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Toxicon 46 (2005) 759–767

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Automated NMR structure determination and disulfide bond identification of the myotoxin crotamine from *Crotalus durissus terrificus*

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Received 12 April 2005; accepted 28 July 2005
Available online 26 September 2005

Abstract

Crotamine is one of four major components of the venom of the South American rattlesnake *Crotalus durissus terrificus*. Similar to its counterparts in the family of the myotoxins, it induces myonecrosis of skeletal muscle cells. This paper describes a new NMR structure determination of crotamine in aqueous solution at pH 5.8 and 20 °C, using standard homonuclear ^{1}H NMR spectroscopy at 900 MHz and the automated structure calculation software ATNOS/CANDID/DYANA. The automatic NOESY spectral analysis included the identification of a most likely combination of the six cysteines into three disulfide bonds, i.e. Cys4–Cys36, Cys11–Cys30 and Cys18–Cys37; thereby a generally applicable new computational protocol is introduced to determine unknown disulfide bond connectivities in globular proteins. A previous NMR structure determination was thus confirmed and the structure refined. Crotamine contains an α-helix with residues 1–7 and a two-stranded anti-parallel β-sheet with residues 9–13 and 34–38 as the only regular secondary structures. These are connected with each other and the remainder of the polypeptide chain by the three disulfide bonds, which also form part of a central hydrophobic core. A single conformation was observed, with Pro13 and Pro21 in the *trans* and Pro20 in the *cis*-form. The global fold and the cysteine-pairing pattern of crotamine are similar to the β-defensin fold, although the two proteins have low sequence homology, and display different biological activities.

Keywords: *Crotalus durissus terrificus*; NMR structure; Crotamine

1. Introduction

Crotamine is a polypeptide present in the venom of the South American rattlesnake *Crotalus durissus terrificus*. First isolated by Goncalves and Vieira (1950), crotamine contains a single polypeptide chain of 42 amino acid residues (Laure, 1975). There are six cysteines, which are all involved in disulfide-bonds (Kawano et al., 1982).

Abbreviations used: COSY, correlation spectroscopy; DLP, defensin-like peptide; E.COSY, exclusive COSY; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; PDB, protein data bank; RMSD, root mean square deviation; TOCSY, total correlation spectroscopy.

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doi:10.1016/j.toxicon.2005.07.018

The globular conformation of crotamine is very stable in solution (Hampe et al., 1978).

Crotamine is part of a family of small basic peptides present in rattlesnake venoms, which are non-enzymatic and have myonecrotic activity. These myotoxins show high amino acid sequence homology (Radis-Baptista et al., 1999). They also show similar action mechanisms during the envenomation (Fletcher et al., 1996), where they reduce the membrane potential and increase the influx of ions through the membrane, thus modifying conductance by a Na^+ channel-mediated mechanism and releasing Ca^{2+} from the heavy fraction of the sarcoplasmatic reticulum (Ownby, 1998).

The myotoxins has been extensively studied, including structural studies by SAXS (Beltran et al., 1990), laser-Raman spectroscopy (Kawano et al., 1982), and ^{1}H NMR (Endo et al., 1989; Nicastro et al., 2003). In these previous studies, the cysteine pairing pattern could not be unambiguously determined by SAX (Beltran et al., 1990) or by the NMR structure determination, which was therefore based on ad hoc disulfide connectivities (Nicastro et al., 2003). In this study, the NMR structure determination of crotamine was repeated, using the highest available field strength for improved NMR sensitivity and resolution. Furthermore, fully automatic interpretation of the 2D [^{1}H,^{1}H]-NOESY data was obtained with the software package ATNOS/CANDID/DYANA (Herrmann et al., 2002a,b; Güntert et al., 1997), which also yielded an automatic determination of the disulfide covalent bond connectivities as a result of the NOE-derived distance information network. The computational approach used here for identification of the unknown disulfide bond connectivities based entirely on [^{1}H,^{1}H]-NOESY distance constraints, will be generally applicable for studies of globular proteins with unknown cysteine pairings, including other polypeptide toxins. A previously determined NMR structure of crotamine (Nicastro et al., 2003) could thus be confirmed and refined.

2. Materials and methods

2.1. Purification of crotamine

Crotalus durissus terrificus venom was extracted from snakes maintained at the FMRP serpentarium of São Paulo University, and dried under vacuum. Six hundred milligrams of crude venom were dissolved in 5 ml of 0.25 M ammonium formate buffer at pH 3.5, and the bulk of crotoxin, the major venom component, was eliminated by low speed centrifugation as a heavy precipitate that formed upon slow addition of 20 ml of cold water to the solution. Dropwise addition of Tris-base solution was then used to raise the pH of the supernatant to 8.8, and the resulting solution was applied to a CM-Sepharose FF (1.5×4.5 cm; Amersham-Pharmacia) column equilibrated with 0.04 M Tris–HCl buffer at pH = 8.8 containing 0.06 M NaCl. After washing the column with 100 ml of equilibrating solution, crotamine was recovered as a narrow protein peak by raising the NaCl concentration of the eluting buffer to 0.6 M. The material was thoroughly dialyzed against water (benzoylated membrane; cut-off MW = 3000) and lyophilized. Amino acid analysis after acid hydrolysis (4 M $MeSO_3$-H + 0.1% tryptamine, 24 h at 115 °C) of a sample indicated a yield of 72 mg (14.7 μmol) of Laure's crotamine (Laure, 1975) and trace amounts of Thr, Ala and Val (purity > 98%).

2.2. NMR spectroscopy

Lyophilized protein was dissolved in 95% H_2O/5% D_2O containing 1 μM NaN_3 at pH 5.8, with a final crotamine concentration of 1.8 mM. The NMR measurements were performed at 20 °C on Bruker DRX600 and Avance 900 spectrometers.

The spectra collected for the backbone and side chain assignments were 2D 2QF-[^{1}H,^{1}H]-COSY (Rance et al., 1983), 2D [^{1}H,^{1}H]-TOCSY (Griesinger et al., 1996) with a mixing time τ_m = 60 ms, and 2D presat-[^{1}H,^{1}H]-NOESY (Wider et al., 1984) with a mixing time τ_m = 60 ms, which was also one of the two data sets used as input for the structure calculation. A 2D [^{1}H,^{1}H]-NOESY spectrum at 40 °C was used to resolve ambiguities caused by chemical shift overlap at 20 °C. In addition, a 2D [^{1}H,^{1}H]-NOESY spectrum in 100% D_2O was used to assign chemical shifts near the water line, and was also the second data set in the input for the structure calculation. 2D [^{1}H,^{13}C]-COSY and 2D [^{1}H,^{15}N]-COSY spectra at natural abundance were collected to verify the resonance assignments. Scalar coupling constants were derived from a 2D [^{1}H,^{1}H]-E.COSY spectrum (Griesinger et al., 1987).

2.3. 3D structure determination and identification of disulfide bonds

The sequence-specific ^{1}H NMR assignments and the ^{1}H chemical shift list for crotamine were generated interactively with the program XEASY (Bartels et al., 1995), following the standard procedure for structure determination using homonuclear NMR spectroscopy (Wüthrich, 1986). Scalar coupling constants were extracted from a 2D [^{1}H,^{1}H]-E.COSY spectrum, using the E.COSY tool of SPSCAN (Ralf Glaser, unpublished).

The structure calculation was performed with the software package ATNOS/CANDID/DYANA, using the amino acid sequence, the chemical shift list and the two aforementioned 2D [^{1}H,^{1}H]-NOESY spectra as input. The standard protocol with seven cycles of peak picking using ATNOS (Herrmann et al., 2002b), NOE assignment using CANDID (Herrmann et al., 2002a) and structure calculation using DYANA (Güntert et al., 1997) was applied. Thereby, in the second and subsequent cycles, the intermediate protein structures were used as an additional guide

for the interpretation of the NOESY spectra. During the first six ATNOS/CANDID cycles, ambiguous distance constraints (Nilges, 1997) were used, while for the final structure calculation in cycle seven, only distance constraints were retained that could be unambiguously assigned based on the protein 3D structure from cycle six. The scalar coupling constants were converted into dihedral angle constraints by the grid search algorithm FOUND (Güntert et al., 1998) and in each ATNOS/CANDID cycle combined with the updated database of NOE upper distance constraints in the input for the DYANA structure calculation.

To determine the disulfide connectivities of the six cysteines, a novel computational protocol consisting of three ATNOS/CANDID/DYANA structure calculations with different input data was applied. A first calculation was exclusively based on NOE-derived distance constraints and served as a reference. A second calculation additionally included disulfide connectivities in an analogous way to the treatment of ambiguous NOE distance constraints (Nilges, 1995, 1997). Three different ambiguous distance constraints to the other five Cys residues were generated for each cysteine, i.e. between its S^{γ} atom and the S^{γ} atoms of all other cysteines, with upper and lower limits of 2.10 and 2.00 Å, between its S^{γ} atom and the C^{β} atoms of all other cysteines, with upper and lower limits of 3.10 and 3.00 Å, and between its C^{β} atom and the S^{γ} atoms of all other cysteines, with limits of 3.10 and 3.00 Å. The $CysS^{\gamma}$–$CysS^{\gamma}$ and $CysC^{\beta}$–$CysS^{\gamma}$ distances obtained in this calculation were then compared with the standard values for disulfide bonds (Williamson et al., 1985), and likely disulfide connectivities were identified from close fits. The resulting connectivities were further evaluated from the convergence of a third ATNOS/CANDID/DYANA calculation, in which each of the thus identified disulfide bridges was constrained by a standard set of three upper and three lower distance constraints (Williamson et al., 1985). The 20 conformers with the lowest residual DYANA target function values, out of 100 starting conformers used in this final calculation, were energy-refined in a water shell with the program OPALp (Luginbühl et al., 1996; Koradi et al., 2000), using the AMBER force field (Cornell et al., 1995), and used to represent the solution NMR structure of crotamine. The program MOLMOL (Koradi et al., 1996) was used to analyze the protein structures and to prepare the figures showing molecular models.

3. Results

3.1. Resonance assignment

The sequential assignment of crotamine was based on homonuclear ^{1}H NMR spectroscopy (Wüthrich, 1986), using 2D 2QF-[^{1}H,^{1}H]-COSY (Rance et al., 1983), 2D [^{1}H,^{1}H]-TOCSY (Griesinger et al., 1996) and 2D [^{1}H,^{1}H]-NOESY (Wider et al., 1984) spectra. Except for Lys2 and Gln3, all expected backbone amide proton resonances were identified and assigned. Fig. 1 contains a survey of the data used for the backbone assignments. All the α-protons and 90% of the carbon-bound side chain protons were assigned. The missing assignments are Lys2 (H_{δ} and $H_{(\varepsilon)}$), His10 (H_{ε}), Lys14 (H_{δ}), Phe25 (H_{ζ}), Lys38 (H_{ε}) and Lys39 (H_{δ} and H_{ε}). The intraresidual H^N–H^{α} cross peak for Lys35 could not be resolved at 20 °C, but was clearly identifiable at 40 °C.

A 2D [^{1}H,^{1}H]-NOESY spectrum in D_2O (Fig. 2) showed strong cross peaks connecting the resonances Leu19H^{α} and Pro20H^{α}, indicating a *cis*-form for Pro20. Strong cross peaks connecting Pro20H^{α} and Pro21H^{δ} indicated the *trans*-form for Pro21. The *trans*-form for Pro13 was similarly evidenced by NOE cross peaks between Phe12H^{α} and Pro13H^{δ} (Fig. 1). Only one conformation was observed for each of the three prolyl residues.

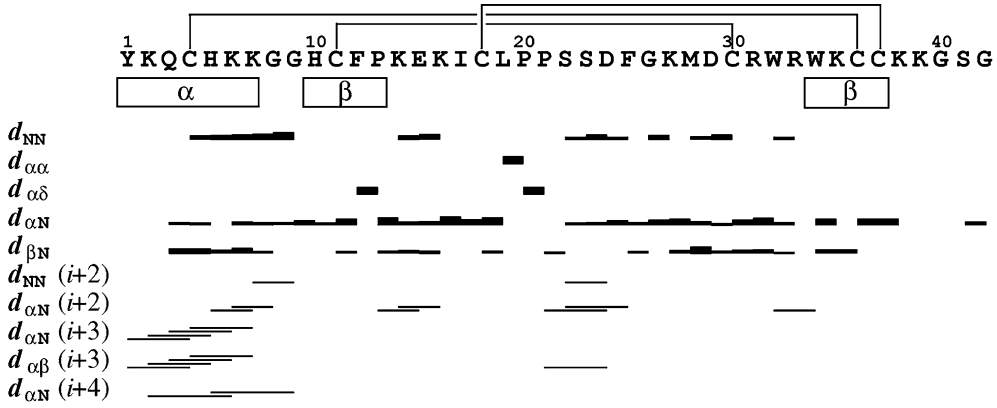

Fig. 1. Amino acid sequence of crotamine and survey of sequential and medium-range NOEs. For the sequential NOE connectivities, thick and thin bars indicate strong and weak NOE intensities. For the Xxx-Pro dipeptide segments, $d_{\alpha\alpha}$ and $d_{\alpha\delta}$ NOEs identify the *cis*- and *trans*-form, respectively. Medium-range NOEs are indicated by lines connecting the two residues that are related by the NOE. The disulfide bridges indicated at the top were determined by the present NMR study.

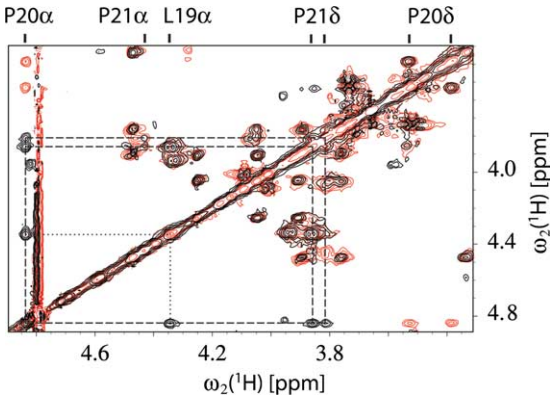

Fig. 2. Superposition of contour plots of a 600 MHz 2D [^{1}H,^{1}H]-TOCSY spectrum (red) and a 900 MHz 2D [^{1}H,^{1}H]-NOESY spectrum (black) of crotamine in ^2H_2O solution. Selected ^{1}H chemical shifts of the residues Leu19, Pro20 and Pro21 are indicated at the top. Dotted lines show the sequential $d_{\alpha\alpha}$ NOE connectivity between residues 19 and 20, which shows that Pro20 adopts the *cis*-conformation, and dashed lines show the sequential $d_{\alpha\delta}$ NOEs between residues 20 and 21, which document the *trans*-conformation of Pro21 (Wüthrich, 1986).

3.2. Structure calculation and identification of the disulfide bonds

The input for the structure calculation package ATNOS/CANDID/DYANA (Herrmann et al., 2002a,b; Güntert et al., 1997) consisted of the amino acid sequence, the chemical shift list, 22 scalar coupling constants collected from a 2D [^{1}H,^{1}H]-E.COSY spectrum, and two homonuclear 2D [^{1}H,^{1}H]-NOESY spectra. To determine the disulfide connectivities of the six cysteines, the results of two ATNOS/CANDID/DYANA calculations with different input data were compared (see Section 2). The calculation based solely on NOE-derived distance constraints (Fig. 3(a)), and a calculation where the updated NOE-derived distance constraints were in each cycle complemented with ambiguous distance constraints to enforce disulfide connectivities, yielded two closely similar NMR structures, with a backbone RMSD value of 0.92 Å between the mean coordinates of the two bundles of 20 conformers. The 3D structure obtained with ambiguous disulfide distance constraints showed near-standard $CysS^{\gamma}$–$CysS^{\gamma}$ and $CysC^{\beta}$–$CysS^{\gamma}$ distances (Williamson et al., 1985) for only one set of three disulfide connectivities, and this result allowed the unambiguous assignment of the cysteine pairing pattern Cys4/Cys36, Cys11/Cys30 and Cys18/Cys37.

In a final ATNOS/CANDID/DYANA structure calculation, the input included unambiguous distance constraints to enforce the three disulfide bonds thus identified. In ATNOS/CANDID/DYANA cycle seven, a total of 1930 NOESY cross peaks were assigned, which yielded 509 meaningful NOE upper distance limits. The low residual DYANA target function value of 0.84 ± 0.25 Å^2 and the average global RMSD value relative to the mean coordinates of 0.50 ± 0.08 Å calculated for the backbone atoms of residues 1–39 represent a high-quality NMR structure determination (Fig. 3(b); Table 1).

The two NMR structures calculated with and without unambiguous disulfide constraints are displayed in Fig. 3, and characteristic NMR parameters are listed in Table 1. For both calculations, a low residual DYANA target function value and a small RMSD value for the final bundle of 20 conformers were obtained. The numbers of meaningful upper distance constraints that resulted from the two calculations show only small differences, indicating that both calculations made similar use of the spectral information. The agreement between the results of the two calculations carries over into structures that are very similar in terms of precision and accuracy. Overall, the combination of similar values for the residual DYANA target function and the number of meaningful upper distance constraints with the RMSD value of 0.93 Å between the mean coordinates of the two bundles of 20 conformers clearly supports that the disulfide bonds were correctly assigned.

3.3. The NMR structure of crotamine

Overall, the new refined structure of crotamine confirms that the previously reported fold (Nicastro et al., 2003) is correct. An α-helix of residues 1–9 and a subsequent hairpin place the residues Tyr1, His5 and His10 into a linear alignment, as was previously pointed out by Endo et al. (1989). The core of the protein is formed by an antiparallel β-sheet constituted by the residues 9–13 and 34–38. A long, non-regular loop connects the two strands of the β-sheet. The disulfide bridges connect the β-sheet to the N-terminal α-helix (Cys4/Cys36) and to this loop (Cys11/Cys30 and Cys18/Cys37). All the nine lysines, with the sole exception of Lys35, and the two arginines are oriented toward the solvent, so that crotamine has extended positively charged molecular surface areas (Fig. 4).

4. Discussion

4.1. Comparison of crotamine with related small disulfide-rich proteins

Crotamine and myotoxin *a*, the most extensively studied members of the myotoxin family, show differences in only three sequence positions (Fig. 4(a)) and have similar biological activities. The present study on crotamine further implies that they have the same disulfide bond patterns, i.e. Cys4/Cys36, Cys11/Cys30 and Cys18/Cys37, which was determined for myotoxin *a* by biochemical methods (Fox et al., 1979).

Structural similarities are also readily apparent when comparing crotamine with the polypeptide DLP-2 extracted from the venom of *platypus* (Torres et al., 2000) (PDB code 1D6B), which has only 25% sequence identity. The most

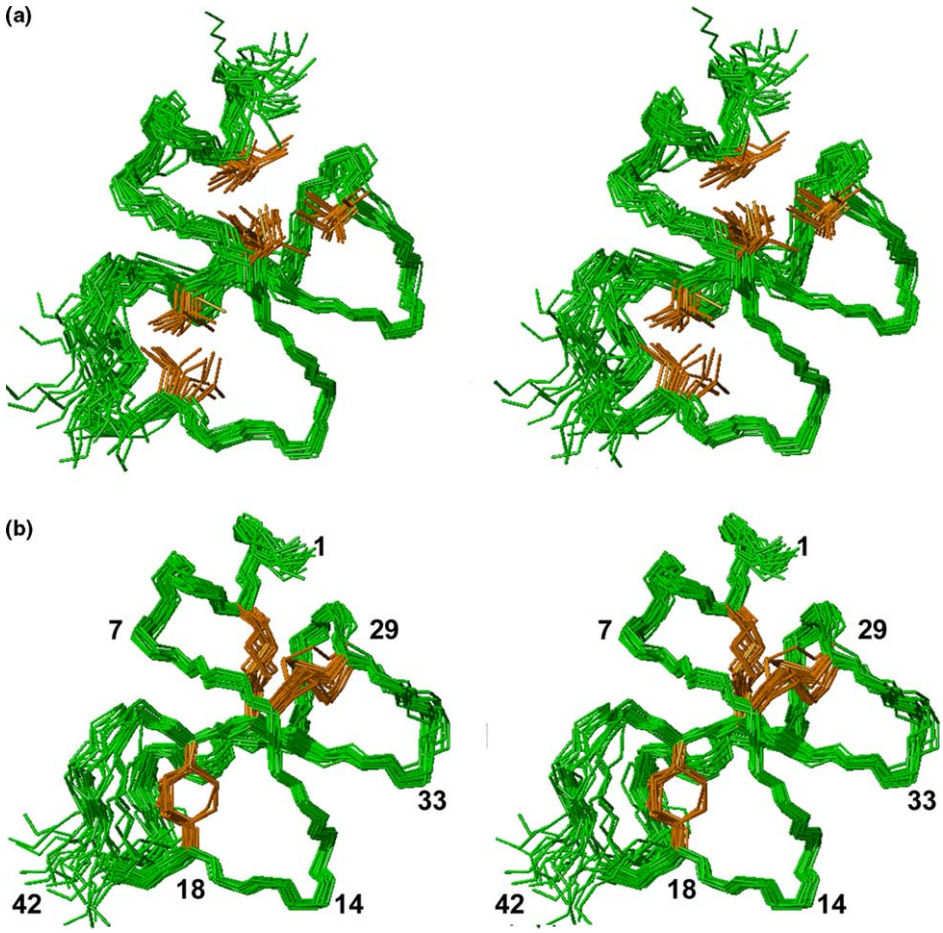

Fig. 3. Stereo views of the NMR structure of crotamine calculated with and without disulfide bond constraints (Table 1). (a) Bundle of 20 DYANA conformers calculated without disulfide bond constraints. The superposition is for best fit of the backbone atoms N, C^{α} and C' of the residues 1–39. The backbone is green and the Cys side chains are yellow. (b) Same as (a), except that the input for the structure calculation included disulfide bond constraints for the Cys pairs 4/36, 11/30 and 18/37 (see text). Selected residue positions are indicated with numbers.

significant differences can be seen near the N- and C-termini, since compared to crotamine, the DLP-2 polypeptide is elongated at the N-terminus and truncated near the C-terminus. The activity of DLP-2 has not yet been well defined, but it is known not to affect the Na^+ channel.

The overall fold of crotamine is the same as presented by the proteins of the β-defensin family (Zimmermann et al., 1995), despite low sequence homology (Fig. 4(a)) and little biological similarity between crotamine and the other members of this family. Defensins are cationic peptides, which are rich in lysine and arginine residues (Dimarcq et al., 1998; Ganz et al., 1985). Six invariant cysteine residues that are conserved throughout the defensin family form three intramolecular disulfide bonds, which provides stability to the defensin tertiary structure. Mammalian α- and β-defensins differ in the pairing of the six cysteine residues, which is 1/6, 2/4 and 3/5 in α-defensins, and 1/5, 2/4 and 3/6 in β-defensins (the numeration from 1 to 6 is in the order of the appearance of the Cys residues in the sequence, see Fig. 4(a)) (Ganz and Lehrer, 1998; Tang and Selsted, 1993). Defensins generally display antimicrobial properties and thus constitute a part of the host defense mechanism of multicellular organisms (Ganz et al., 1985). The high net positive charge of these proteins facilitates the electrostatic interaction with negatively charged bacterial cell surfaces, and can thus lead to permeabilization of bacterial membranes. Although many defensins display common or very similar global folds (Ganz and Lehrer, 1998), they may individually have greatly different physiological activities. Overall, inspection of Fig. 4(c) indicates that the wide variation of the surface charge distribution in the presently considered, similarly folded toxins might be an important factor for their biological functions and specificities.

Finally, we should refer here to the previous claim that crotamine displays analgesic activity (Mancin et al., 1998), since this effect was recently shown to be due to the presence of a crotoxine fragment, which is another major

Table 1
Input for the NMR structure determination of crotamine with and without disulfide bond constraints, and statistics of the bundle of 20 energy-minimized conformers used to represent the solution structure

	-With disulfide bond constraints	-Without disulfide bond constraints
NOE upper distance limits[a]	509	497
Scalar coupling constants	22	22
Dihedral angle constraints	146	147
Residual DYANA target function value (Å^2)	0.84±0.25	1.56±0.48
Residual distance constraint violations		
Number >0.1 Å	6±2	5±2
Maximum (Å)	0.12±0.01	0.12±0.01
Residual dihedral angle constraint violations		
Number >2.5°	1±1	1±1
Maximum (°)	3.07±1.22	2.91±1.07
AMBER energies (kcal/mol)		
Total	−676.68±64.69	−665.84±69.09
van der Waals	−84.25±6.03	−81.22±8.06
Electrostatic	−996.24±58.34	−984.72±68.43
RMSD from ideal geometry		
Bond lengths (Å)	0.0082±0.0003	0.0082±0.0002
Bond angles (°)	2.20±0.09	2.19±0.06
RMSD to the mean coordinates (Å)[b]		
Backbone atoms N, C^{α}, C′ (1–39)	0.50±0.08	0.68±0.09
All heavy atoms (1–39)	1.00±0.08	1.17±0.15
RMSD between mean coordinates (Å)[c]	0.93	
Ramachandran plot statistics[d]		
Most favored regions (%)	61	66
Additional allowed regions (%)	26	25
Generously allowed regions (%)	9	6
Disallowed regions (%)	4	3

[a] Except for the top four entries, the data characterize the group of 20 energy-minimized conformers that is used to represent the NMR structure; the mean value and the standard deviation are given.
[b] The numbers in parentheses indicate the residues for which the RMSD was calculated.
[c] The RMSD between the mean coordinates of the two bundles of 20 conformers obtained with and without disulfide bond constraints was calculated for the backbone atoms N, C^{α}, C′ of residues 1–39.
[d] As determined by PROCHECK (Morris et al., 1992).

component of the *Crotalus durissus terrificus*' venom that copurifies with the crotamine (Itagaki, personal comm.).

4.2. Automated identification of disulfide connectivity

The software package ATNOS/CANDID/DYANA offers an objective avenue to determine otherwise unknown cysteine pairing patterns by simultaneously performing NOESY spectral analysis and determination of disulfide connectivity. In order to include unknown disulfide connectivities in the calculation, ambiguous distance constraints between the S^{γ} atom of a specified Cys residue and the S^{γ} atoms of the other cysteines, and between a specified CysC^{β} atom and the S^{γ} atoms of all other cysteines are generated (see Section 2). Depending on the information content of the available NOE data, such ambiguous distance constraints could in principle be satisfied by more than one set of cysteine pairings, or even by clustering of three or more cysteines. In the present study, no such ambiguities remained, and for the identified three disulfide bonds quite exact S^{γ}–S^{γ} and S^{γ}–C^{β} distances were found in all 20 conformers used to describe the NMR structure (Wüthrich, 1986). This observation indicates that as long as a sufficiently dense NOE network can be experimentally established, as was done here by exhaustive NOE identification and network-anchoring using the ATNOS/CANDID modules, there is a good chance that ambiguous disulfide distance constraints converge into a single cysteine-paired solution structure of the protein.

4.3. Data bank accession numbers

Chemical shift assignments and coupling constants have been deposited with BioMagResBank[1] (accession number 6576). The atomic coordinates of the NMR structure has been deposited with the Protein Data Bank[2] (PDB ID code 1Z99).

[1] www.bmrb.wisc.edu.
[2] www.rcsb.org.

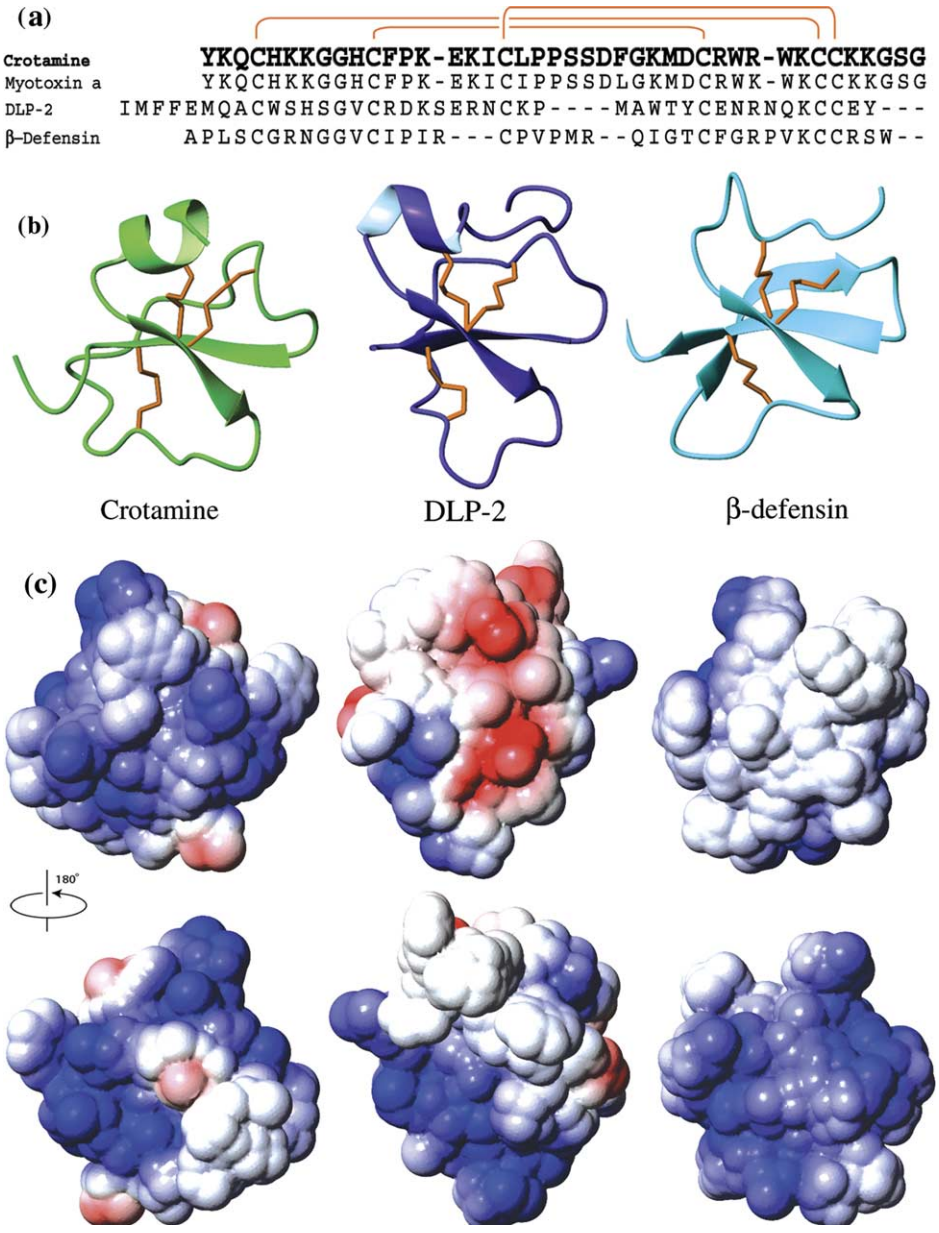

Fig. 4. (a) Amino acid sequence alignment for crotamine, myotoxin *a,* DLP-2 (Torres et al., 2000) and β-defensin (Zimmermann et al., 1995), using CLUSTALW (Thompson et al., 1994). At the top, the disulfide bonds in crotamine are drawn in yellow. (b) Ribbon presentations of crotamine, DLP-2 and β-defensin, with the disulfide bonds in yellow. The orientation of the DLP-2 and β-defensin structures was determined by superposition for best fit with the cysteine heavy atoms in crotamine. No 3D structure for myotoxin *a* is available. (c) Protein surface views in the same orientation as in (b) (upper trace), and after rotation by 180° about a vertical axis (lower trace). Color code: negative charges red, positive charges blue, uncharged residues white.

Acknowledgements

We thank L.H.A. Pedrosa and C.J. Laure for a generous supply of *Crotalus durissus terrificus* venom, and F. Fiorito for help in performing the structure calculations. Financial support was obtained from Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES-PDEE (process # 1243-012), FAPESP (SMOLBNet, 01/07532-0), CNPq (300851-98-7), and the Schweizerischer Nationalfonds (projects

31.49047.96 and 3100A0-105570/1). The use of the computational facilities of the Center of Competence in Computational Chemistry of the ETH Zürich is gratefully acknowledged.

References

Bartels, C., Xia, T.H., Billeter, M., Güntert, P., Wüthrich, K., 1995. The program XEASY for computer-supported NMR-spectral analysis of biological macromolecules. J. Biomol. NMR 6, 1–10.

Beltran, J.R., Mascarenhas, Y.P., Craievich, A.F., Laure, C.J., 1990. SAXS study of the snake toxin alpha-crotamine. Eur. Biophys. J. 17, 325–329.

Cornell, W.D., Cieplak, P., Bayly, C.I., Gould, I.R., Merz, K.M., Ferguson, D.M., Spellmeyer, D.C., Fox, T., Caldwell, J.W., Kollman, P.A., 1995. A 2nd generation force-field for the simulation of proteins, nucleic-acids, and organic molecules. J. Am. Chem. Soc. 117, 5179–5197.

Dimarcq, J.L., Bulet, P., Hetru, C., Hoffmann, J., 1998. Cysteine-rich antimicrobial peptides in vertebrates. Biopolymer 47, 465–477.

Endo, T., Oya, M., Ozawa, H., Kawano, Y., Giglio, J.R., Miyazawa, T., 1989. A proton nuclear magnetic resonance study on the solution structure of crotamine. J. Protein Chem. 8, 807–815.

Fletcher, J.E., Hubert, M., Wieland, S.J., Gong, Q.H., Jiang, M.S., 1996. Similarities and differences in mechanisms of cardiotoxins, melittin and other myotoxins. Toxicon 34, 1301–1311.

Fox, J.W., Elzinga, M., Tu, A.T., 1979. Amino acid sequence and disulfide bond assignment of myotoxin a isolated from the venom of Prairie rattlesnake (*Crotalus viridis viridis*). Biochem. 18, 678–684.

Ganz, T., Selsted, M.E., Szklarek, D., Lehrer, R.I., 1985. Antimicrobial Properties of Human Defensins - Major Peptides of Human Neutrophils. Fed. Proc. 44, 907.

Ganz, T., Lehrer, R.I., 1998. Antimicrobial peptides of vertebrates. Curr. Opin. Immunol. 10, 41–44.

Goncalves, J.M., Vieira, L.G., 1950. Estudos sobre venenos de serpentes brasileiras I, Análise eletroforética. An. Acad. Bras. Cienc. 22, 141–150.

Griesinger, C., Sørensen, O.W., Ernst, R.R., 1987. Practical aspects of the E.COSY technique - measurement of scalar spin coupling-constants in peptides. J. Magn. Reson. 75, 474–492.

Griesinger, C., Otting, G., Wüthrich, K., Ernst, R.R., 1988. Clean-TOCSY for ^{1}H spin system identification in macromolecules. J. Am. Chem. Soc. 110, 7870–7872.

Güntert, P., Mumenthaler, C., Wüthrich, K., 1997. Torsion angle dynamics for NMR structure calculation with the new program DYANA. J. Mol. Biol. 273, 283–298.

Güntert, P., Billeter, M., Ohlenschläger, O., Brown, L.R., Wüthrich, K., 1998. Conformational analysis of protein and nucleic acid fragments with the new grid search algorithm FOUND. J. Biomol. NMR 12, 543–548.

Hampe, O.G., Vozari-Hampe, M.M., Goncalves, J.M., 1978. Crotamine conformation: effect of pH and temperature. Toxicon 16, 453–460.

Herrmann, T., Güntert, P., Wüthrich, K., 2002a. Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA. J. Mol. Biol. 319, 209–227.

Herrmann, T., Güntert, P., Wüthrich, K., 2002b. Protein NMR structure determination with automated NOE-identification in the NOESY spectra using the new software ATNOS. J. Biomol. NMR 24, 171–189.

Kawano, Y., Laure, C.J., Giglio, J.R., 1982. Laser Raman study on crotamine. Biochim. Biophys. Acta 705, 20–25.

Koradi, R., Billeter, M., Wüthrich, K., 1996. MOLMOL: a program for display and analysis of macromolecular structures. J. Mol. Graph. 14, 51–55.

Koradi, R., Billeter, M., Güntert, P., 2000. Point-centered domain decomposition for parallel molecular dynamics simulation. Comp. Phys. Commun. 124, 139–147.

Laure, C.J., 1975. Die Primärstruktur des Crotamins. Hoppe-Seyler's Z. Physiol. Chem. 356, 213–215.

Luginbühl, P., Güntert, P., Billeter, M., Wüthrich, K., 1996. The new program OPAL for molecular dynamics simulations and energy refinements of biological macromolecules. J. Biomol. NMR 8, 136–146.

Mancin, A.C., Soares, A.M., Andriao-Escarso, S.H., Faca, V.M., Greene, L.J., Zuccolotto, S., Pela, I.R., Giglio, J.R., 1998. The analgesic activity of crotamine, a neurotoxin from Crotalus durissus terrificus (South American rattlesnake) venom: A biochemical and pharmacological study. Toxicon 36, 1927–1937.

Morris, A.L., Macarthur, M.W., Hutchinson, E.G., Thornton, J.M., 1992. Stereochemical Quality of Protein-Structure Coordinates. Proteins-Struct. Funct. Genet. 12, 345–364.

Nicastro, G., Franzoni, L., de Chiara, C., Mancin, A.C., Giglio, J.R., Spisni, A., 2003. Solution structure of crotamine, a Na^{+} channel affecting toxin from *Crotalus durissus terrificus* venom. Eur. J. Biochem. 270, 1969–1979.

Nilges, M., 1995. Calculation of protein structures with ambiguous distance restraints - automated assignment of ambiguous NOE crosspeaks and disulfide connectivities. J. Mol. Biol. 245, 645–660.

Nilges, M., 1997. Ambiguous distance data in the calculation of NMR structures. Folding Design 2, 53–57.

Ownby, C.L., 1998. Structure, functions and biophysical aspects of the myotoxins from snake venoms. J. Toxicol. Toxin. Rev. 17, 213–238.

Radis-Baptista, G., Oguiura, N., Hayashi, M.A., Camargo, M.E., Grego, K.F., Oliveira, E.B., Yamane, T., 1999. Nucleotide sequence of crotamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*). Toxicon 37, 973–984.

Rance, M., Sørensen, O.W., Bodenhausen, G., Wagner, G., Ernst, R.R., Wüthrich, K., 1983. Improved spectral resolution in COSY ^{1}H NMR spectra of proteins via double quantum filtering. Biochem. Biophys. Res. Commun. 117, 479–485.

Tang, Y.Q., Selsted, M.E., 1993. Characterization of the Disulfide Motif in Bnbd-12, an Antimicrobial Beta-Defensin Peptide from Bovine Neutrophils. J. Biol. Chem. 268, 6649–6653.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence

alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Torres, A.M., de Plater, G.M., Doverskog, M., Birinyi-Strachan, L.C., Nicholson, G.M., Gallagher, C.H., Kuchel, P.W., 2000. Defensin-like peptide-2 from platypus venom: member of a class of peptides with a distinct structural fold. Biochem. J. 348, 649–656.

Wider, G., Macura, S., Kumar, A., Ernst, R.R., Wüthrich, K., 1984. Homonuclear two-dimensional ^{1}H NMR of proteins, Experimental procedures. J. Magn. Reson. 56, 207–234.

Williamson, M.P., Havel, T.F., Wüthrich, K., 1985. Solution conformation of the proteinase Inhibitor-IIa from bull seminal plasma by H-1 nuclear magnetic resonance and distance geometry. J. Mol. Biol. 182, 295–315.

Wüthrich, K., 1986. NMR of Proteins and Nucleic Acids. Wiley, New York.

Zimmermann, G.R., Legault, P., Selsted, M.E., Pardi, A., 1995. Solution structure of bovine neutrophil Beta-Defensin-12 - the peptide fold of the Beta-Defensins is identical to that of the classical defensins. Biochem. 34, 13663–13671.