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Structural studies on the cobra venom factor: isolation, purification, crystallization and preliminary crystallographic analysis

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Cobra venom factor (CVF) is the complement-activating protein in cobra venom. It is a three-chain glycoprotein with a molecular weight of 149 000 Da. In serum, CVF forms a bimolecular enzyme with the Bb subunit of factor B. The enzyme cleaves C3 and C5, causing complement consumption in human and mammalian serum. CVF is frequently used to decomplement serum to investigate the biological functions of complement and serves as a tool to investigate the multifunctionality of C3. Furthermore, CVF bears the potential for clinical application to deplete complement in situations where complement activation is involved in the pathogenesis of disease. CVF was isolated from Indian cobra (*Naja naja naja*) venom. The protein was crystallized at room temperature using the sitting-drop vapour-diffusion technique. The crystals diffract to 2.7 Å resolution and belong to the tetragonal space group $P4_1$, with unit-cell parameters $a = b = 62.7$, $c = 368.1$ Å.

1. Introduction

Cobra venom factor (CVF) is the complement-activating protein in cobra venom (Vogel, 1991; Vogel *et al.*, 1996). It is a 149 000 Da glycoprotein consisting of three disulfide-linked chains with molecular masses of ~68 500 (α-chain), ~48 500 (β-chain) and ~32 000 Da (γ-chain) (Eggertsen *et al.*, 1981; Vogel & Müller-Eberhard, 1984). The γ-chain shows size heterogeneity, which appears to be a consequence of differential processing at the C-terminus (Vogel & Müller-Eberhard, 1984). The carbohydrate portion [~7.4%(*w*/*w*)] consists of three N-linked oligosaccharide chains, two of which are in the α-chain and one of which in the β-chain (Vogel & Müller-Eberhard, 1984; Grier *et al.*, 1987; Gowda *et al.*, 1992; Fritzinger *et al.*, 1994). The major oligosaccharide could be identified as a symmetric fucosylated biantennary complex-type chain with an unusual α-galactose residue at its non-reducing end (Gowda *et al.*, 1992, 1994). Minor oligosaccharide chains include tri- and tetra-antennary complex-type chains as well as a small percentage of high-mannose chains (Gowda *et al.*, 1999).

When CVF is added to human or mammalian serum it activates the complement system. CVF binds to factor B of the alternative pathway. When factor B is in complex with CVF, factor B is cleaved by factor D into the activation peptide Ba and the serine protease domain Bb which remains bound to CVF. The bimolecular complex CVF–Bb is a C3 convertase which cleaves C3 (Müller-Eberhard & Fjelström, 1971; Vogt *et al.*, 1974; Hensley *et al.*, 1986). In addition to the binding site for factor B, CVF has a binding site for C5 (von Zabern *et al.*, 1980). C5, when bound to CVF, becomes susceptible to cleavage by the CVF–Bb enzyme. Accordingly, the CVF–Bb enzyme exhibits not only C3-cleaving activity but also C5-cleaving activity and is referred to as C3/C5 convertase (E.C. 3.4.21.47).

CVF is a functional and structural analogue of complement component C3. The functional similarity of CVF and C3b correlates with many structural similarities including immunological cross-reactivity, circular dichroism spectra and secondary structure and terminal amino-acid sequences (Alper & Balavitch, 1976; Eggertsen *et al.*, 1983; Vogel *et al.*, 1984). The full extent of the structural similarity of C3 and CVF became apparent when the primary structure of both proteins became available from molecular cloning (De Bruijn & Fey, 1985; Fritzinger *et al.*, 1992, 1994). Both proteins are synthesized as single-chain pre-pro-proteins with a protein sequence identity of ~51% between CVF and mammalian C3s and 85% in the case of cobra C3. Pro-C3 is processed by removing four Arg residues into the mature two-chain protein, whereas CVF is processed into its mature three-chain form by proteolytically removing the C3a and C3d domains (Fig. 1). Surprisingly, this proteolytic processing is not required for CVF function; recombinant single-chain pro-CVF exhibits identical functional activity to native CVF (Kock *et al.*, 1996).

CVF has become a frequently used tool to deplete complement in laboratory animals in order to investigate the role of complement in

Table 1
Crystallographic data and data-collection statistics.

Crystal data	
Space group	$P4_1$
Unit-cell parameters (Å)	$a = b = 62.7$, $c = 368.1$
V_M (Å^3 Da^{-1})	2.4 (one molecule per asymmetric unit)
Data collection	
Resolution (Å)	20.0–3.0
Wavelength (Å)	1.5418
X-ray source	Rotating anode (operating at 50 kV, 100 mA)
No. of observations	33265
No. of unique reflections	13644
R_{merge} (%)	7.8 (18.3)†
Completeness (%)	89.7 (81.4)†
$I/\sigma(I)$	7.6 (4.5)†

† Values in parentheses refer to the outermost shell.

host defence or disease pathogenesis (Vogel, 1991). Because CVF can be safely administered to laboratory animals, the protein or a humanized derivative may be useful as a therapeutic agent for complement depletion in clinical conditions where activation is involved in the pathogenesis of disease. This includes such diverse clinical conditions as reperfusion injury, xenograft rejection, rheumatoid arthritis and retroviral gene therapy. Another potential application of CVF is its use in antibody conjugates for targeted complement activation to induce tumour-cell killing (Vogel & Müller-Eberhard, 1981).

Although several proteins of the complement system have been crystallized and their three-dimensional structures determined, the crystallization of C3 has not been reported. This is presumably a consequence of the great flexibility of the molecule, which exhibits a multitude of biological functions and can interact with at least ten plasma proteins or cellular receptors. Three-dimensional structures have only been reported for two C3 fragments, the C3a anaphylatoxin (Huber *et al.*, 1980) and C3d (Nagar *et al.*, 1998; Zanotti *et al.*, 2000). In order to understand the various molecular functions of CVF and its differences from C3 and to exploit the potential clinical applications, it is important to elucidate the three-dimensional structure of CVF, its three chains and its complex with factor B. Here, we report the preliminary X-ray crystallographic data for CVF.

2. Experimental

2.1. Isolation and purification of CVF

Lyophilized venom of the Indian cobra (*N. naja naja)* was obtained from Irula cooperative snake farm in Tamilnadu, India. Purification of CVF was carried out using the method of Vogel & Müller-Eberhard (1984). The procedure was further modified to suit the crystallization requirements (Sharma & Singh, 1999). Initially, 0.2 g of cobra venom was dissolved in 1 ml 50 m*M* Tris–HCl, 100 m*M* NaCl pH 7.5. This solution was centrifuged at 293 K for 10 min to remove the insoluble material. The supernatant was applied to a Sephadex G-150 gel-filtration column (150 × 1.5 cm) which was equilibrated with the same buffer. The gel-filtration profile showed a total of five major peaks. The second peak was identified as containing CVF through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The fractions corresponding to the second peak were pooled, ultrafiltered and dialysed against 50 m*M* Tris–HCl pH 8.5.

This was further applied to a DEAE–Sephacel column (30 × 2 cm) equilibrated with 50 m*M* Tris–HCl pH 8.5 and eluted with a linear gradient of 0–0.5 *M* NaCl. The CVF bound to this column eluted at 0.3 *M* NaCl and was confirmed by determining the sequence of the first 20 N-terminal residues. The CVF peak was pooled, dialysed against deionized water and lyophilized. The SDS–PAGE of the CVF peak under non-reducing conditions showed a single band at 149 kDa. The purified and lyophilized CVF was used in the following crystallization experiments.

2.2. Crystallization of CVF

Initially, crystallization experiments for CVF were performed by screening a variety of precipitating agents together with different salts and at different pH values at 277 K and room temperature. However, microcrystals of the native intact protein were observed only with ammonium sulfate in screening setups using the hanging-drop vapour-diffusion method. Further refinement of these conditions including seeding did not yield crystals of a suitable size for diffraction. These crystallization conditions were carried out in hanging-drop experiments with protein concentrations ranging from 20 to 25 mg ml^{-1} in 10 m*M* sodium phosphate buffer pH 7.0. All subsequent efforts to improve crystal size did not bear fruit.

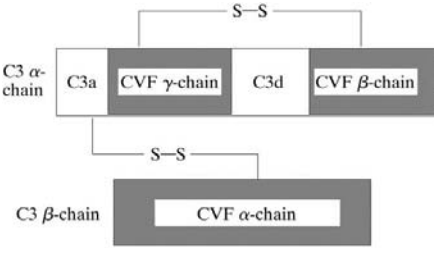
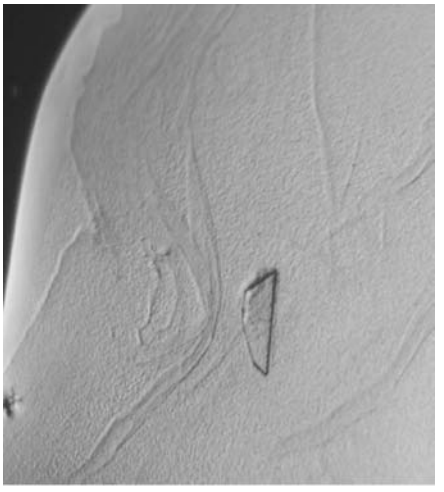
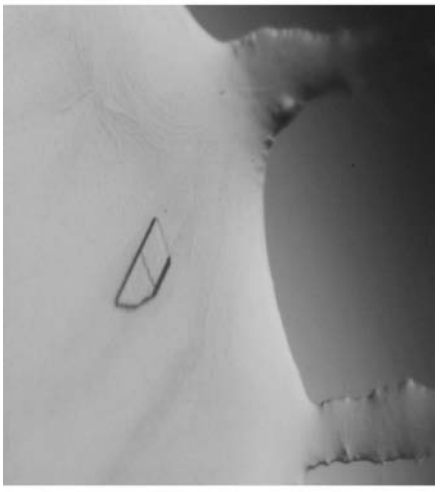


Figure 1
Schematic drawing showing the chain structures of C3 and CVF and their relationship.

In an independent set of crystallization experiments, the dissolved protein samples were exposed to a warm water bath (303 K) before crystallization. The protein concentration was varied between 5 and 10 mg ml^{-1} and the sitting-drop method was employed. The final drop volume of 10 µl was prepared from 5–10 mg ml^{-1} protein solution in 10 m*M* sodium phosphate buffer pH 7.0–8.0. The precipitant solution was placed in the reservoir. In the initial experiments, crystals of slightly irregular shape were obtained at 293 K under two conditions: (*a*) 1.5 *M* ammonium sulfate, 1 m*M* calcium chloride, 1 m*M* sodium



(*a*)



(*b*)

Figure 2
Crystals used for X-ray experiments are shown in (*a*) and (*b*). These crystals grow up to 0.2 × 0.15 × 0.05 mm and diffract to a maximum resolution of 2.7 Å when synchrotron radiation was used, but were not stable in the synchrotron beam.

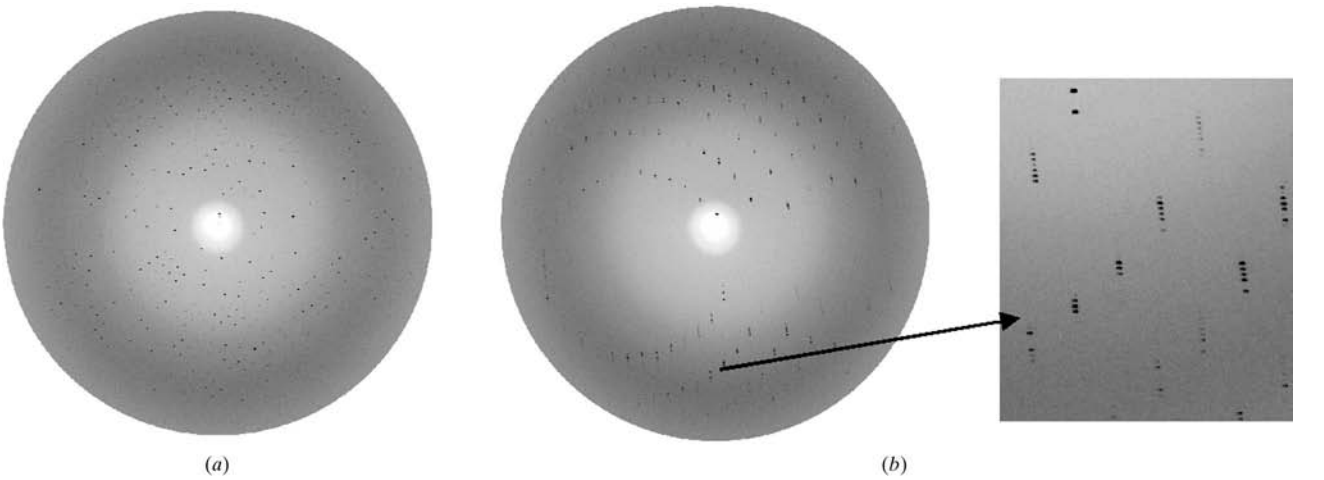


Figure 3
Diffraction pattern of a CVF crystal presented in two crystal orientations: (*a*) showing the *a** and *b** section of the reciprocal lattice and (*b*) showing the long unit-cell axis *c*. A section of the diffraction pattern has been enlarged (right).

phosphate pH 8.0 and (*b*) 1.8 *M* ammonium sulfate, 1 m*M* calcium chloride, 1 m*M* sodium phosphate pH 7.0. Subsequently, experiments were carried out with slight variations of the concentrations of the precipitating agents around these conditions to improve crystal size and quality. Finally, crystals suitable for X-ray analysis appeared after 6–8 d using well solutions consisting of 1.5–1.6 *M* ammonium sulfate, 5 m*M* calcium chloride, 0.5–1.0 m*M* sodium phosphate pH 8.2 and 1.8–1.85 *M* ammonium sulfate, 5 m*M* calcium chloride, 0.5–1.0 m*M* sodium phosphate pH 6.8. The crystals grew as relatively thin plates and had dimensions of approximately 0.2 × 0.15 × 0.03 mm, as shown in Figs. 2(*a*) and 2(*b*). The crystals obtained under these two conditions were similar in size and quality.

2.3. X-ray diffraction, data collection and analysis

An initial set of diffraction data to 3.0 Å resolution was collected at room temperature on a MAR 300 imaging-plate scanner with monochromated Cu $K\alpha$ radiation from a rotating-anode source operating at 50 kV and 100 mA from one crystal of dimensions 0.2 × 0.11 × 0.05 mm. The resolution extended to 2.7 Å when synchrotron radiation at ELETTRA and DESY-Hamburg were used. Considering that the crystals were very small and one unit-cell parameter was extremely large, the overall quality of the diffraction pattern was reasonably good, with sharp reflections (Fig. 3). The data were processed using the *HKL* package (Otwinowski & Minor, 1997). Crystal data and data-collection statistics are given in Table 1. Work on the optimization of cryoconditions and a heavy-atom derivative search are in progress. Furthermore, crystallization experiments on deglycosylated CVF and also on recombinant CVF are under way.

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