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Crystallization and preliminary X-ray analysis of *Clostridium botulinum* neurotoxin type B

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Single crystals of *Clostridium botulinum* neurotoxin type B have been obtained by the vapor-diffusion method. These crystals belong to space group $P2_1$, with unit-cell parameters a = 76.08, b = 123.11, c = 95.86 Å, β = 113.03° and diffract to at least 1.8 Å resolution. Native data have been collected from flash-frozen crystals at the National Synchrotron facility of Brookhaven National Laboratory. These crystals often tend to be non-isomorphic.

1. Introduction

C. botulinum, first isolated over 100 years ago, is a Gram-positive spore-forming bacterium which grows anaerobically at 303–310 K under pH 6.5–7.0 conditions. *C. botulinum* neurotoxins (BoNT) are the most potent toxins to humans and cause the disease known as botulism or flaccid paralysis. Food-borne botulism is caused by ingestion of food containing the potent BoNT secreted during anaerobic growth of *C. botulinum*. Clostridial neurotoxins bind specifically to neuronal cells, enter the cytosol and block neurotransmitter release (Simpson, 1989). The LD_{50} of these neurotoxins is about 1 ng kg^{-1} for humans (Schiavo, Rossetto & Montecucco, 1994). *C. botulinum* produces seven (A–G) antigenically distinct serotypes. The neurotoxin is synthesized and remains as an inactive single chain of 150 kDa until cleaved by proteases before being released as a dichain – a heavy chain of 100 kDa and a light chain of 50 kDa held together by a disulfide bond. BoNT/E is the only neurotoxin released as a single chain which, however, can be cleaved by exogenous proteases like trypsin to form a dichain (Dasgupta & Rasmussen, 1983; Sathyamurthy & Dasgupta, 1985). Dichain BoNTs are much more toxic than single-chain BoNT. The seven serotypes of *C. botulinum* and the tetanus toxin of *C. tetani* (TeNT) share a low sequence homology. However, there are regions where the homology is very good. For example, all of them share a common motif, HE*xx*H – a zinc-binding motif in the light chain. This motif has helped in identifying these toxins as zinc endopeptidases (Schiavo, Poulain *et al.*, 1992; Schiavo, Rosetto *et al.*, 1992; Schiavo, Shone *et al.*, 1993). Clostridial neurotoxins are classified as AB toxins consisting of an active domain and a binding domain. *C. botulinum* toxins consist of three functional domains (Menestrina *et al.*, 1994), *viz*. binding, translocating and catalytic domains. The toxic activity consists of a four-step mechanism (Montecucco *et al.*, 1994): (*a*) binding to the surface, (*b*) internalization into vesicles, (*c*) translocation into cytosol and (*d*) attacking the substrates. Di- and trisialogangliosides have been identified as receptors for the binding domain (Kozaki *et al.*, 1987; Nishiki *et al.*, 1996; Pierce *et al.*, 1986). A double-receptor binding gangliosides and a protein, has also been suggested (Montecucco, 1986). The translocation mechanism is not yet very well understood. A change in conformation of the translocation domain induced by pH changes which would expose the hydrophobic core to the membrane has been suggested (Schiavo, Rossetto & Montecucco, 1994). Each neurotoxin has a specific target component in the neuroexocytosis apparatus. BoNT/B, D, F and G specifically cleave the vesicle-associated membrane protein (VAMP, also called synaptobrevin), BoNT/A and E cleave synaptosomal associated protein of 25 kDa (SNAP-25) by specific hydrolysis and BoNT/C cleaves syntaxin (Blasi *et al.*, 1992, 1993; Schiavo, Benfenati *et al.*, 1992; Schiavo, Rosetto, Benfenati *et al.*, 1994; Schiavo, Rosetto *et al.*, 1993; Schiavo, Santucci *et al.*, 1993; Schiavo *et al.*, 1995; Schiavo, Shone *et al.*, 1993). When the substrate is cleaved by the catalytic domain, acetylcholine release is blocked causing flaccid paralysis and finally death. BoNTs and TeNT have different symptoms. While BoNTs cause flaccid paralysis, TeNT causes spastic paralysis. This difference arises from the different sites of action. After being internalized, BoNT-containing vesicles remain at the neuromuscular junction, while TeNT-containing vesicles undergo retrograde axonal transport to enter the inhibitory neurons of the central nervous system (CNS; Schiavo, Rossetto & Montecucco, 1994). TeNT blocks the release of γ-aminobutyric acid (GABA) or glycine. These toxins have gained

attention recently because of their potential use as biowarfare agents. The US Food and Drug Administration has licensed BoNT/A for therapeutic uses and BoNT/B is also being used for medicinal purposes (Hambleton, 1992).

2. Materials and methods

Purified *C. botulinum* neurotoxin type B (E.C. 3.4.24.69) precipitated with 60% ammonium sulfate was purchased from Food Research Institute, Madison, Wisconsin, USA (Evans *et al.*, 1986). It was stored at 283 K until the toxin was prepared for crystallization. The SDS gel of the native toxin showed a single band of 150 kDa; a reduced SDS gel showed two bands of 100 and 50 kDa corresponding to the heavy and light chains, respectively. Except for about 5%, the toxin was in unnicked form. The precipitate was centrifuged in a refrigerated microfuge for about 5 h at 5000 rev min^{-1}. The toxin was recovered by removing the supernatant and dissolving the precipitate in 50 m*M* HEPES buffer and 100 m*M* NaCl at pH 7.0. This was dialyzed against 50 m*M* HEPES buffer and 100 m*M* NaCl at pH 7.0 for 2 d to completely remove ammonium sulfate. The dialysate was changed four times during this period. The toxin was concentrated with Millipore concentrators (10 kDa cutoff) to a final concentration of about 8 mg ml^{-1}. Our experience has shown that the above steps have to be carried out very carefully, with minimal disturbance to the toxin. When the toxin was dissolved in a solution, care was taken not to shake the vial but to let it sit for a while until the entire toxin went into solution.

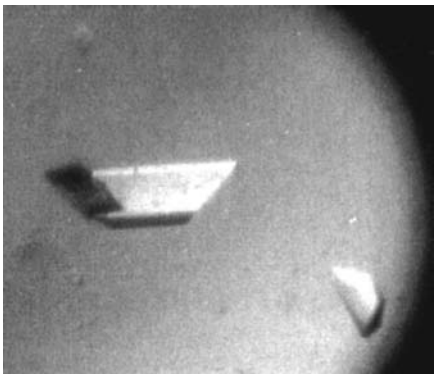

Figure 1
Single crystals of *C. botulinum* neurotoxin type B photographed under a polarizing microscope.

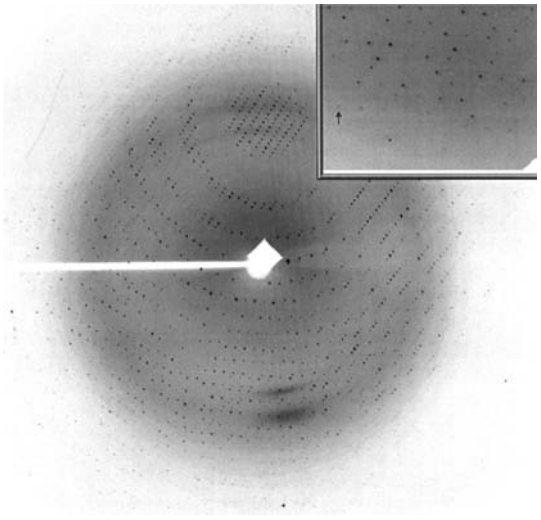

Figure 2
Diffraction image of a single oscillation frame as obtained from Brandeis B4 detector. The oscillation corresponds to 1°. The inset shown is from the left bottom part of the detector. The diffraction limit, marked with a small arrow in the inset, corresponds to 1.8 Å.

Initial crystallization conditions were obtained with the use of the Hampton Research Crystal Screen, restricting the pH value close to the pI (5.23) value of the toxin. 4 µl of a droplet containing a 1:1 ratio of protein to precipitant was placed in a microbridge, which was in turn placed inside Linbro plate wells containing appropriate precipitants. The wells were sealed with clear tape and left at room temperature. Initial trials gave thin plate-like crystals with PEG 6000 and 100 m*M* MES at pH 6.0 in 7–10 d and diffracted to about 3.0 Å resolution. Several additives were tried to improve the crystal quality but none worked better than the initial condition. Finally, seeding with cat whiskers gave good quality crystals (Fig. 1). Plate-like crystals were transferred to 200 µl mother liquor, crushed well in a vial and stirred well with a Vortex stirrer. The crystallization droplets were allowed to equilibrate overnight and then seeded gently with a cat whisker briefly dipped in the crushed-crystal solution (Stura & Wilson, 1991). These crystals diffracted at least to 1.8 Å (Fig. 2). Initial attempts to flash-freeze the crystal by adding 15% glycerol to the mother liquor were unsuccessful. However, when the crystals were transferred to mother liquor containing sodium cacodylate buffer instead of MES the flash-freezing worked very well. Crystals had average dimensions of 0.3 × 0.3 × 0.1 mm, but crystals twice this size were also occasionally obtained.

Single-crystal X-ray diffraction data at liquid-nitrogen temperature were collected at beamline X12C of the National Synchrotron Light Source, Brookhaven National laboratory. Crystals diffracted to 1.8 Å resolution. A Brandeis B4 CCD-based detector was used to collect data with a crystal-to-detector distance of 145 mm with λ = 1.1 Å. Data were collected using the software *MARMAD* (Skinner & Sweet, 1998) and were reduced with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystals belong to space group $P2_1$, with unit-cell parameters a = 76.08, b = 123.11, c = 95.86 Å, β = 113.03°. Assuming one molecule of 150 600 Da per asymmetric unit, the Matthews coefficient (Matthews, 1968) is 2.743 Å^3 Da^{-1}, which translates to an estimated solvent content of 55% by volume of the unit cell.

3. Results and discussion

Unit-cell parameters for the initially obtained plate-like crystals were similar but not identical to the one from which data were collected. Comparison of structure factors of these two crystals gave an $R_{\rm merge}$ of 30%, clearly indicating that they belong to different crystal forms. A total of 553 505 reflections were collected and reduced to 145 662 unique reflections with no σ cutoff on intensity. The overall $R_{\rm sym}$ was 8.3%, with 30% for the highest resolution shell. The data were 97.1% complete to 1.8 Å resolution, with 81.4% completeness and an average $I/\sigma(I)$ of 3 in the resolution range 1.86–1.8 Å. In spite of high-quality diffraction, non-isomorphism was observed between crystals from the same well, which was not evident until the data were merged. Such non-isomorphism has been observed previously in other crystals (Sixma *et al.*, 1993; Swaminathan *et al.*, 1992).

In the case of BoNT/B, it was relatively easy to obtain a large number of small crystals, but these were of poor quality. However, a small number of crystals, separated from one another and of fairly good morphology and size, were obtained when spot seeding was used rather than streak seeding. The protein also had to be handled very carefully, avoiding vigorous tapping or shaking, prior to setting up crystallization. It was also found that flash-freezing was improved when about 5% glycerol was present in the crystallization drop.

Recently, interest in the three-dimensional structure of these toxins has grown enormously. The crystal structure of *C. botulinum* neurotoxin type A has been reported to 3.3 Å resolution (Lacy *et al.*, 1998). To the best of our knowledge, this is

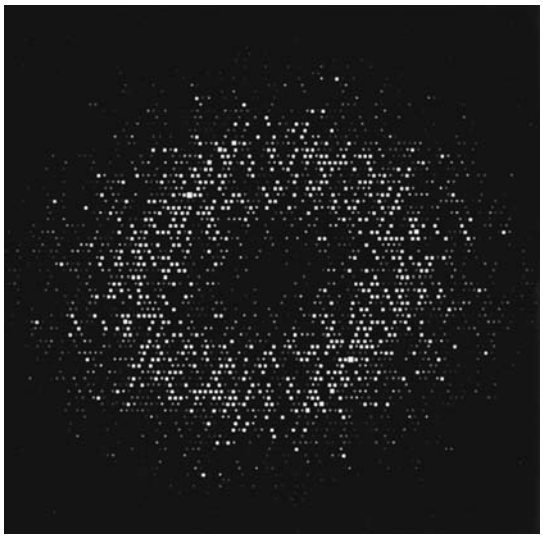

Figure 3
A pseudo-precession photograph of the *h*0*l* zone of *C. botulinum* neurotoxin type B crystals. Data to 1.8 Å resolution are included. This picture was generated by *PRECESS* from the *PHASES* package (Furey & Swaminathan, 1997).

the first time such high diffraction quality crystals have been obtained and the structure determination should reveal high-resolution details. We have identified several heavy-atom derivatives and will solve the phase problem by a combination of the multiple anomalous dispersion (MAD; Hendrickson, 1991) and multiple isomorphous replacement with anomalous scattering (MIRAS; Blow & Crick, 1959) methods.

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