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Isolation, purification, crystallization and preliminary X-ray analysis of β_1-bungarotoxin from *Bungarus caeruleus* (Indian common krait)

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β-Bungarotoxin is a heterodimeric neurotoxin consisting of a phospholipase A_2 subunit linked by a disulfide bond to a K^+ channel binding subunit, which is a member of of the Kunitz protease-inhibitor subfamily. Purified β_1-bungarotoxin was crystallized using microdialysis techniques. The rectangular-shaped crystals are orthorhombic, space group $C222_1$, with unit-cell dimensions a = 80.7, b = 82.5, c = 56.9 Å. The crystals have a calculated V_m of 2.35 Å^3 Da^{-1} for one molecule in the asymmetric unit. This corresponds to a solvent content of 48%. X-ray intensity data were collected to 2.6 Å resolution. The data set is 97.4% complete.

1. Introduction

Phospholipases found in some snake venoms are potent neurotoxins which target their enzymatic action to the synaptic membrane. One of these is the heterodimeric neurotoxin β-bungarotoxin, which acts by inhibiting the release of acetylcholine from motor nerve endings, one of the most investigated steps in neuromuscular transmission (Lee *et al.*, 1972; Kelly & Brown, 1974; Abe *et al.*, 1977). Structurally, β-bungarotoxin is a heterodimeric neurotoxin consisting of a phospholipase A_2 (PLA_2) subunit linked by a disulfide bond to a K^+ channel binding subunit, which is a member of the Kunitz protease-inhibitor superfamily (MacDermot *et al.*, 1978). It acts presynaptically by binding *via* the protein-inhibitor-like subunit to a presynaptic potassium channel and then blocking neurotransmission with the second subunit, which has phospholipase A_2 activity, thus altering acetylcholine release in both the peripheral and central nervous systems (Ueno & Rosenberg, 1996).

Although the presynaptic action of β-bungarotoxin has been established, there is also evidence for postsynaptic action of β-bungarotoxin in mammalian skeletal muscle (Storella *et al.*, 1992).

β-Bungarotoxin may be a useful tool in the study of the physiological role of phosphorylation of synaptosomal proteins in neurotransmitter release. Insight into the mechanism of β-bungarotoxin may lead to the development of therapeutic strategies against pathological cells and enveloped viruses, and might serve as a useful tool to characterize the ion-translocating device, the ionophore, associated with the cholinergic receptor site (Bon & Changeux, 1977).

Six isoforms of β-bungarotoxin (β_1–β_6) from the venom of *Bungarus multicinctus* (Kondo *et al.*, 1982) and five isoforms of β-bungarotoxin (β_1–β_5-caeruleotoxin) from the venom of *B. caeruleus* (Abe *et al.*, 1977) have been identified and characterized. However, so far the only crystal structure to be determined is that of β_2-bungarotoxin isolated from the banded krait *B. multicinctus*, at 2.45 Å resolution (Kwong *et al.*, 1995). The structure reveals a partially occluded substrate-binding surface, reduced hydrophobicity of the phospholipase subunit and an ion channel binding region in the Kunitz protease inhibitor, which may mimic the regulatory interaction of endogenous neuropeptides. It appears that the phospholipase subunit may be suitably adapted for targeting the toxin.

In the present study, we report the isolation, purification, crystallization and preliminary X-ray analysis of crystals of β_1-bungarotoxin from *B. caeruleus* (Indian krait).

2. Experimental

2.1. Isolation and purification of β_1-bungarotoxin

β_1-Bungarotoxin was isolated and purified using the method cited by Kondo *et al.* (1982) with several modifications introduced in our laboratory. Lyophilized *B. caereulus* venom was purchased from the Irula Cooperative Snake Farm, Tamil Nadu, India. Approximately 1 g of lyophilized venom was dissolved in distilled water at a concentration of 50 mg ml^{-1}. This was centrifuged at 8000*g* for 15 min to remove insoluble material. The supernatant was applied to a cation exchanger, CM-Sephadex C-25 (1 × 60 cm), which had been equilibrated with 0.05 *M* ammonium acetate pH 5.0 at 277 K. Proteins adsorbed on the column were eluted with a linear gradient from 0.05 *M* pH 5.0 to 0.5 *M* pH 7.0 ammonium

acetate. The flow rate was maintained at 20 ml h^{-1} and 5 ml fractions were collected. Fraction VII was further fractionated on a Sephadex G-75 column (1 × 100 cm) with 0.1 *M* ammonium acetate pH 5.0 at 277 K. The flow rate was maintained at 5 ml h^{-1} and 5 ml fractions were collected. The major fraction was pooled and lyophilized. This contained homogenous β_1-bungarotoxin. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and native polyacrylamide gel electrophoresis (native PAGE) indicated the molecular weight to be approximately 20 kDa.

2.2. Crystallization of β_1-bungarotoxin

Crystallization of β_1-bungarotoxin was achieved with the microdialysis method at 277 K. Purified β_1-bungarotoxin was dissolved at a concentration of 10 mg ml^{-1} in 0.05 *M* Tris–HCl containing 1.4 *M* NaCl, 0.5 m*M* EDTA and 0.01% NaN_3 at pH 8.0. This protein solution was equilibrated against a reservoir solution containing 2.6 *M* NaCl in 0.05 *M* Tris–HCl at pH 10.0. Rectangular-shaped crystals with regular faces appeared within 4 d.

2.3. Data collection

X-ray data have been collected to 2.6 Å from a single crystal flash-frozen at 100 K in harvesting solution containing 21% glycerol, using synchrotron radiation at beamline BW-7B, EMBL, Hamburg Outstation, DESY, Germany, with λ = 0.8345 Å and a MAR Research image-plate detector. The data were processed using *DENZO* (Otwinowski & Minor, 1997) and scaled together with *SCALEPACK*. Subsequent calculations were performed with the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

3. Results

Rectangular-shaped crystals of β_1-bungarotoxin grew in 4 d, with maximum crystal dimensions 0.3 × 0.05 × 0.04 mm. The crystals were mounted in loops and flash-frozen at 100 K in 21% glycerol. Although the crystals were small, they diffracted beyond 2.5 Å and were stable in the X-ray beam. The data set was complete to 97.4% with $R_{\rm merge}$ = 8.2% for data between 20 and 2.6 Å resolution. The crystals were orthorhombic and belonged to space group $C222_1$ with unit-cell dimensions a = 80.7, b = 82.5, c = 56.9 Å. The molecular weight of β_1-bungarotoxin is approximately 20130 Da (PLA_2, 13200 Da and protease inhibitor, 6930 Da) as determined by extrapolation using SDS–PAGE and native PAGE. Assuming that there is one molecule of bungarotoxin in the asymmetric unit, the crystals have a calculated V_m of 2.35 $Å^3$ Da^{-1} (Matthews, 1968) and an estimated solvent content of 48%. Attempts to solve the structure by the molecular-replacement method are underway; the sequence homology between β_2-bungarotoxin and β_1-bungarotoxin is ∼65%.

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