crystallization papers

Crystallization and preliminary X-ray analysis of candoxin, a novel reversible neurotoxin from the Malayan krait *Bungarus candidus*

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Candoxin, a novel three-finger toxin from *Bungarus candidus*, is a reversible antagonist of muscle ($\alpha\beta\gamma\delta$) but a poorly reversible antagonist of neuronal $\alpha7$ nicotinic acetylcholine receptors. It has a molecular weight of 7344 Da, with 66 amino-acid residues including ten half-cystines. The fifth disulfide bridge is located at the tip of loop I (Cys6–Cys11) instead of in loop II as found in other α-neurotoxins. Interestingly, candoxin lacks the segment cyclized by the fifth disulfide bridge at the tip of the middle loop of long-chain neurotoxins, which was reported to be critical for binding to $\alpha7$ receptors. As a first step to determining its three-dimensional structure, candoxin was crystallized by the hanging-drop vapour-diffusion technique in conditions around 1.5 M sodium chloride, 10%(v/v) ethanol. The crystals formed belonged to the hexagonal system, space group $P6_222$, with unit-cell parameters $a = 54.88$, $b = 54.88$, $c = 75.54$ Å, $\alpha = \beta = 90$, $\gamma = 120°$, and diffract to a resolution of 1.80 Å. The crystallographic asymmetric unit contains one molecule of candoxin, with an estimated solvent content of 44.6%. Attempts to solve these structures by molecular-replacement methods have not been successful and a heavy-atom derivative search has been initiated.

1. Introduction

The venom from a single species of snake contains about 100 different proteins and peptides, all of which appear to fall into only a small number of superfamilies represented by a common fold or scaffold. One such family of venom proteins is the three-finger toxin family (MW range 6000–8000 Da), wherein all members adopt a 'three-fingered' fold: three adjacent loops (forming a β-sheet) that emerge from a globular core confined by four conserved disulfide bridges (Endo & Tamiya, 1991; Menez, 1998; Tsetlin, 1999; Kini, 2002). These toxins are rather flat and leaf-shaped, with two faces defined by the plane formed by the β-sheet of the loops, which often adopts a slightly concave shape. The similar overall fold notwithstanding, three-finger toxins exhibit a diverse range of pharmacological activities. The family includes the potent curaremimetic neurotoxins which confer lethality to Elapidae and Hydrophidae venoms. Other members include muscarinic toxins with selectivity towards distinct types of muscarinic receptors (Jerusalinsky & Harvey, 1994), fasciculins that inhibit acetylcholinesterase (Cervenansky *et al.*, 1991), calciseptins that block L-type calcium channels (De Weille *et al.*, 1991; Albrand *et al.*, 1995), cardiotoxins (cytotoxins) that exert their toxicity by forming pores in cell membranes (Bilwes *et al.*, 1994) and dendroaspins, which are antagonists of various

cell-adhesion processes (McDowell *et al.*, 1992). Hence, the 'three-finger' scaffold is used by the snake to 'hang' different combinations of functional groups on, generating an array of target specificities, an example of

> the prodigality of ... toxic functions remain(ing) clearly associated with a structural economy

(Menez, 1998) seen with most snake-venom proteins. Within the three-finger toxin family itself, there are some structural variations. Certain classes of neurotoxins, such as the long-chain α-neurotoxins (α-bungarotoxin from *Bungarus multicinctus*) and the neuronal κ-bungarotoxin (*B. multicinctus*) also have an additional (fifth) disulfide bridge that is located in the middle loop (loop II; Endo & Tamiya, 1991; Menez, 1998; Tsetlin, 1999). This fifth bridge is located in loop I in a group of poorly characterized toxins called 'weak toxins' (Utkin *et al.*, 2001), which are typically of a lower order of toxicity (50–1000 times less potent) compared with prototypical α-neurotoxins (Mebs & Claus, 1991). We have recently purified and sequenced two three-finger toxins, bucandin and candoxin, from Malayan krait (*B. candidus*) venom, which are structurally related to weak toxins. The three-dimensional structure of bucandin, a novel toxin with a yet unknown molecular target, has been determined (Kuhn *et al.*, 2000; Torres *et al.*, 2001).

Recently, Nirthanan *et al.* (2002) reported candoxin to be a novel reversible antagonist of muscle ($\alpha\beta\delta$) nicotinic acetylcholine receptors. Interestingly, it is also a potent antagonist of neuronal $\alpha7$ nicotinic acetylcholine receptors, but in this case is poorly reversible. Interestingly, candoxin lacks the segment cyclized by the fifth disulfide bridge at the tip of the middle loop of long-chain neurotoxins which has been reported to be critical for binding to $\alpha7$ receptors (Servent *et al.*, 1997; Antil-Delbeke *et al.*, 2000). It is therefore a novel toxin that shares a common scaffold with long-chain α-neurotoxins, but possibly utilizes additional functional determinants that assist in the recognition of neuronal $\alpha7$ receptors (Nirthanan *et al.*, 2002). Determination of its crystal structure will enable the elucidation of these determinants and will enhance its utility as a tool for studies of neuronal acetylcholine receptors in the central nervous system. As a first step to determining its structure, as presented in this paper, we crystallized candoxin and recorded its preliminary diffraction data.

2. Materials and methods

2.1. Materials

Lyophilized *B. candidus* venom was obtained from Venom Supplies (Tanunda, SA, Australia). Prepacked chromatography columns were purchased from Amersham Biosciences (Buckinghamshire, UK) and Phenomenex (Torrance, CA, USA). Acetonitrile (ACN) was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and trifluoroacetic acid (TFA) from Fluka Chemika-Biochemika (Buchs, Switzerland); all other chemicals were purchased from Sigma (St Louis, MO, USA).

2.2. Purification of candoxin

Candoxin was purified from *B. candidus* venom as described previously (Nirthanan *et al.*, 2002). Crude venom was fractionated by gel filtration on a Superdex 30 column (1.6 × 60 cm) in 50 m*M* Tris–HCl buffer pH 7.5. The fraction containing candoxin was loaded onto a reverse-phase Jupiter C18 (0.21 × 25 cm) column equilibrated with 0.1% TFA; proteins were eluted with a linear gradient of 80% ACN in 0.1% TFA (buffer *B*) (20–45% *B* over 80 min). The peak containing candoxin was re-chromatographed on the same column using a shallower gradient (28–36% *B* over 50 min). The purity of candoxin was assessed by electrospray ionization mass spectrometry (ESI–MS), matrix-assisted laser desorption ionization–time of flight

Table 1
X-ray diffraction data-collection statistics of the candoxin crystal.

Values for parameters in the highest resolution shell, 1.86–1.80 Å, are given in parentheses.

Space group	$P6_222$
Unit-cell parameters	
a (Å)	54.88
b (Å)	54.88
c (Å)	75.54
$\alpha = \beta$ (°)	90
γ (°)	120
Resolution of data set (Å)	47.673–1.800
No. of observed reflections	134406
No. of unique reflections	6707 (644)
Redundancy	20.0
R_{merge}† (%)	6.2 (50.9)
Completeness (%)	99.9 (99.8)
Matthews coefficient‡ (Å^3 Da^{-1})	2.2
Solvent content (%)	44.6
No. of molecules per asymmetric unit	1
$I/\sigma(I)$	12.5 (6.7)

† $R_{merge} = \sum[(I - \langle I \rangle)^2]/\sum(I^2)$. ‡ Matthews (1968).

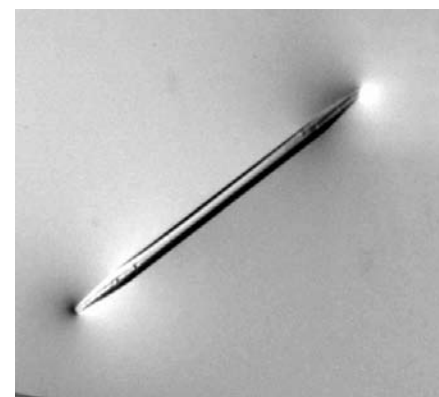mass spectrometry (MALDI–TOF MS) and capillary electrophoresis.

2.3. Crystallization and X-ray analysis

A lyophilized sample of candoxin was dissolved in Milli-Q water to a concentration of 10 mg ml^{-1}. Crystallization was performed by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates. Initial trials were carried out at room temperature with Hampton Research Crystal Screens I and II. Typically, 2 µl drops of protein solution were mixed with an equal volume of the screening solution and equilibrated over 500 µl of the latter as reservoir solution. Small crystals formed in condition No. 8 of Crystal Screen II [1.5 *M* NaCl, 10%(*v/v*) ethanol]. The conditions were subjected to two rounds of optimization after which one condition (among several; 1.75 *M* NaCl, 15% ethanol) gave single crystals at room temperature after about a week, which continued to grow to maximum dimensions of about 0.1 × 0.1 × 0.5 mm over the next 2–4 weeks. One such crystal (0.1 × 0.1 × 0.4 mm) was transferred to mother-liquor solution (1.75 *M* NaCl, 15% ethanol) containing 25% glycerol and was flash-frozen in liquid nitrogen. An X-ray diffraction data set was collected at the crystallographic beamline X12B at the National Synchrotron Light Source, Brookhaven National Laboratory (NSLS, Upton, New York). The beam-

line was set to a wavelength of 0.96110 Å. Using the oscillation method, 180 images of diffraction data were collected from the native crystal. Individual frames consisted of an oscillation of 1° with the detector distance set to 150 mm. The crystal diffracted X-rays to a resolution of 1.80 Å; even after 6 h of exposure, there was no significant radiation damage. Diffraction intensities were measured using an ADSC Quantum 4 Detector (a 2 × 2 array CCD detector) and were processed and scaled using the *HKL*2000 suite of programs (Otwinowski & Minor, 1997). Data-processing statistics are presented in Table 1.

3. Results

Candoxin was purified by gel filtration and two consecutive reverse-phase HPLC steps (Nirthanan *et al.*, 2002). It was found to be free of detectable contaminants by ESI–MS,

Figure 1
Photograph of a crystal of candoxin used for the diffraction experiment.
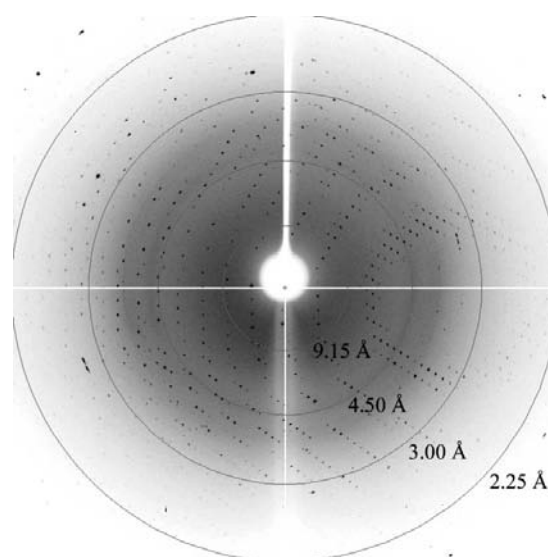
Figure 2
A diffraction image of the candoxin crystal.

MALDI–TOF MS and capillary electrophoresis (Nirthanan *et al.*, 2002). A crystal of candoxin ($0.1 \times 0.1 \times 0.4$ mm; Fig. 1) belonging to space group $P6_222$, with unit-cell parameters $a = 54.88$, $b = 54.88$, $c = 75.54$ Å, $\alpha = \beta = 90$, $\gamma = 120°$, diffracted to a resolution of 1.80 Å (Fig. 2). The completeness was high (99.8%) even in the highest resolution shell (1.86–1.80 Å). There is only one molecule per asymmetric unit.

Attempts were made to solve the structure of candoxin by molecular replacement using several homology models and the native data in different resolution ranges. The programs *AMoRe* (Navaza, 1994), *MOLREP* (Vagin & Teplyakov, 1997), *CNS* (Brünger *et al.*, 1998), *EPMR* v.2.5 (Kissinger *et al.*, 2001) and *Queen of Spades* (Glykos & Kokkinidis, 2000) were used, but no solution could be found. We are currently preparing heavy-atom derivatives of candoxin in an attempt to solve its structure by single or multiple anomalous dispersion techniques.

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