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Crystallization and preliminary crystallographic studies of the HA3 subcomponent of the type B botulinum neurotoxin complex

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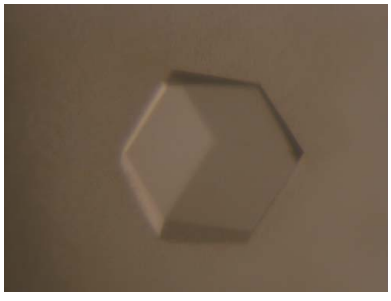


The haemagglutinin subcomponent HA3 of the type B botulinum neurotoxin complex, which is important in toxin absorption from the gastrointestinal tract, has been expressed, purified and subsequently crystallized in two crystal forms at different pH values. Form I belonged to space group $R32$, with unit-cell parameters $a = b = 357.4$, $c = 249.5$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Form II belonged to space group $I4_132$, with unit-cell parameters $a = b = c = 259.0$ Å, $\alpha = \beta = \gamma = 90^\circ$. Diffraction data were collected from these crystals to a resolution of 3.0 Å for both form I and form II.

1. Introduction

Botulinum neurotoxin (BoNT) is one of the most toxic proteins and elicits flaccid paralysis (Schiavo *et al.*, 2000). Of the seven serotypes, types A, B, E and F cause botulism in both humans and animals, while types C and D cause botulism in animals but very rarely in humans. Type G toxin-producing organisms have been experimentally isolated from soil, but there have not been any naturally occurring outbreaks caused by this type of toxin. The BoNT produced by *Clostridium botulinum* forms a complex by associating with nontoxic proteins, including a haemagglutinin (HA) component and a nontoxic protein lacking HA activity (nontoxic non-HA; NTNH; Sakaguchi, 1982; Oguma *et al.*, 1999). This neurotoxin complex causes food-borne intoxication. To cause disease, orally ingested BoNTs in the complex must take a long journey to reach their targets, the peripheral nerves. The initial and strong obstacle to the entrance of orally ingested BoNT (150 kDa) to the body is the intestinal epithelial barrier. The mechanism by which this large protein toxin crosses the intestinal epithelial barrier remains elusive.

Recently, we have found a novel activity of the HA component: the type B HA directly binds to E-cadherin and disrupts the paracellular barrier of the intestinal epithelium, facilitating the transepithelial delivery of BoNT and other macromolecules (Matsumura *et al.*, 2008; Sugawara *et al.*, 2010). We also showed that type A HA in the toxin complex has a similar barrier-disrupting activity and a greater potency than type B HA in the human intestinal epithelial cell lines Caco-2 and T84 and in the canine kidney epithelial cell line MDCK I. In contrast, type C HA in the toxin complex had no detectable effect on the paracellular barrier in these human cell lines, but showed barrier-disrupting activity and potent cytotoxicity in MDCK I (Jin *et al.*, 2009). These findings indicate that types A, B and C HAs possess a potent activity to disrupt epithelial paracellular barrier function, with distinct features in their modes of action. However, the molecular mechanisms by which each type of HA disrupts the epithelial barrier remained unclear.

The HA components of types A–D consist of three different subcomponents: HA1, HA2 and HA3 (Oguma *et al.*, 1999). Recently, several structural studies of HA subcomponents have been reported. X-ray crystallographic structures of HA1 [type C (Inoue *et al.*, 2003; Nakamura *et al.*, 2008) and type A (Arndt *et al.*, 2005)], the HA1–HA2 complex (type D; Hasegawa *et al.*, 2007) and HA3 (type C; Nakamura *et al.*, 2009) have been determined. Furthermore, recent transmission electron-microscopy studies of the type D BoNT complex (L toxin, 16S toxin) suggested an ellipsoidal-shaped structure

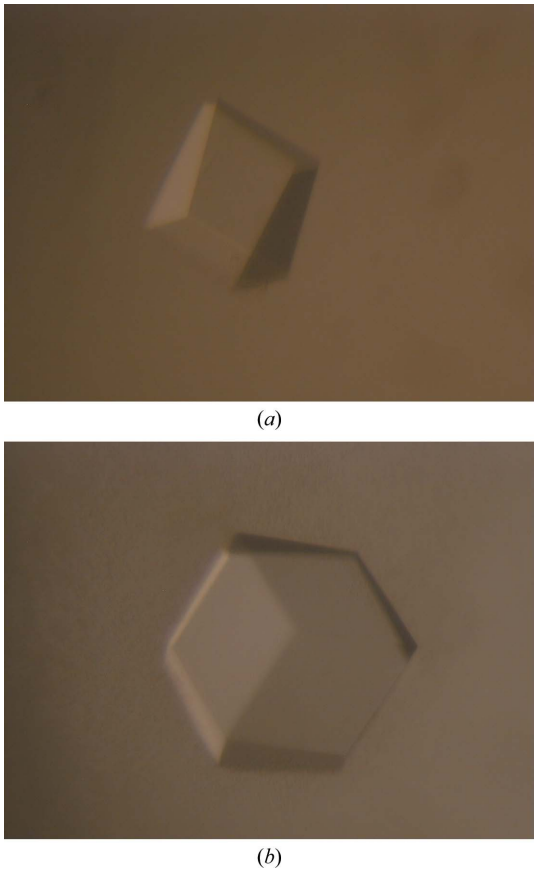
with three extended arms formed by the HA component (Hasegawa *et al.*, 2007).

With the aim of shedding light on the as yet undiscovered structural basis of the type B HA3 subcomponent of the BoNT complex, we present here the crystallization and preliminary X-ray diffraction analysis of the recombinant type B HA3 subcomponent.

2. Methods and results

2.1. Expression and purification of rBHA3

A 1.8 kbp type B HA3-encoding DNA fragment, digested with *Kpn*I and *Sal*I from PCR products amplified from *C. botulinum* type B (Lamanna) genomic DNA, was cloned into the *Kpn*I and *Sal*I sites of pET-52b (Novagen). The consequent plasmid pStrep-BHA3 includes the sequence encoding the mature type B HA3 protein (amino acids 19–626 of the BHA3 sequence) with an initial methionine and an additional 23-amino-acid sequence (MAS**WSHPQFEK**-GALEVLFQGPGYQ) including a *Strep*-tag II sequence (shown in bold) at the N-terminus of the HA3 protein. The recombinant protein (rBHA3) was expressed in *Escherichia coli* strain Rosetta 2 (DE3) (Novagen). The cells were disrupted by sonication with a model 250 Analog Sonifier (Branson) at 273 K. The disrupted cell suspension was centrifuged at 25 000 rev min^{-1} (SW41Ti, Beckman) for 60 min at 277 K to pellet insoluble material. The rBHA3 protein with a *Strep*-tag II in the supernatant was purified using *Strep*-Tactin MacroPrep resin (Novagen) according to the manufacturer's protocols.



(*a*)

(*b*)

Figure 1
Crystals of rBHA3: (*a*) form I (pH 8.0), (*b*) form II (pH 5.5).

Table 1
Data-collection statistics for rBHA3 native data sets.

Values in parentheses are for the outer resolution shell.

	Form I (pH 8.0)	Form II (pH 5.5)
Beamline	BL44XU	BL44XU
Wavelength (Å)	0.80	0.80
Space group	*R*32	$I4_132$
Unit-cell parameters (Å)		
a	357.4	259.0
b	357.4	259.0
c	249.5	259.0
Resolution (Å)	3.0	3.0
Total reflections	695328	647209
Unique reflections	119017	29842
$R_{\rm merge}$† (%)	8.4 (49.0)	7.4 (42.3)
Completeness (%)	97.4 (99.3)	99.9 (100.0)
Multiplicity	5.8	21.7

† $R_{\rm merge} = 100\sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of all symmetry-related reflections $I_i(hkl)$.

The protein concentration was determined with the BCA protein-assay kit (Pierce) using bovine serum albumin as a standard. The rBHA3 fraction was concentrated to 10 mg ml^{-1} using a Vivaspin 20 (Sartorius) and sterile-filtrated using 0.1 µm Ultrafree-MC (Millipore). The homogeneity of the purified preparation was confirmed by 12.5% SDS–PAGE and native PAGE.

2.2. Crystallization

For crystallization experiments, a 6 mg ml^{-1} solution of rBHA3 in 0.01 *M* Tris–HCl pH 8.0 was employed. Crystallization trials were set up at room temperature as sitting-drop vapour-diffusion experiments in Cryschem plates (Hampton Research). Initial screening was performed using the sparse-matrix method (Jancarik *et al.*, 1991) with the commercial crystal screening kits Wizard I and II (Jena Bioscience). Crystals of rBHA3 were readily grown from sodium chloride solutions. The crystallization droplets consisted of 2 µl protein solution and 2 µl reservoir solution and were equilibrated against 500 µl reservoir solution. Two different crystal forms were obtained by equilibration against a solution consisting of 2.4 *M* sodium chloride at 293 K in sitting-drop vapour-diffusion setups at different pH conditions. Form I crystals were obtained using 2.4 *M* sodium chloride, 0.1 *M* Tris–HCl pH 8.0. Triangle-shaped tabular crystals appeared within a few days and grew to maximum dimensions of about 0.5 × 0.5 × 0.2 mm (Fig. 1*a*). Form II crystals were obtained using 2.4 *M* sodium chloride, 0.1 *M* Bis-Tris propane pH 5.5. Diamond-shaped crystals were obtained with approximate dimensions of 0.5 × 0.3 × 0.3 mm (Fig. 1*b*).

2.3. Data collection and processing

X-ray diffraction data were collected from rBHA3 crystals at 100 K in a nitrogen stream, supplementing the mother-liquor solution with 20% glycerol as a cryoprotectant. All diffraction data were collected on a Rayonix MX225-HE charge-coupled device detector on beamline BL44XU at SPring-8 (Table 1). The X-ray wavelength was 0.8 Å, the oscillation range was 0.5° and the crystal-to-detector distance was 380 mm. Diffraction data were indexed, integrated and scaled with the *HKL*-2000 program package (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

3. Discussion

The form I crystals belonged to the rhombohedral space group *R*32, with unit-cell parameters $a = b = 357.4$, $c = 249.5$ Å, $\alpha = \beta = 90$, $\gamma = 120°$

(see Table 1). A total of 119 017 unique reflections were obtained using the *HKL*-2000 program package. Intensity data in the resolution range 50.0–3.0 Å were processed with an R_{merge} of 8.4%. The acceptable range of the volume-to-weight ratio (V_M; Matthews, 1968) indicated that the form I crystal contained 4–12 protein molecules per asymmetric unit assuming an M_r of 67 kDa for the expressed rBHA3 domain (V_M = 1.91–5.72 Å^3 Da^{-1}). This corresponds to a solvent fraction of about 35.5–78.5%, typical values for protein crystals. The form II crystals belonged to the cubic space group $I4_132$, with unit-cell parameters $a = b = c = 259.0$ Å, $\alpha = \beta = \gamma = 90^\circ$ (Table 1). A total of 29 842 unique reflections were obtained. Intensity data in the resolution range 50.0–3.0 Å were processed with an R_{merge} of 7.4%. The acceptable range of the volume-to-weight ratio (V_M) indicated that the form II crystal contained 1–3 protein molecules per asymmetric unit assuming an M_r of 67 kDa for the expressed rBHA3 domain (V_M = 1.80–5.41 Å^3 Da^{-1}). This corresponds to a solvent fraction of about 31.8–77.3%, typical values for protein crystals. The crystallographic analysis of rBHA3 will be based on the multiple isomorphous replacement method/MAD method; a search for heavy-atom derivatives is presently in progress.

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References

Arndt, J. W., Yu, W., Bi, F. & Stevens, R. C. (2005). *Biochemistry*, **44**, 9574–9580.
Hasegawa, K., Watanabe, T., Suzuki, T., Yamano, A., Oikawa, T., Sato, Y., Kouguchi, H., Yoneyama, T., Niwa, K., Ikeda, T. & Ohyama, T. (2007). *J. Biol. Chem.* **282**, 24777–24783.
Inoue, K., Sobhany, M., Transue, T. R., Oguma, K., Pedersen, L. C. & Negishi, M. (2003). *Microbiology*, **149**, 3361–3370.
Jancarik, J., Scott, W. G., Milligan, D. L., Koshland, D. E. & Kim, S.-H. (1991). *J. Mol. Biol.* **221**, 31–34.
Jin, Y., Takegahara, Y., Sugawara, Y., Matsumura, T. & Fujinaga, Y. (2009). *Microbiology*, **155**, 35–45.
Matsumura, T., Jin, Y., Kabumoto, Y., Takegahara, Y., Oguma, K., Lencer, W. I. & Fujinaga, Y. (2008). *Cell. Microbiol.* **10**, 355–364.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Nakamura, T., Kotani, M., Tonozuka, T., Ide, A., Oguma, K. & Nishikawa, A. (2009). *J. Mol. Biol.* **385**, 1193–1206.
Nakamura, T., Tonozuka, T., Ide, A., Yuzawa, T., Oguma, K. & Nishikawa, A. (2008). *J. Mol. Biol.* **376**, 854–867.
Oguma, K., Inoue, K., Fujinaga, Y., Yokota, K., Watanabe, T., Ohyama, T., Takeshi, K. & Inoue, K. (1999). *J. Toxicol. Toxin Rev.* **18**, 17–34.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Sakaguchi, G. (1982). *Pharmacol. Ther.* **19**, 165–194.
Schiavo, G., Matteoli, M. & Montecucco, C. (2000). *Physiol. Rev.* **80**, 717–766.
Sugawara, Y., Matsumura, T., Takegahara, Y., Jin, Y., Tsukasaki, Y., Takeichi, M. & Fujinaga, Y. (2010). *J. Cell Biol.* **189**, 691–700.