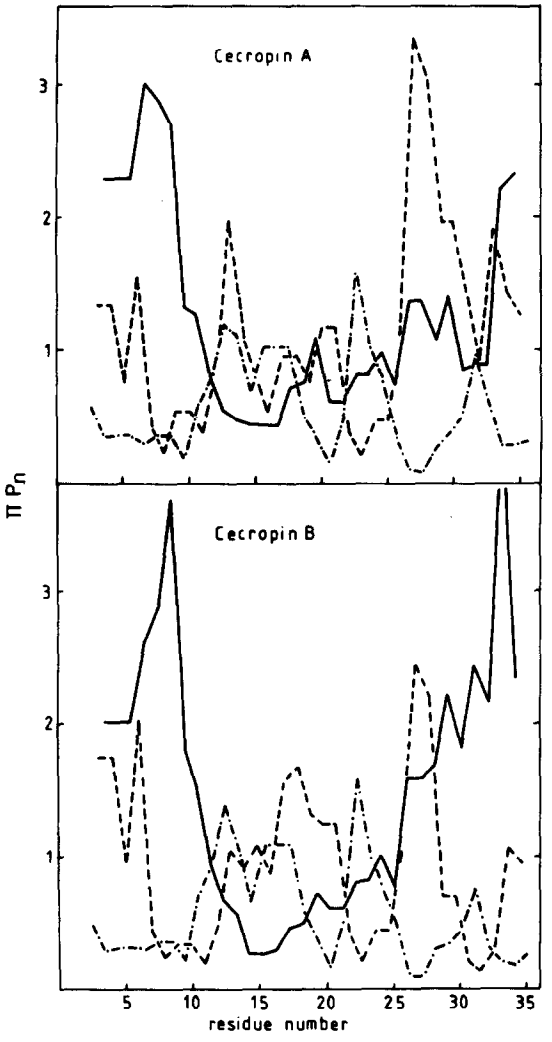

Fig.3. Prediction of secondary structures of cecropin A and B: (——) the product of the conformational parameter P_α for 6 adjacent amino acid residues (α-helix probability); (– – –) the product of P_β-values for 5 amino acid residues (β-sheet probability); (–·–·) the product of 4 P_t-values (β-turn probability). These πP_n-values are plotted at the middle of the respective amino acid intervals.

surface of globular proteins. However, a support that the β-strands in cecropin B are not joined by a turn comes from the rules of Lifson and Sanders for prediction of parallel or antiparallel β-strands [14]. The regions 16–20 and 25–29 have an average pair probability of 0.72 if joined by a turn to an antiparallel β-sheet. The small size of the cecropins makes it unlikely that an intramolecular parallel β-sheet could be formed. The average pair probabilities for the above regions in parallel sheets with themselves are 1.5 and 1.1. The sheets are then intermolecular. This possibility has to be considered as a simple calculation and indicates that the number of cecropin molecules needed to kill a single *E. coli* cell could be as high as 10^6 (data from [3]). Considering the surface area of the

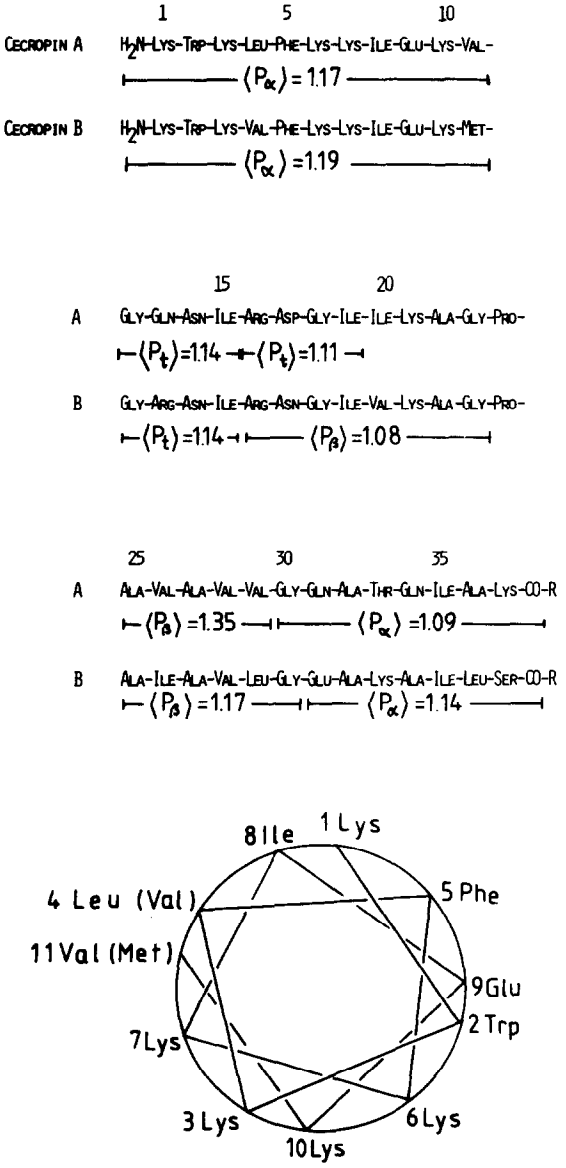

Fig.4. The amino acid sequences of cecropin A and B. The arithmetic mean values of the empirical parameters P_α, P_β and P_t for α-helix, β-sheet and β-turn are given for the predicted conformation. The projection along the α-helical axis for residues 1–11 of cecropin A and B (in parenthesis) is showing the amphipathic nature of such a helix (bottom).

bacterium this means that the average binding area available for a cecropin molecule could be as small as the expected dimensions of the cecropins. Thus, multimer formation can not be ruled out.

The main component in bee venom, melittin, is a well-characterized lytic peptide consisting of 26 amino acid residues ordered in an amphiphilic way. Both melittin and cecropin effect bacterial membranes [3] and their CD spectra are similar [15]. The secondary structures here described also suggest that the attachment of cecropin to a membrane may be similar to what has been proposed for melittin; an anchoring of the amphipathic helix to the surface of the membrane and a penetration of the central part of the molecule deeper into the lipid bilayer [15]. This could give rise to a wedge effect. The consequent disordering of the lipid molecules might be the rationale of cell lysis.

Acknowledgements

I wish to thank Dr K. E. Falk, Department of Biochemistry and Biophysics, Chalmers University of Technology, Göteborg, for running NMR-spectra. The CD-spectra were run in the same department. Financial support was obtained from the Swedish Natural Science Research Council (B2453).

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