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

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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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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