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Crystallization and preliminary X-ray diffraction studies of α-toxin from two different strains (NCTC8237 and CER89L43) of *Clostridium perfringens*

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Abstract

The α-toxin of *Clostridium perfringens* is the major virulence determinant for gas gangrene in man. The gene encoding the α-toxin has been cloned into *E. coli* from two strains of the bacterium (NCTC8237 and CER89L43) and subsequently purified to homogeneity. The two strains of α-toxin differ by five amino acids, resulting in the toxin from NCTC8237 being sensitive to chymotrypsin digestion while that from CER89L43 is resistant. The α-toxin from each of these strains has been crystallized in two different forms by the hanging-drop vapour-diffusion method at 293 K. CER89L43 form I crystals belong to space group *R*32 and have two molecules in the crystallographic asymmetric unit and a unit cell with $a = b = 151.4$, $c = 195.5$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The crystals diffracted to $d_{min} = 1.90$ Å. The characteristics of the NCTC8237 form I crystals have already been reported. The form II crystals from both strains belong to space group $C222_1$ with one molecule in the crystallographic asymmetric unit and, for strain CER89L43, have cell dimensions $a = 61.05$, $b = 177.50$, $c = 79.05$ Å, $\alpha = \beta = \gamma = 90^\circ$, while for strain NCTC8237 the cell dimensions are $a = 60.50$, $b = 175.70$, $c = 80.20$ Å, $\alpha = \beta = \gamma = 90^\circ$. The crystals diffracted to maximum resolutions of 1.85 and 2.1 Å for the CER89L43 and the NCTC8237 strains, respectively.

1. Introduction

The α-toxin of *Clostridium perfringens* has a place in the history of microbiology as it was the first bacterial toxin shown to have an enzymatic (phospholipase C) activity (MacFarlane & Knight, 1941). It is one of a number of toxins produced by *C. perfringens* and plays a significant role in the pathogenesis of many diseases in man and animals (McDonel, 1986; Williamson & Titball, 1993). The α-toxin, a two-domain protein, is a member of a family of bacterial zinc-metallophospholipases which include the *C. perfringens* phospholipase C, the *C. bifermentans* phospholipase C and the *C. novyi* phospholipase C. The *Bacillus cereus* phosphatidylcholine-preferring phospholipase C (PC-PLC) and the *Listeria monocytogenes* phospholipase (PLC-B) are single-domain proteins (Titball, 1993) with homologies to the N-terminal domain of α-toxin. All of these phospholipases are inactivated by zinc-chelating agents and are relatively thermostable, but have different substrate specificities and toxicological properties (Titball *et al.*, 1993). An alignment of the deduced amino-acid sequences of these proteins reveals that they fall into two groups, depending on the presence or absence of the C-terminal domain (Titball, 1993). This C-terminal domain of α-toxin shows sequence homology with arachidonate-5-lipoxygenase (HA5L), a eukaryotic lipid-metabolizing enzyme (Titball *et al.*, 1991). The function of the C-terminal domain is not clear and studies with a truncated form of the *C. perfringens* α-toxin have shown that the phospholipase C activity resides in the N-terminal domain. However, the isolated N-terminal domain is non-toxic and has a markedly reduced ability to hydrolyse sphingomyelin (Titball *et al.*, 1991), while the phosphatidylcholine-hydrolysing activity remains unaltered. The isolated N-terminal domain also possesses similar properties to the *B. cereus* PC-PLC. These observations lead to the hypothesis that the C-terminal domain could enable the enzyme to interact with membrane phospholipids, hydrolysis of which results in the toxic effects of α-toxin (Titball *et al.*, 1993).

The cloning and expression of the gene encoding the toxin into *E. coli* have already been reported (Titball *et al.*, 1989). The α-toxin produced in *E. coli* was shown to have similar biophysical and enzymatic properties to the toxin produced by *C. perfringens*. Previously, Basak *et al.* (1994) reported the purification, crystallization and preliminary X-ray diffraction studies of *C. perfringens* α-toxin produced by *E. coli* containing the cloned α-toxin gene from strain NCTC8237, which was isolated from a case of gas gangrene in man. Recently it has become apparent that α-toxin produced by strains of *C. perfringens* isolated from the intestine of diseased animals is more resistant to proteases such as chymotrypsin. The primary amino-acid sequence of this form of α-toxin shows several differences compared with the amino-acid sequence of the NCTC8237 strain. We now report the purification of α-toxin produced in *E. coli* containing the cloned α-toxin gene from a strain of *C. perfringens* (CER89L43) isolated from a diseased calf (Ginter *et al.*, 1996).

2. Methods and results

2.1. *Cell culture and protein purification*

2.1.1. *CER89L43 α-toxin*. *E. coli* containing plasmid pL43-2, which encodes the *C. perfringens* CER89L43 α-toxin (Ginter *et al.*, 1996), was cultured in brain heart infusion broth containing ampicillin (50 μg ml^{-1}) at 310 K for 24 h with shaking (170 r min^{-1}). The culture was harvested by centrifugation at 9000 r min^{-1} for 45 min at 277 K. Cells were resuspended in ice-cold 20 m*M* Tris–HCl, pH 8.0, containing 0.58 *M* sucrose, 10 m*M* EGTA, 0.5 mg ml^{-1} lysozyme, 0.5 m*M* $ZnCl_2$ and 1 m*M* $CaCl_2$ [all buffers contained 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF)]. The suspension was stirred, on ice, for 15 min, followed by centrifugation at 15000 rev min^{-1} for 30 min at 277 K. The supernatant was then filtered using a 22 μm filter. The α-toxin was purified from the filtrate using a two-step process. The crude extract was loaded onto a DEAE-Sephacel (Pharmacia) column and bound protein was eluted using a gradient of 0–1 *M* NaCl in the same buffer (20 m*M*

Tris–HCl, pH 8.0). Fractions containing α-toxin were analysed by SDS–PAGE and Western blotting, and tested for phospholipase C activity (Titball *et al.*, 1989). These fractions were then concentrated and loaded on to a HiLoad 26/60 Superdex 200 column which was equilibrated with PBS, pH 7.2. The α-toxin was eluted with PBS and the fractions containing pure α-toxin were analysed as before and then dialysed overnight against 10 m*M* Tris–HCl, pH 8.0, at 277 K. The protein was stored at 253 K with 50%(*v*/*v*) glycerol.

2.1.2. *Strain NCTC8237 α-toxin*. The protein was produced by the method described above after culture of *E. coli* containing plasmid pKSa3 (Basak *et al.*, 1994). Filtered crude extract was first loaded on to a DEAE-Sephacel column equilibrated with 20 m*M* Tris–HCl, pH 8.0, at a flow rate of 1 ml min^{-1} and bound protein was eluted with 0–1 *M* NaCl gradient in the same buffer. The eluted protein was analysed, concentrated using a stirred ultrafiltration cell with PM10 membrane and dialysed overnight against the same buffer at 277 K. Concentrated protein was filtered and loaded on to a HiLoad MonoQ HR 10/10 column (Pharmacia), which had been previously equilibrated with the same buffer. The protein was eluted with a continuous NaCl gradient from 0–0.5 *M*, again in the same buffer. Fractions containing pure α-toxin were analysed as before and stored at 253 K with 50%(*v*/*v*) glycerol.

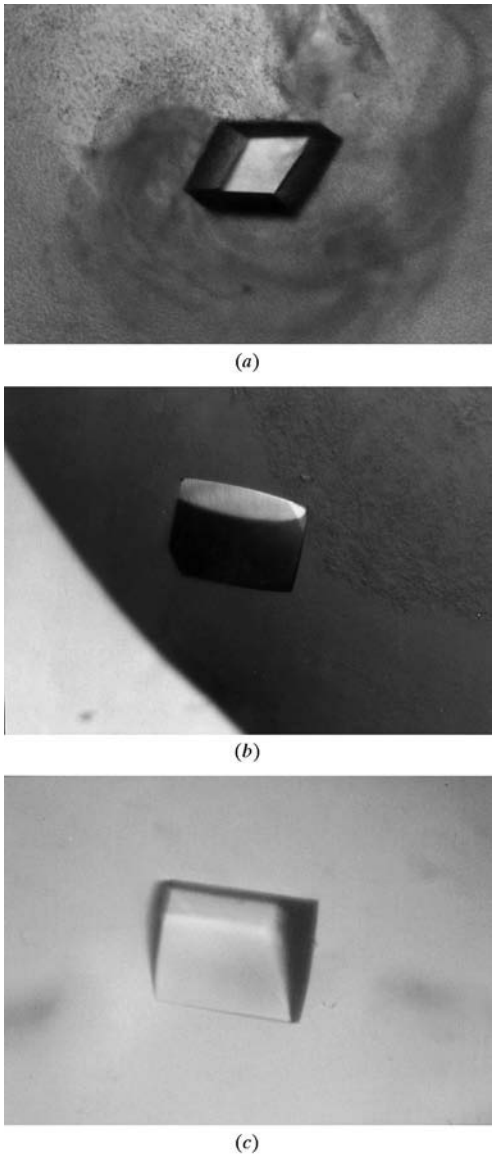


Fig. 1. (*a*) Crystals of α-toxin (strain CER89L43), rhombohedral form, with dimensions of approximately 0.6 × 0.6 × 0.4 mm. (*b*) Crystals of α-toxin (strain CER89L43), orthorhombic form, with dimensions of approximately 0.6 × 0.4 × 0.3 mm. (*c*) Crystals of α-toxin (strain NCTC8237), orthorhombic form, with dimensions of approximately 0.5 × 0.4 × 0.3 mm.

2.2. *Crystallization*

Protein from both strains was concentrated using solvent precipitation, as described by Basak *et al.* (1994). Crystals were grown using the hanging-drop vapour-diffusion method (McPherson, 1982) at 293 K.

2.2.1. *Strain CER89L43 α-toxin*. Two different forms of crystals were grown.

Form I. The optimal conditions for reproducible crystal growth were found to be 4 µl of protein solution containing 9–10 mg ml^{-1} protein in 10 m*M* Tris–HCl, pH 7.5, and an equal volume of well buffer containing 1.7 *M* NaCl in sodium acetate, pH 4.6. Well defined truncated-rhombohedral crystals (Fig. 1*a*) with maximum dimensions of 0.6 × 0.6 × 0.4 mm grew in 3–4 weeks.

Form II. These crystals were grown using 1 *M* sodium acetate as precipitant in 100 m*M* Na-HEPES, pH 7.6, with 0.05 *M* cadmium sulfate. The protein was mixed with well buffer in a 1:1 ratio and left to equilibrate with reservoir solution. Rectangular plates with maximum dimensions of 0.6 × 0.4 × 0.3 mm (Fig. 1*b*) grew in 2–3 weeks.

2.2.2. *Strain NCTC8237 α-toxin*. The optimal conditions for crystals of this strain were very similar to those for form II crystals of the CER89L43 strain. These crystals, however, could not be reproduced as reliably as those from CER89L43. The biggest truncated-rhombohedral crystal was 0.5 × 0.4 × 0.3 mm (Fig. 1*c*) and grew in 4–5 weeks.

2.3. *X-ray analysis, data collection and processing*

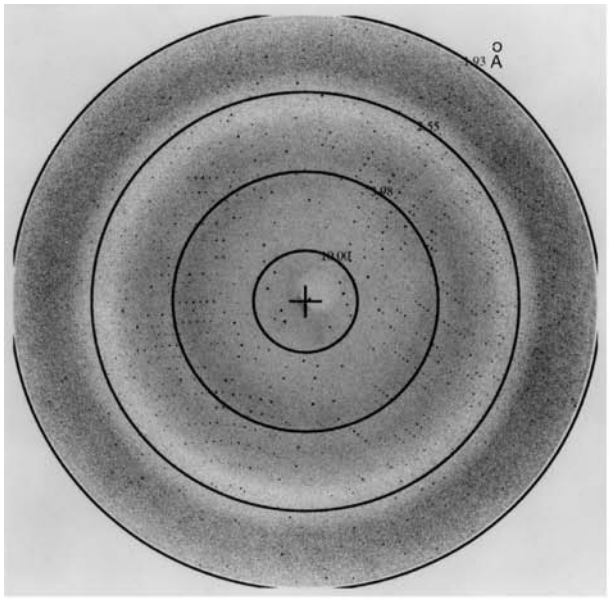
Initial characterization for crystal forms I and II of strain CER89L43 was carried out using an in-house MAR Research 90 mm-radius image plate mounted on a rotating-anode X-ray generator operating at 40 kV and 90 mA. The form I and form II crystals diffracted to $d_{\rm min}$ = 2.7 and 2.6 Å, respectively. Subsequently, higher resolution data sets were collected for both crystal forms using the CCLRC synchrotron facility at Daresbury.

2.3.1. *Form I*. These crystals diffracted to a Bragg spacing of 1.90 Å (Fig. 2*a*) at station 9.5 of the CCLRC synchrotron facility at Daresbury. Analysis of the data sets indicate that the crystals exhibit *R*32 symmetry with unit-cell parameters $a = b = 151.38$, $c = 195.3$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Assuming two subunits in the crystallographic asymmetric unit, V_m = 2.5 Å^3 Da^{-1}, which corresponds to a solvent content of 50% (Matthews, 1977). No significant peaks were seen in the self-rotation function at $\chi = 180^\circ$; however, there was a peak in the

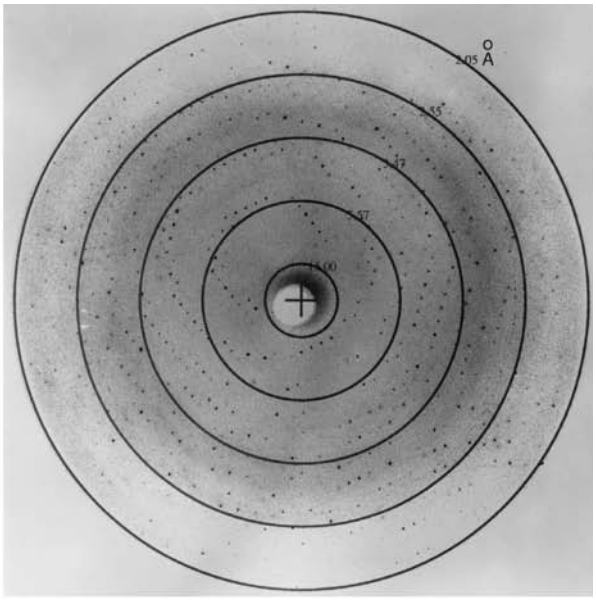
native Patterson consistent with two molecules in the asymmetric unit related by a translation of approximately 0.5z.

2.3.2. *Form II*. The diffraction limit for this crystal form was 1.85 Å (Fig. 2*b*) at station 9.5 of the CCLRC synchrotron facility at Daresbury. Analysis of the data reveals that the crystals are orthorhombic with unit-cell parameters a = 61.30, b = 177.30, c = 79.20 Å, $\alpha = \beta = \gamma = 90°$. Reflections $h + k = 2n + 1$ and $00l = 2n + 1$ are absent, indicating that the space group is $C222_1$. Assuming one molecule in the crystallographic asymmetric unit, the solvent content of these crystals is 50% (Matthews, 1977). Datasets for both crystal forms were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

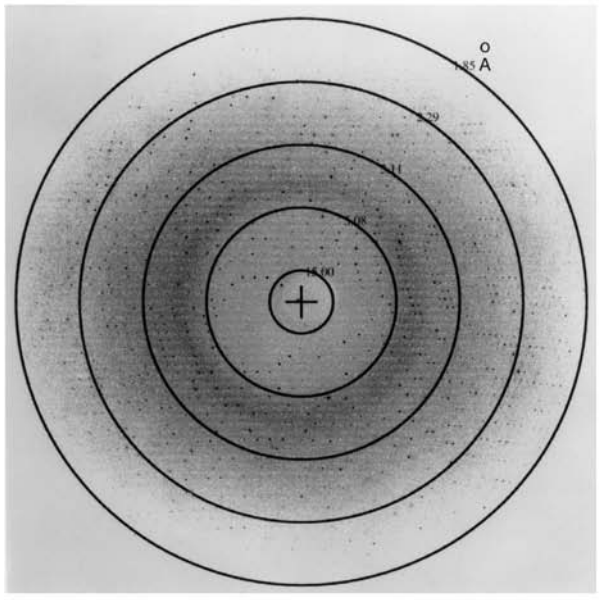
2.3.3. *New crystal form of NCTC8237 strain*. All data were collected from a single crystal cryo-cooled to 100 K at station 9.6 of the CCLRC synchrotron facility at Daresbury. The crystals diffracted to a maximum resolution of 2.10 Å (Fig. 2*c*). Analysis of the data indicated that the crystal belonged to space group $C222_1$ with unit-cell parameters a = 60.49, b = 175.68, c = 80.19 Å, $\alpha = \beta = \gamma = 90°$. Assuming one subunit in the crystallographic asymmetric unit, the solvent content of these crystals is 50% (Matthews, 1977). These data were processed with *MOSFLM* (Leslie *et al.*, 1986). Scaling and merging were performed using the programme *SCALA* (Collaborative Computational Project, Number 4, 1994).



(*a*)



(*c*)



(*b*)

Fig. 2. (*a*) Oscillation photograph of α-toxin (CER89L43) rhombohedral crystals diffracting to 1.9 Å at the edge. (*b*) Oscillation photograph of α-toxin (CER89L43) orthorhombic crystals diffracting to 1.85 Å at the edge. (*c*) Oscillation photograph of α-toxin (NCTC8237) orthorhombic crystals diffracting to 2.0 Å at the edge.

Table 1. *Native data for different crystal forms*

Strain	N_{ref}	Resolution (Å)	R_{merge} (%)	Completeness (%)
CER89L43, form I	56901	40–1.95	5.8 (19.3 from 2.02–1.85 Å)	90.9
CER89L43, form II	30175	40–1.90	6.5 (15.7 from 1.96–1.90 Å)	87.5
NCTC8237	16808	40–2.20	12.2 (20.3 from 2.32–2.20 Å)	76.3

3. Discussion

Well diffracting crystals of α-toxin were grown in two very different conditions, from two different strains. These crystals were stable in the X-ray beam and diffracted to 1.8–2.0 Å resolution at the CLRC synchrotron facility at Daresbury. Native data sets for all crystal forms were collected. The details of the native data for the different crystal forms are summarized in Table 1.

We are at present working on crystals from the CER89L43 strain, and the search for heavy-atom derivatives of both the crystal forms is in progress to facilitate the determination of the three-dimensional structure of α-toxin.

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