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Preliminary X-ray crystallographic study of the receptor-binding domain of the D/C mosaic neurotoxin from *Clostridium botulinum*

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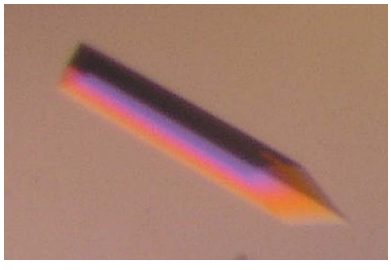

Botulinum toxin (BoNT) from *Clostridium botulinum* OFD05, isolated from bovine botulism, is a D/C mosaic-type BoNT. BoNTs possess binding, translocation and catalytic domains. The BoNT/OFD05 binding domain exhibits significant sequence identity to BoNT/C, which requires a single ganglioside as a binding receptor on neuronal cells, while BoNT/A and BoNT/B require two receptors for specific binding. To determine the binding mechanism of BoNT/OFD05 and its ganglioside receptors on neuronal cells, recombinant BoNT/OFD05 receptor-binding domain has been expressed, purified and crystallized. Native and SeMet-derivative crystals showed X-ray diffraction to 2.8 and 3.1 Å resolution, respectively. The crystals belonged to space group $P2_12_12_1$.

1. Introduction

Clostridium botulinum, an anaerobic soil bacterium, produces seven botulinum toxin serotypes (BoNTs) designated A–G (Simpson, 1986). Seven types of *C. botulinum* have been distinguished by differences in the antigenic specificities of their pharmacologically similar neurotoxins. The bacterial spores are widely present in the environment and are resistant to various physical and chemical agents. Therefore, they can contaminate food and germinate to yield the vegetative bacterial cells that produce one or more of the toxins under suitable anaerobic conditions. BoNTs cause three forms of botulism: foodborne botulism, infant botulism and wound botulism. The signs and symptoms of botulism are essentially the same for all forms of the disease and are the consequence of sustained blockade of acetylcholine release at somatic and autonomic nerve terminals (Montecucco & Molgo, 2005). In addition, BoNTs are amongst the most poisonous biological toxins identified to date. The toxicity of BoNT/A has been estimated to be ∼0.2 ng per kg of body weight and as little as 0.1–1 µg is lethal in humans (Schantz & Johnson, 1992). Nonetheless, these extremely poisonous molecules have become useful therapeutic agents in an expanding number of applications in human medicine as well as in the cosmetics industry (Montecucco & Molgo, 2005).

BoNTs are large neurotoxic proteins of ∼150 kDa that consist of a light chain (L-chain; 50 kDa) and a heavy chain (H-chain; 100 kDa) linked by a disulfide bond (Schiavo *et al.*, 2000). They are synthesized as single-chain polypeptides and then activated by proteolytic cleavage into L- and H-chains. The three-dimensional structures of BoNT types A, B and E revealed three functionally distinct domains: catalytic (L-chain), translocation (amino-terminus of the H-chain; H_N) and binding domains (carboxy-terminus of the H-chain; H_C) (Lacy *et al.*, 1998; Swaminathan & Eswaramoorthy, 2000; Kumaran *et al.*, 2009). The L-chain inhibits the exocytosis of acetylcholine by cleaving soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) using its zinc-dependent endopeptidase activity, whereupon the Ca^{2+}-triggered fusion of a synaptic vesicle with the presynaptic membrane is disrupted (Montecucco & Schiavo, 1994). Different types of BoNT target respective SNARE-protein families. The H-chain is composed of the two remaining domains and serves as the vehicle that delivers the L-chain into the cytosol of

neuronal cells. The H_N is responsible for translocating the L-chain. In contrast, the H_C exhibits highly selective binding for neurons, which is the first step in intoxication. It is well known that the adhesion between neurotoxins and nerve endings involves gangliosides and the H_C. However, it is believed that gangliosides offer nonspecific binding to BoNTs. To bind to the target neuronal cells specifically, BoNTs require other protein receptors, such as synaptotagmin I and II and synaptic vesicle glycoprotein 2 (SV2). Unlike the other types, gangliosides are predicted to play a significant role in the binding of BoNT/C to neurons, as a deficiency of gangliosides produced little sensitivity to BoNT/C *in vivo* (Tsukamoto *et al.*, 2005, 2008).

The botulinum serotypes differ in pathogenicity in humans and animals (Johnson & Bradshaw, 2001). In contrast to human botulism, which mainly involves BoNT/A, BoNT/B and BoNT/E, BoNT/C and BoNT/D are causative agents of animal and avian botulism. *C. botulinum* strain OFD05 was isolated from bovine botulism in Japan. It contains two-thirds of the BoNT/D gene and one-third of the BoNT/C gene, indicating that it is a mosaic form of BoNT types D and C (Nakamura *et al.*, 2010). The H_C region of BoNT from OFD05 (OFD05H_C) shows 77% sequence identity to type C BoNT, despite its 30–39% sequence identity to BoNTs other than type C, and shows type C-like receptor recognition. To address how BoNT/OFD05 recognizes gangliosides as its specific receptors, we are studying the binding mechanism of OFD05H_C from a structural viewpoint. This report describes the initial attempt to obtain protein crystals of OFD05H_C.

2. Materials and methods

2.1. Cloning, expression and purification

The DNA fragment encoding OFD05H_C was amplified using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) with the genomic BoNT/OFD05 gene as a template and the primers OFD05H_C-forward (5′-NNNN**CCATGG**AATATTTCAATAATAT TAATGAATATTTCAATAG-3′) and OFD05H_C-reverse (5′-NNN-N**CTCGAG**TTCACTTGCAGGTACAAAAACCC-3′); *Nco*I and *Xho*I sites were included at the ends of the forward and reverse primers, respectively (restriction-enzyme sites are shown in bold). These primers were designed based on the respective DNA sequences in GenBank (accession No. AB461915). After purification and digestion with *Nco*I and *Xho*I, the PCR products were ligated into the *Nco*I and *Xho*I sites of the pET28b vector (Merck, Darmstadt, Germany). The DNA sequence was verified using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). In the resultant plasmid, the His tag is attached at the C-terminus. The recombinant plasmid was introduced into *Escherichia coli* strain B843 (DE3) harbouring the pRARE2 helper plasmid. The cells were grown at 310 K in 1000 ml Luria–Bertani (LB) broth containing 25 µg ml^{-1} kanamycin and 34 µg ml^{-1} chloramphenicol until the optical density reached 0.6–0.8. After addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 m*M*, bacterial culture was continued at 298 K for an additional 24 h. The selenomethionine (SeMet) derivative was obtained by the same method as described above, except that M9 medium supplemented with 1 m*M* selenomethionine was used instead of LB medium. There was a slight modification of the expression conditions in that after adding IPTG to a final concentration of 0.5 m*M* bacterial growth was continued for an additional 48 h.

The cells were collected and suspended in 20 ml sonication buffer (20 m*M* Tris–HCl pH 8.0 and 300 m*M* NaCl). The cells were then disrupted by sonication and centrifuged at 45 000*g* for 30 min at 283 K. The supernatant was loaded onto an Ni–NTA Superflow column (Qiagen, Hilden, Germany). After washing the column with 10 ml sonication buffer, the adsorbed protein was eluted with stepwise increases in imidazole concentration: 15, 32, 62.5, 125 and 500 m*M*. Fractions containing target protein were collected and dialyzed against 20 m*M* Tris–HCl pH 8.0 and 200 m*M* NaCl at 277 K. The collected fractions were further purified by gel filtration on HiLoad 26/60 Superdex 200 columns (GE Healthcare, Buckinghamshire, England) pre-equilibrated with the dialysis buffer described above. The homogeneity of the purified protein was confirmed by SDS–PAGE.

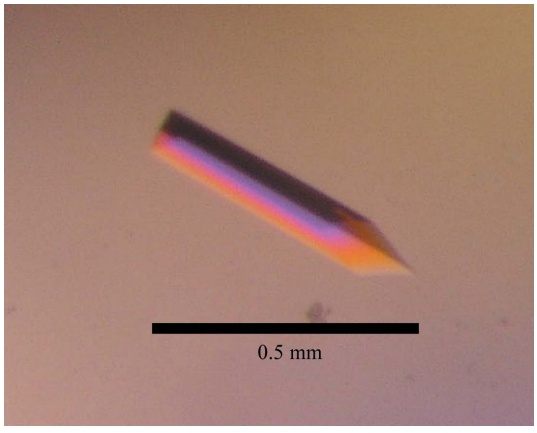

Figure 1
Native crystal of the H_C domain from *C. botulinum* strain OFD05 used for X-ray data collection. The crystal dimensions are approximately 0.7 × 0.1 × 0.1 mm.

2.2. Crystallization

The purified protein was concentrated to approximately 1 mg ml^{-1} using an Amicon Ultra-15 ultrafiltration device (Millipore, Billerica, Massachusetts, USA) for crystallization. The protein concentration was determined by monitoring the absorption at a wavelength of 280 nm with a molar extinction coefficient of 85 760 M^{-1}, as determined from the amino-acid sequence. The absorption was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Crystallization screening of the native protein was performed with NeXtal Classics Suite, NeXtal Classics Lite Suite, NeXtal JCSG$^+$ Suite and NeXtal JCSG Core Suite I–IV (Qiagen), giving a total of 672 conditions, using the sitting-drop method by mixing 1 µl protein solution (approximately 1 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 and 200 m*M* NaCl) with 1 µl reservoir buffer and equilibrating against 100 µl reservoir buffer at 293 K. The crystals of native protein that were most suitable for further diffraction experiments were grown with 0.2 *M* potassium/sodium tartrate, 0.1 *M* trisodium citrate pH 5.6 and 1 *M* ammonium sulfate (Fig. 1). The crystallization conditions for the SeMet-derivative protein were optimized based on the conditions that gave crystals of native protein using StockOptions Salt (Hampton Research, Aliso Viejo, California, USA), in which individual salt candidates were mixed with the original solution in a 1:5 ratio (salt:original solution). The best crystals of SeMet protein were grown by crystallization in the presence of 0.1 *M* MES pH 6.5, 1.6 *M* magnesium sulfate and 1 *M* sodium chloride.

Table 1
Data-collection statistics.

Values in parentheses are for the last shell.

	SeMet	Native
Beamline	SPring-8 BL41XU	PF NW12A
Wavelength (Å)	0.9770	1.0000
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters		
a (Å)	57.9	57.8
b (Å)	138.7	139.0
c (Å)	162.4	160.9
Resolution limits (Å)	50–3.1(3.21–3.1)	50–2.8 (2.9–2.8)
No. of observed reflections	306056	223250
No. of unique reflections	24212	32077
Multiplicity	12.6 (8.7)	7.0 (6.1)
Completeness (%)	99.5 (96.0)	98.0 (86.2)
R_{merge} (%)	16.4 (47.1)	5.8 (31.0)

2.3. Data collection and initial phase determination

X-ray diffraction data sets were collected from SeMet-substituted and native OFD05H$_C$ on beamline BL41XU of SPring-8 (Hyogo, Japan) and beamline NW12A of Photon Factory (PF; Tsukuba, Japan), respectively, under cryogenic conditions at 100 K. Crystals were soaked in mother liquor containing 30% glycerol and flash-cooled in a stream of liquid nitrogen. A total range of 360° was covered with 1.0° oscillation and 30 s exposure per frame. The wavelength of 0.9770 Å for the collection of Se single-wavelength anomalous diffraction (Se-SAD) data from SeMet-substituted OFD05H$_C$ was chosen based on the fluorescence spectrum of the Se *K* absorption edge (Rice *et al.*, 2000). All diffraction data were indexed, integrated, scaled and merged using the *HKL*-2000 software package (Otwinowski & Minor, 1997). The statistics are shown in Table 1. The initial phases were determined with the program *SHELX* (Sheldrick *et al.*, 2001) with Se-SAD data at 4 Å resolution and then improved with the program *DM* (Schuller, 1996; Wang, 1985; Cowtan, 1994). The modified 4 Å phases were then applied to the native data set for phase extension to 2.8 Å resolution.

3. Results and discussion

The BoNT/OFD05H$_c$ protein was overexpressed as a soluble protein in *E. coli*, purified and crystallized for structural studies. The yield of recombinant protein was approximately 1.7 mg per litre of culture for native protein. However, the yield was reduced when the protein was expressed in M9 minimal media.

The diffraction data set of native OFD05H$_C$ was collected to a resolution of 2.8 Å. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 57.8, b = 139.0, c = 160.9 Å (Table 1). The Matthews coefficient and solvent content, which were estimated as 3.21 Å^3 Da^{-1} and 61.7%, respectively, were consistent with the presence of two molecules in the asymmetric unit. Initially, we attempted to determine the crystal structure using the molecular-replacement method with PDB entries 2nyy (Garcia-Rodriguez *et al.*, 2007) and 1epw (Swaminathan & Eswaramoorthy, 2000) as search models for the N- and C-terminal regions of H$_C$, respectively. Although they have 31% and 30% sequence identity with OFD05H$_C$, no obvious solution was obtained. Therefore, we prepared crystals of SeMet-substituted protein. Initial phasing was performed by the single-wavelength anomalous diffraction (SAD) method at 4 Å resolution with 19 of 28 selenium sites. The initial phases were transferred into the diffraction data of the native crystal and then expanded to 2.8 Å resolution with the program *DM*. Further model building is currently under way in our laboratory.

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