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Preliminary crystallographic studies of a protease-resistant botulinum neurotoxin associated protein Hn-33

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Botulinum neurotoxin (BoNT) is one of the most potent toxins known. BoNT is also a food poison, which means that the toxin must survive the protease action and acidity of the gut. A group of neurotoxin-associated proteins which are only beginning to be identified and characterized are believed to be responsible for this protection. Hn-33 is a 33 kDa polypeptide which is a major component of the type A botulinum neurotoxin complex. Crystals of Hn-33 have been grown by vapour-diffusion techniques. They belong to a primitive orthorhombic space group and diffract to a resolution of 2.6 Å, with unit-cell parameters a = 130.3, b = 122.2, c = 37.2 Å.

1. Introduction

Botulinum neurotoxin (BoNT) is one of the most potent toxins known. Seven serologically distinguishable neurotoxins (A–G) are produced by the anaerobic bacterium *Clostridium botulinium*. Botulinum neurotoxin (BoNT) is a large protein ($M_r \simeq 150$ kDa), composed of an ∼100 kDa heavy chain (HC) linked to an ∼50 kDa light chain (LC) by a disulfide bond and non-covalent interactions (Singh, 1996). BoNT neurotoxin shares many characteristics with tetanus neurotoxin, including amino-acid sequence similarity and mode of action. However, only BoNT is a food poison. In order to cause botulism, BoNT has to survive the low pH and the action of proteases in the gastrointestinal tract, which it does with the help of essential neurotoxin-associated proteins (NAPs). From the gut, it is translocated across the intestinal mucosal layer, adsorbed by the epithelial layer and enters general circulation.

Whilst the toxin itself has proved to be an indispensable tool in examining the process of exocytosis, the roles of the neurotoxin-associated proteins (NAPs) have been less well investigated. The NAPs have to protect the toxin from proteolysis and also to assist in translocation of the neurotoxin through the epithelial layer and into general circulation, a role similar to that of bacterial adhesins such as the filamentous haemagglutinin (FHA) produced by *Bordetella pertussis* and high molecular-weight surface-exposed proteins from non-typeable *Haemophilus influenzae*.

A 33 kDa haemagglutinating protein (Hn-33) is a common feature in the toxic complex from a number of the serotypes. BoNT A Hn-33, with a predicted sequence of 293 amino acids, has been shown to be resistant to proteolytic cleavage, consistent with a role in protection of the toxin (Fu *et al.*, 1998; Sharma & Singh, 1998). It has been demonstrated that BoNT C Hn-33 plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine, leading to the efficient absorption of toxin (Fujinaga *et al.*, 1997). BoNT A (East *et al.*, 1994), BoNT B (Yang *et al.*, 1996) and BoNT C Hn-33 (Tsuzuki *et al.*, 1990) have been cloned. The Hn-33 components from types A and B show high (>85%) identity, but share lower identity with the Hn-33 component from type C (around 36%). It is thought that the binding properties of different Hn-33 serotypes to epithelial cell receptors may be responsible for the specificity of botulinum serotypes for different animal hosts.

In addition to explaining the binding of the toxic complex to the gut wall, the NAPs make attractive non-toxic targets for botulinum

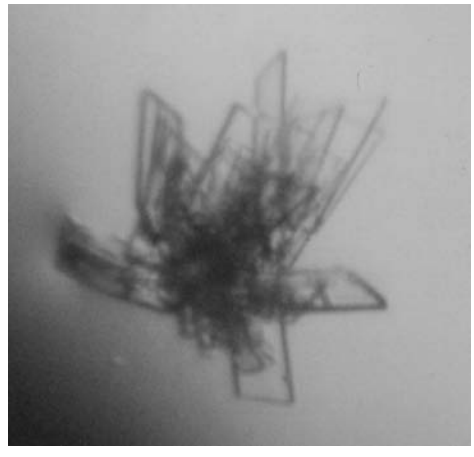

Figure 1
Crystals of Hn-33.

Table 1
Analysis of diffraction from Hn-33 type A crystals.

Unit-cell parameters (Å)	a = 130.3, b = 122.2, c = 37.2
Packing density V_m (Å^3 Da^{-1})	2.24; one dimer per asymmetric unit
Diffraction limit (Å)	2.6

Partial data set (20°)

Diffraction limit (Å)	3.5
Total observations	2301
Unique reflections	1576
Average $I/\sigma(I)$ (all data)	4.1
R_{sym} (all data)	0.13
Completeness (all data) (%)	19
Average $I/\sigma(I)$ (outer shell)	0.9
R_{sym} (outer shell)	0.61

neurotoxin vaccine development and also for the development of toxin preparations for use in therapeutic applications. An understanding of the protective role of the NAPs may also assist in the design of protection, perhaps by the same proteins, for other oral vaccines. Hn-33 is a unique protein which exhibits resistance to several proteases despite the presence of cleavage sites in its amino-acid sequence. The molecular basis of the protease resistance must therefore originate from the three-dimensional folding of the polypeptide. Knowledge of the three-dimensional structure will greatly enhance the utility of Hn-33, which has recently been isolated in its functional form (Fu *et al.*, 1998). Here, we report preliminary results from X-ray crystallographic analysis.

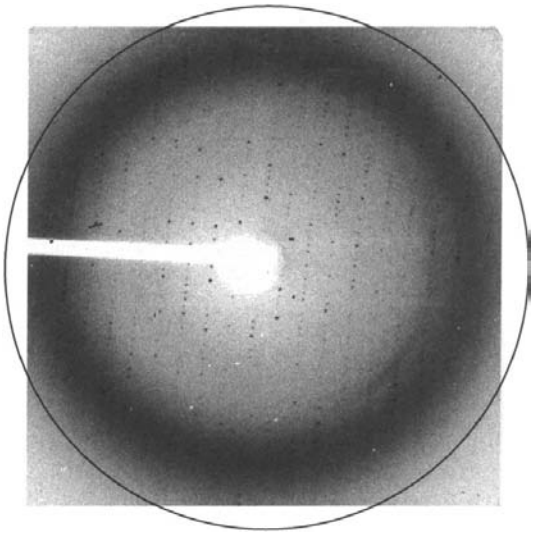

Figure 2
Diffraction pattern recorded from a crystal of Hn-33. The bold circle represents the 2.6 Å diffraction limit. Crystal-to-film distance, 8 cm; image-plate radius, 5 cm; Cu $K\alpha$ radiation; oscillation range, 1°; exposure time, 1 h; temperature, 298 K.

2. Methods

Hn-33 was isolated and purified from an anaerobic culture of *Clostridium botulinum* type A as described by Fu *et al.* (1998). An additional purification step was performed prior to crystallization. Protein was loaded onto a gel-filtration column (S200, Pharmacia) and eluted with HEPES buffer pH 7.0, 100 m*M* NaCl. The fractions containing Hn-33 were pooled and the protein was concentrated and exchanged into a buffer of 10 m*M* HEPES pH 7.0 using a microconcentrator (Amicon). Crystals were obtained by the hanging-drop vapour-diffusion method, using PEG 4000 and 2-propanol as precipitants. A CCD detector (Brandeis) was used for data collection at both X-ray sources, and the *HKL* package (Otwinowski & Minor, 1997) was used for indexing, integration and scaling.

3. Results

The best crystals were obtained using a reservoir containing 16%(*w*/*v*) PEG 4000 and 10%(*v*/*v*) 2-propanol and mixing protein with a concentration of 5 mg ml^{-1} in a 1:1 ratio with reservoir solution. Plate-like crystals grew in bunches after about 7 d (Fig. 1), with individual crystals reaching maximum dimensions of 0.6 × 0.2 × 0.1 mm.

The diffraction limit of such a crystal was about 2.6 Å employing Cu $K\alpha$ graphite-monochromated radiation from a Rigaku RU-200 rotating-anode source operated at 50 kV and 100 mA (Fig. 2) and the CCD detector for data collection. However, this fragile plate was cracked with the result that the diffraction pattern was twinned.

The unit-cell dimensions and space group were determined using a partial data set (20° of data) collected from a small single crystal (<0.1 × <0.1 × <0.1 mm) at beamline X12C, Brookhaven Laboratory National Synchrotron Light Source at room temperature, again using the CCD detector (Table 1). Data collection was terminated by a loss of the X-ray beam. The diffraction pattern revealed an orthorhombic space group, with unit-cell dimensions a = 130.3, b = 122.2, c = 37.2 Å. Systematic absence of reflections of the type $h00$ (h odd) indicate the presence of a screw axis. There is insufficient data along the other two directions for a complete analysis, but the space group is most likely to be $P2_12_12$ or $P2_12_12_1$, although $P222_1$ is possible. The incomplete data and the plate-shaped crystal both contributed to a high R_{merge} for those data which were recorded twice or more. The asymmetric unit is likely to contain two monomers of Hn-33, giving a Matthews constant V_m = 2.24 Å^3 Da^{-1} (Matthews, 1974). From this, the solvent content of the asymmetric unit is estimated to be 45%.

4. Conclusions

In both binding and immunogenic properties, the BoNT NAPs resemble the filamentous haemagglutinin (FHA) produced by *B. pertussis*, the agent responsible for whooping cough. A hairpin model of the FHA has been proposed (Makhov *et al.*, 1994) in which the head is composed of the terminal domains, the shaft consists mainly of hyper-elongated β-sheets and the tail is composed of the intervening sequence. However, a crystal structure of another *B. pertussis* virulence factor, P69, shows the 69 kDa protein is a single domain folded into a 16-stranded β-helix (Emsley *et al.*, 1996). Circular dichroism measurements indicate a high percentage of β-structure in BoNT A Hn-33 (Sharma *et al.*, 1999) suggesting that the structures as well as the functions of these two proteins may be similar. Our prediction of two monomers in each asymmetric unit for these Hn-33 crystals is consistent with the dimeric structure of Hn-33 in solution (Sharma *et al.*, 1999). It is a structure which has been proposed to play a critical role in the large complex structure of BoNT type A (Oguma & Fijinaga, 1996).

A necessary step in successful colonization and, ultimately, production of disease by microbial pathogens is the ability to adhere to host surfaces. In the case of botulinum neurotoxin, the pathogen is a protein rather than an organism. Structural studies on the NAPs and on functionally related bacterial adhesins can inform us about this process at a molecular level. There is still a lot to be learned about the ability of bacteria to mimic host molecules and to use this mimicry to enhance their pathogenesis.

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