Solution structure of crotamine, a Na\(^+\) channel affecting toxin from *Crotalus durissus terrificus* venom

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Crotamine is a component of the venom of the snake *Crotalus durissus terrificus* and it belongs to the myotoxin protein family. It is a 42 amino acid toxin cross-linked by three disulfide bridges and characterized by a mild toxicity (LD\(_{50}\) = 820 \(\mu\)g per 25 g body weight, i.p. injection) when compared to other members of the same family. Nonetheless, it possesses a wide spectrum of biological functions. In fact, besides being able to specifically modify voltage-sensitive Na\(^+\) channel, it has been suggested to exhibit analgesic activity and to be myonecrotic. Here we report its solution structure determined by proton NMR spectroscopy. The secondary structure comprises a short N-terminal \(\alpha\)-helix and a small antiparallel triple-stranded \(\beta\)-sheet arranged in an \(\alpha\beta_1\beta_2\beta_3\) topology never found among toxins active on ion channels. Interestingly, some scorpion toxins characterized by a biological activity on Na\(^+\) channels similar to the one reported for crotamine, exhibit an \(\alpha\beta\) fold, though with a \(\beta_1\alpha\beta_2\beta_3\) topology.

In addition, as the antibacterial \(\beta\)-defensins, crotamine interacts with lipid membranes. A comparison of crotamine with human \(\beta\)-defensins shows a similar fold and a comparable net positive potential surface.

To the best of our knowledge, this is the first report on the structure of a toxin from snake venom active on Na\(^+\) channel.

**Keywords:** \(\beta\)-defensin; myotoxin; NMR; scorpion toxin; structure.

Despite the fact that Na\(^+\) channels are affected by a large variety of toxins from arthropods, coelenterates, microorganisms, fish and plants, they are seldom the targets of toxins from snake venom [1]. One exception is crotamine (Crt), a protein of 42 amino acids present in the venom of *Crotalus durissus terrificus* [2,3] and characterized by a wide spectrum of biological activities. This toxin, in fact, has been known for a long time to be able to induce membrane depolarization dependent muscle contractions by increasing the Na\(^+\) permeability of skeletal muscle membranes [4–9] and to affect, in anallostericfashion, the action ofother Na\(^+\) channel neurotoxins (i.e. tetrodotoxin, veratridine, batrachotoxin and grayanotoxin) [4,5,7–10]. In addition, while loose patch-clamp recording of macroscopic sodium currents in frog skeletal muscle has revealed that Crt inhibits the inactivation of the Na\(^+\) channel in a fashion similar to that of scorpion \(\alpha\)-toxins [11], other experiments have shown that, at low doses, it has an analgesic activity involving both central and peripheral mechanisms [12]. Moreover, Crt actively interacts with lipid membranes being able to form vacuoles and exhibiting myonecrotic activity [13,14].

Crt belongs to a family of small basic rattlesnake venom myotoxins that includes myotoxin a [15], peptide C [16], myotoxin I and II [17] and the CAM toxin [18]. They exhibit high primary sequence identity (Fig. 1) and, in addition, they are antigenically related [19]. However, when compared to the other members of the family, Crt exhibits a reduced toxicity (intraperitoneal injection LD\(_{50}\) = 820 \(\mu\)g per 25 g body weight) [12]. Interestingly, the three-dimensional (3D) structure of all these toxins is yet unknown.

As for Crt, the absence of direct binding studies using a radioactively labelled toxin, as well as the lack of structural information, has strongly hampered the possibility either to identify the receptor site on the Na\(^+\) channel or to suggest some precise hypothesis about the molecular mechanisms associated with the multiplicity of its biological functions.

In the attempt to answer these questions, we have been prompted to solve its 3D solution structure. The results reveal that Crt is characterized by an \(\alpha\beta_1\beta_2\beta_3\) structural topology, so far, never found in toxins active on ion channels. This observation and the high sequence identity with other myotoxins suggest that the 3D fold of Crt may represent a canonical structure of that protein family.

In addition, we show that both its fold and potential surface partially resemble the structural features of the antimammalian scorpion \(\alpha\)-neurotoxins and of the human antibacterial \(\beta\)-defensins with which Crt shares some biological properties.

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**Abbreviations:** AaHII, scorpion toxin from *Androctonus australis* Hector II; Crt, crotamine.

**Note:** The atomic coordinates of crotamine have been deposited in the RCSB Protein Data Bank, with accession code 1H5O.

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Materials and methods

Isolation of crotamine

Native crotamine was isolated and purified from the yellow venom of the snake *Crotalus durissus terrificus* as described elsewhere [12]. Identity and purity were confirmed by MALDI-TOF mass spectrometric analysis.

Circular dichroism

CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier system PTC-348 WFI for temperature control. The amplitude of the CD signal was calibrated using a 0.1% (w/v) solution of d-(+)-camphor-sulfonic acid (Aldrich). The spectra were collected at 20 ± 0.1 °C from 190 to 260 nm using a 1-mm path length cell. The concentration of the toxin ranged between 3 and 9 · 10⁻⁵ M. Data are the average of five separate recordings and ellipticity is reported as the mean residue molar ellipticity, (θ; deg-cm²-dmol⁻¹).

NMR spectroscopy

The two-dimensional proton NMR spectra were acquired at a protein concentration of approximately 1 mM in a mixture of 10 mM potassium phosphate buffer/trifluoroethanol-d₃ (70 : 30, v/v, pH ≈ 4) in order to discard any aggregation.

All spectra were recorded at 600 MHz on a Bruker DMX spectrometer equipped with a triple-resonance probe and pulsed field gradient unit. Spectra were obtained at various temperatures ranging from 5 °C to 35 °C.

The proton assignments were achieved by recording Double Quantum Filtered Correlation Spectroscopy (DQF-COSY) [20,21], Total Correlation Spectroscopy (clean-TOCSY) [22,23] (spin lock duration 18, 44, and 80 ms) and Nuclear Overhauser Effect Spectroscopy (NOESY) [24] (mixing time 100, 150 and 200 ms). Typical acquisition consisted of 512 t₁ increments (64 scans per increment) with 2048 complex points in t₂ over a spectral width of 9000 Hz. The water signal was suppressed using either the WATER-GATE sequence [25] or a low-power presaturation during the relaxation delay with the carrier frequency centred on the solvent signal. Quadrature detection was achieved using hypercomplex data acquisition [26]. The data were zerofilled and Fourier transformed to yield 4096 (F₂) × 2048 (F₁) real data point matrices.

All 2D spectra were processed on a Silicon Graphics O2 workstation using the program *FELIX-ND* data processing package (MSI, Molecular Simulations Inc., San Diego, CA).

Structure calculations

Distance constraints were obtained by measuring the intensity of the NOE cross peaks in the spectrum recorded with a mixing time of 100 ms at 35 °C. To relate the NOE data with interproton distances, a calibration was made using the program CLUSTALW [75].
using well defined geminal H_p-H_b connectivities. NOEs were classified as strong, medium, weak and very-weak, corresponding to interproton upper distance restraints of 3.0 Å, 4.0 Å, 5.0 Å and 6.0 Å, respectively. Upper distance restraints involving nonstereo-specifically assigned methylene, aromatic, and methyl protons were replaced by appropriate pseudoatoms [27]. The long- and medium-range restraints involving side-chains protons were further relaxed by an additional 0.5 Å to account for internal motions.

NH-C_H coupling constants were estimated in DQF-COSY spectrum from the measurements of extrema separations in dispersive and absorptive plots of rows through cross peaks [28]. In this experiment, the digital resolution after zero-filling along F2 was 0.56 Hz per point. A total of 24 ψ angle restraints were derived from the 3J_NH-Ci coupling constants using the Karplus relation [29], and were used for structure calculations with an allowed tolerance of ±30°.

Disulfide bonds between cysteine residues 4–36, 11–30, 18–37, identified on the basis of the known disulfide pairing pattern of the myotoxin a [15], were defined as covalent linkages and introduced after the first cycle of structure calculation. All peptide bonds were constrained to the trans position (Ω = 180°) with the exception of the one between Leu19 and Pro20 that was constrained to the cis position (Ω = 0°), as indicated by the presence of characteristic NOEs.

Structures were computed using the simulated annealing method [30] in the NMR-Refine module of the INSIGHTII package (MSI Molecular Simulations Inc., San Diego, CA, USA). The 26 best structures were further refined using a dynamic simulated annealing protocol. Final minimizations were performed with full Consistent Valence Force Field (CVFF), to a maximum derivative of 0.001 kcal Å⁻¹. Solvation effects were simulated by using a distance-dependent dielectric constant.

The Crt ensemble was inspected by the program INSIGHTII and analyzed by PROCHECK-NMR [31]. The program MOLMOL [32] was used to analyze the structures in terms of rmsd values, hydrogen bonds, regular secondary structures, solvent-accessible surface areas, angular order parameters, and electrostatic potential.

### Results and discussion

#### Circular dichroism

The far-UV CD spectrum of Crt at pH 4.0 shows, as for many toxins [33], two main bands at 197 nm (positive) and at 207 nm (negative) together with a less intense positive band at approximately 222 nm (Fig. 2). This last spectral feature, generally unusual for globular proteins, is shared by a number of other proteins, including toxins such as cobra toxin, erabutoxin b, myotoxins and α-neurotoxins [34,35].

The presence of a positive band at approximately 195 nm and of a negative one in the range 210–215 nm, usually indicates a dominant antiparallel β-sheet folding [33]. In this case, however, the observed blue shift to 207 nm of the negative band suggests the presence of some helical contribution.

![CD spectra of crotamine at 20 °C in 3 mM potassium phosphate buffer as a function of various trifluoroethanol concentrations (% v/v, pH ≈ 4).](image)

The band in the region 221–231 nm is consistent with a β_s transition arising from the Trp residues and/or with the L_a(0 → 1) transition associated with the Tyr residues [35]. Nonetheless, we cannot exclude the possibility that the band originates as a contribution from the disulfide bridges [36].

As for a large number of toxins [33], probably because of the constraints imposed by the disulfide bonds, we found that the secondary structure of Crt is independent on pH in the range 3.7–9.0 (data not shown), thus confirming previous results obtained by SAXS [37,38] and ORD [39].

The titration with trifluoroethanol performed at pH 4.0 (Fig. 2), shows that the CD spectrum remains almost unchanged up to 90% trifluoroethanol. Only at the highest cosolvent concentration did we observe a minor perturbation of the toxin secondary structure.

#### NMR data

A number of studies using polyacrilamide gel electrophoresis [40] and SAXS [37,38] have indicated that Crt in aqueous solution may dimerize or, more in general, aggregate. Indeed, the NMR proton spectra obtained at a protein concentration of approximately 1 mM in water were indicative of aggregation (data not shown). Recognizing that trifluoroethanol, besides stabilizing secondary structures is able to disrupt aggregates [41–43] and having verified by CD spectroscopy that moderately low concentrations of trifluoroethanol do not induce any significant alteration of the toxin secondary structure, the NMR experiments have been carried out in H_2O/trifluoroethanol (70:30, v/v) in order to shift the dimerization equilibrium towards the monomeric form [44]. Under these experimental conditions, we could observe only a few and very weak
additional peaks that did not interfere with spectra analysis, which differed from previous reports for both Crt [45] and myotoxin a [46].

The sequence-specific resonance assignment of Crt has been carried out according to the sequential assignment procedure [27]. The combined analysis of the fingerprint regions of the TOCSY and DQF-COSY spectra, recorded at 35 °C, revealed all the expected NH-CαH cross-peaks. The sequential connectivities were obtained from the NOESY spectrum recorded with a mixing time of 100 ms. Spectra recorded at different temperatures were also used to confirm assignments in cases of peak overlap or proximity to the water resonance.

A comparison of amide and α-protons chemical shifts of Crt with respect to the corresponding ones reported for myotoxin a in water [46] revealed a large identity (within ± 0.3 p.p.m.) for almost all the residues. This finding is not surprising if we keep in mind that the two molecules share 39 out of the 42 amino acids. Furthermore, this indicates that the amount of cosolvent used did not affect the protein structure. A major difference was observed only for Phe25 αH and Lys38 NH. As for residue 25, the reason could be that while myotoxin a carries a leucine, in Crt there is a phenylalanine that, in addition, could be also responsible for the difference in the Lys38 NH chemical shifts. In fact, in the structures of Crt, the side-chain of Phe 25 is close enough to the amide proton of Lys38 to induce a ring current effect.

Description of the toxin structure and potential surface

The characteristic NOE connectivities, reported in Fig. 3A, and the analysis of the φ and ψ angle distribution allowed the identification of the type and boundaries of the secondary structure elements. Crt consists of three antiparallel β-strands (involving residues 9–12, 24–25, 35–38), one α-helix (residues 3–7), and three β-turns (residues 13–16, 27–30 and 31–34). The first β-strand is connected to the second one by a long and solvent exposed loop, Pro13–Ser23. The loop comprises the first turn which, based on the φ/ψ angles, can be classified as a distorted type-I β-turn. The second and third β-strands are connected by a loop presenting two consecutive β-turns of which the first can be classified as a type-VIII and the second one as a type-I. The +2x, −1 topology [47] of the triple-stranded antiparallel β-sheet was

![Fig. 3. Summary of data used in the determination of the secondary structure of crotamine.](image)

(A) Primary sequence of Crt with the sequential and medium-range NOE pattern. For sequential connectivities, the thickness of the bars indicates the NOE intensities; medium-range NOEs are identified by lines connecting the two-coupled residues. (B) Schematic representation of Crt secondary structure showing the three antiparallel β-strands together with the intra- and interstrand NOE connectivities (double-arrow lines) defining the topology of the β sheet. Broken lines indicate the expected intrachain hydrogen bonds.
identified based on the presence of the characteristic intra- and interstrand NOEs (Fig. 3B). As a result, the toxin shows an αβ1β2β3 topology where both the first and the second strands run antiparallel to the third one. The β-sheet is twisted in a right-handed fashion and it is stabilized by four hydrogen bonds between strands β1 and β3, involving residues 10–37 and 12–35, and by two hydrogen bonds between strands β2 and β3, involving residues 25–36. The hydrogen bonds were identified in the final structures even though no constraints for those interactions had been introduced during the structure calculation.

As for the three disulfide bridges, while Cys4–Cys36 and Cys18–Cys37 connect the strand β3 with the α-helix and the first loop (Pro13–Ser23), respectively, Cys11–Cys30 connects β1 with the second loop (Gly26–Trp34), Fig. 3A.

Among the three prolines, Pro13 and Pro21 form peptide bonds in trans conformation, as inferred by the presence of strong d_{i,i+1} connectivities. In the case of Pro20, instead, a strong NOE between Leu19 and Pro20 H protons indicates that the peptide bond is in cis conformation, thus creating a kink in the polypeptide chain.

A final set of restraints consisting of 580 non-redundant interproton distances and 24 φ dihedral angles was derived from spectral analysis, Table 1. A plot showing the total number of NOE-derived interproton distance restraints for each residue is given in Fig. 4 (top).

A total of 50 structures was determined and the best 26 were selected (Fig. 5) as they satisfy the criteria of systematic residual distance violations not greater than 0.5 Å and dihedral violations not more than 5°. As already suggested by previous studies [37,38] the molecule has a flat shape and the molecular plane corresponds approximately to the triple-stranded β-sheet with the short α-helix packed against the sheet.

The superposition of the 26 selected structures over residues 2–39 (Fig. 5), showed a global rmsd, with respect to the mean structure, of 0.91 ± 0.15 Å for backbone atoms and 1.47 ± 0.15 Å for all heavy atoms. Further details of the statistics of the NMR models are listed in Table 1. Analysis of the Ramachandran plot, performed with the PROCHECK_NMR program [31], shows that 64% of the residues lie in the most favoured regions, 30% in the additional allowed regions and 6% in the generously

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Table 1. Structural statistics of the 26 selected solution structures of crotamine.

<table>
<thead>
<tr>
<th>Restraints statistics</th>
<th>Number</th>
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</thead>
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<td>i, i + 3</td>
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<tr>
<td>i, i + 4</td>
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</tr>
<tr>
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<td>129</td>
</tr>
<tr>
<td>φ dihedral angles</td>
<td>24</td>
</tr>
</tbody>
</table>

Mean rmsd from idealized covalent geometry

| bonds (Å)                          | 0.002  |
| angles (°)                         | 0.90   |

Ramachandran angle distribution

| %                                  |
| most favoured regions              | 63.6   |
| additional allowed regions         | 30.3   |
| generously allowed regions         | 6.1    |
| disallowed regions                 | 0      |

rmsd (Å) from the mean structure

| backbone (residues 2–39)           | 0.91 ± 0.15 |
| heavy atoms (residues 2–39)        | 1.47 ± 0.15  |
| backbone (residues 3–7, 9–12, 24–25, 35–38) | 0.44 ± 0.06 |
| heavy atoms (residues 3–7, 9–12, 24–25, 35–38) | 1.03 ± 0.10 |
| backbone (residues 9–12, 24–25, 35–38) | 0.39 ± 0.09  |
| heavy atoms (residues 9–12, 24–25, 35–38) | 0.99 ± 0.15  |
| backbone (residues 3–7)            | 0.19 ± 0.10  |
| heavy atoms (residues 3–7)         | 0.81 ± 0.18  |

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allowed regions. The low mean rmsd values from the idealized covalent bond (0.002 Å) and covalent angles (0.90°) indicate the absence of stereochemical distortions.

An assessment of the local variability of backbone and heavy atom positions is provided by the histogram of the rmsd (Fig. 4, bottom). As expected, the regions presenting elements of regular secondary structure are better defined. In fact, the backbone rmsd value restricted to residues 3–7, 9–12, 24–25 and 35–38, is 0.44 ± 0.06 Å with respect to the mean structure and the corresponding φ and ψ order parameters, S(φ) and S(ψ), are generally 1.

The disulfide bonds show a certain conformational disorder exhibiting two sets of conformations corresponding to γ3 = ± 90°. This disorder is consistent with the lack of specific Hβ2–Hβ3 NOEs across the bond.

The segment Ile17–Ser23 and the C-terminal region (residues Gly40–Gly42) exhibit a poor backbone definition (Fig. 5) due to the absence of assignable long-range NOEs.

Figure 6A,B shows a representation of the electrostatic potential associated with the solvent-accessible surface of Crt and evidences the presence of a large positive patch (blue) with only three negative small regions (red).

In addition, it is interesting to note that Lys35, out of the nine Lys residues, is not exposed to the solvent and its Hζ is within H-bonded salt-bridge distance to the Asp24 carboxyl oxygen.

Comparison with scorpion α-toxins and β-defensins

The NMR-derived models show that Crt exhibits an αβ motif, Fig. 7A, a structural feature observed not only in other toxins that affect ion channels, such as the α- and β-toxins from scorpion venom (Fig. 7B) but also in non-related proteins, such as β-defensins (Fig. 7C) and thionins [48–55].

As for the scorpion α- and β-toxins, their biological activity consists of the ability to modify the Na⁺ permeability by modulating the gating of the Na⁺ channel. In particular, while α-toxins slow or inhibit the sodium current inactivation [56,57], β-toxins shift the activation voltage to more negative potentials [58]. Studies on the α-toxin present in the venom of the scorpion Leirus quinquestratus suggested that the binding site for that toxin overlaps to a considerable extent to the one for Crt [7]. The hypothesis of a common binding site [7] has been further supported by the observation that Crt, similarly to scorpion α-toxins, enhances the depolarizing effect of the lipid soluble toxins veratrine, batrachotoxin and grayanotoxin [8]. Indeed, recent studies on the inactivation kinetics of the Na⁺ current [11] have evidenced that Crt and scorpion α-toxins act in a very similar fashion.

Based on these observations and looking for the presence of common structural determinants that could justify the observed functional similarities, we have compared Crt with the most representative member of the scorpion α-toxins family, the antimammalian AaHII [57,60] (PDB accession no. 1PTX).

Despite the low sequence homology and a distinct sequential arrangement of the secondary structure elements (αβββ for Crt vs. ββαβββ in the case of AaHII), the global fold of the two toxins appears quite similar, Fig. 7A,B. Crt could be considered a structurally simplified form of AaHII, with a truncated N-terminal portion of the α-helix, shorter β-strands and the lack of the relatively long C-terminal region, thus suggesting a possible common ancestral origin.

To compare the two toxins better, Crt and AaHII have been aligned with respect to their secondary structure elements, i.e. β2 of Crt with β1 of AaHII, the two α-helices with respect to each other, β1 and β3 of Crt with β2 and β3 of AaHII, respectively, Fig. 8. As a result, introducing some gaps in order to maximize similarities among residues, a certain degree of homology in the primary sequence becomes apparent.

In particular, it is worthwhile to point out that Tyr1 of Crt corresponds to Tyr21 in AaHII, located in the first turn of the helix and Cys4 of Crt, connecting the α-helix to the β3 strand, corresponds to Cys26 of AaHII that, similarly, connects the α-helix to the strand β3.

In Crt, Gly8 is located between the helix and strand β1 and has its correspondent in Gly31, belonging to the loop that connects the α-helix to strand β2 of AaHII. This can be
considered an example of structural simplification. In fact, while in Crt a single glycine residue forms this junction, in AaHII the connection is composed of three residues, Leu29–Gly31.

Analyzing the strand $\beta_1$ of Crt, we find that Gly9, His10, and Cys11 have their counterpart in residues Gly34, Tyr35 and Cys36 in the strand $\beta_2$ of AaHII. Noteworthy, the residues Gly9–Cys11 in Crt and Gly34–Cys36 in AaHII form a consensus sequence, GXC, that, in scorpion toxins as well as in $\beta$-defensins and thionins, appears to be related to a specific structural requirement [49]. In fact, it has been suggested that glycine must be conserved in the $\alpha$/$\beta$ motif due to steric hindrance between the helix and the sheet and it is essential for a correct folding [49,50].

The Phe12 residue, located at the end of strand $\beta_1$ of Crt, presents its counterpart in Trp38 similarly located at the end of strand $\beta_2$ of AaHII. Interestingly, chemical modifications of scorpion $\alpha$-toxins have demonstrated that the aromatic residues Trp38 and Tyr21 (corresponding to Tyr1 in Crt), may have an important role for their toxicity and efficiency in binding to Na$^+$ channels [61,62].

Asp24 and Phe25, located in strand $\beta_2$ of Crt, have their structural correspondent in Asp3 and Tyr5; the latter residue is highly conserved among scorpion $\alpha$-toxins [63] and it is arranged in a ‘herringbone’ fashion in the aromatic cluster present in the $\beta_1$ strand of AaHII [59,60].

The disulfide bridge Cys11–Cys30 of Crt, that anchors the strand $\beta_1$ to the loop Gly26–Trp34, has its correspondent in disulfide Cys36–Cys16 of AaHII that, similarly, connects the topologically equivalent $\beta_2$ strand to the loop Tyr5–Arg18. Overall, it is noteworthy that out of the six cysteine residues present in Crt, five match the ones in AaHII.

Besides all these similarities, the alignment also reveals that the residues responsible for the formation of the hydrophobic patch, common to the scorpion toxins, are not structurally conserved in Crt. However, in the vicinity of the Crt hydrophobic patch we find the salt-bridge Asp24–Lys35, similar to that observed in AaHII, ion-pair Glu32–Lys50, and in other scorpion $\alpha$-toxins.

Large regions of the Crt and AaHII surface are characterized by a positive electrostatic potential (Fig. 6A–D). Clearly, this feature is more pronounced for Crt that
possesses a net charge of +10 with respect to AaHII whose
total charge is +3. The residues in the positive region have
been indicated to be critical for AaHII toxicity [59,60] and it
has been proposed they are involved in defining the binding
specificity [64].

The correlation between positive electrostatic potential
and biological activity has been verified by the decrease in
toxicity produced both by chemical modification of the
basic residues Lys2, Lys28, Lys58 and Arg62 in AaHIII
[65] and by point mutation of the corresponding residues
in the anti-insecticidal Lqh4aIT [62,66–68]. Moreover,
mutagenesis of the receptor site 3 of recombinant rat
brain Na⁺ channels [69] supported such a correlation
indicating the existence of negative and neutral residues
that are essential for the high affinity binding of scorpion α-toxins.

Fig. 7. Cartoon representation of the backbone structure of (A) Crt, (B) AaHII (PDB accession no. 1PTX), (C) HBD-1 (human β-defensin-1, PDB accession no. 1IJU). Disulfide bonds are also shown (yellow stick lines). The figure was prepared using the program MOLMOL [32].

Fig. 8. Secondary structure of AaHII (A) and
Crt (B) together with an alignment of segments
of AaHII (A) and Crt (B) based on secondary
structure elements.
Recently, much attention has been given to the human basic antimicrobial polypeptides β-defensins [70,71]. Interestingly, they display a 3D fold similar to Crt (Fig. 7A,C) and comparable potential surface (Fig. 6A, B,E,F). The superposition of the Cα-trace atoms of the secondary structure regions of the three human β-defensins HBD1, HBD2 and HBD3, residues 3–8, 10–13, 23–24, 33–36, for HBD1 (PDB 1JJU), residues 6–11, 13–16, 26–27, 36–39, for HBD2 (PDB 1FD3) and residues 10–13, 17–19, 28–29, 39–42 for HBD3 (PDB 1KJ6), onto the corresponding ones of the Crt solution structure, yields average rmsd values of 0.9, 1.0 and 1.5 Å, respectively. Although β-defensins and Crt show a relatively low sequence identity (30%) (Fig. 1), the sequence alignment reveals the conservation of the elements of secondary structure as well as of residues responsible for the formation of the β-defensin fold, such as the 6 Cys residues, the Gly residues 8, 9, 26 and Pro20 [51].

It has been proposed that β-defensins exhibit their activity by disrupting the bacterial membranes as a result of polar interactions and oligomer formation [51,52,54]. This antibacterial activity as well as the ability to form dimers in solution appears to be directly proportional to their net positive surface charge [51,52,54].

Considering that, similarly to these proteins, Crt exhibits a highly positive potential surface (Fig. 6A,B,E,F) and a clear tendency to form aggregates [37,38,40], we can hypothesize that, as the β-defensins, it can interact electrostatically with the negative surface of the membranes inducing the formation of gaps through which ions and/or other molecules can move. Indeed, such a possibility might justify the observed Crt myonecrotic activity mediated by the formation of vacuoles [13,14].

In conclusion, Crt is an example where structural homologies with proteins deriving from different species provide the key to interpret its complex biological activity.

Indeed, the presence of the α/β scaffold and the existence of a surface characterized by a positive electrostatic potential seem to justify the functional similarity with the Na⁺ channel affecting scorpion α-toxins. However, significant structural differences such as the shorter size of the secondary structure elements might be responsible for the reduced toxicity of Crt when compared to other members belonging to the same family [12]. One example could be the reduced length of the α-helix that does not allow the formation of the cysteine-stabilized α-helix motif [72] common to most ion channel blocking polypeptides.

Similarly, the strong resemblance with the β-defensins fold [52–54], the conservation of some structural key residues as well as the arrangement of the disulfide bridges, might justify the common tendency to aggregate and interact with lipid membranes.

Note

A proposed structure of Crt, based on homology building and molecular dynamics simulations, has been published, recently [73]. The most significant difference between the theoretical 3D model and the NMR-derived structures is the lack of the N-terminal α-helix segment. A reason for that could be the choice of the bovine β-defensin, BNBD12 [74], as a template.

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