Cloning, expression and purification of botulinum neurotoxin type A heavy chain – crystallographic evidence for a putative tetrameric pore

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Abstract: *Clostridium botulinum* produces seven antigenically distinct serotypes of botulinum neurotoxin (BoNTs, A-G) the most potent toxins to humans. They cause paralysis at less than picomolar concentration by blocking neurotransmitter release. BoNT consists of a heavy chain and a light chain. The C-terminal half of the heavy chain allows the toxin to bind to the presynaptic membrane while the N-terminal half helps in forming a channel in the endosomal membrane to allow the light chain to escape into the cytosol. While the binding and catalytic mechanisms are well understood now, the details of translocation still remain a mystery. A full length BoNT/A heavy chain (BAHC) has been cloned, over expressed and purified from inclusion bodies by solubilising with a detergent. Preliminary crystallographic results show that BAHC forms a tetramer in the crystal lending experimental support for tetrameric pore formation for the light chain to pass through the endosomal membrane.

Keywords: botulinum neurotoxin; BoNT; translocation; oligomerisation; pore formation; heavy chain; HC; auto induction; inclusion bodies.

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1 Introduction

Clostridium botulimum produces botulinum neurotoxins (BoNTs), the most potent toxins to humans. There are seven serologically distinct BoNTs (BoNT A-G). BoNTs are produced as a single polypeptide chain (150 kDa) and cleaved exogenously or endogenously into a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked through a disulfide bond (Lacy and Stevens, 1999). BoNTs function via three steps, extra cellular binding through gangliosides and specific protein receptors at the presynaptic

membrane, internalisation via energy mediated endocytosis, and endosomal membrane translocation of the LC into the cytosol. Once in the cytosol, the LC cleaves one of the three proteins of the SNARE complex and blocks the neurotransmitter release leading to flaccid paralysis (Simpson, 1986, 1989).

Neuronal cell surfaces contain low affinity gangliosides and high specificity protein receptors for BoNTs. Accordingly, a double receptor model has been proposed (Chai et al., 2006; Montecucco, 1986). In the absence of gangliosides the clostridial neurotoxins do not penetrate the cell (Yowler et al., 2002). Earlier reports show that BoNT/A and BoNT/B have single ganglioside binding site. Also, it is known that the translocation domain is able to form channels in cell membranes (Sheridan, 1998) and artificial bilayers (Blaustein et al., 1987; Donovan and Middlebrook, 1986; Hoch et al., 1985).

Crystal structures of BoNT/A and its catalytic and binding domains are available (Fu et al., 2009; Kumaran et al., 2008; Lacy et al., 1998; Pless et al., 2001; Segelke et al., 2004; Stenmark et al., 2008). While the binding and catalytic mechanisms are understood fairly well, the details about the translocation mechanism are only now slowly emerging (Fischer and Montal, 2007b; Fischer et al., 2008, 2009; Montal, 2009; Montal et al., 1990). The essential mechanism underlying the LC translocation through the endosomal membrane can be better understood with detailed information of the three-dimensional structure of the BoNT HC that forms the translocation channel. So far, structural information has been limited to low resolution electron microscopy for BoNT/B (Schmid et al., 1993), channel current studies, substrate proteolysis experiments (Koriazova and Montal, 2003) and single molecule detection of an intermediate during BoNT translocation across membranes (Fischer and Montal, 2007a). Recent fluorescence-labelled dextran studies show that the width of the membrane channel is at the most 24.2 Å (Parikh and Singh, 2007). Since the dimension of the LC is ~50 Å, it is not possible for it to penetrate the endosomal membrane without unfolding. Accordingly, it is suggested that it unfolds while passing through the channel (or before) and refolds once it is in the cytosol and is ready for proteolytic attack of its substrates. Since the HC alone is involved in translocation and formation of channel, we have chosen the BoNT/A heavy chain (hereafter BAHC) to study the pore/channel formation of these neurotoxins by X-ray diffraction. Lipid bilayer studies and channel current studies have shown that the HC forms a channel. The BAHC protein was expressed in *E. coli* as inclusion bodies and solubilised with anionic detergent LSN. Crystals obtained in the presence of detergent diffracted to better than 6.0 Å and a self-rotation Patterson map showed a four-fold symmetry indicating a tetrameric association. Hence, the present findings suggest possible tetrameric state of BAHC in the presence of a detergent partly mimicking the lipid environment.

2 Materials and methods

2.1 Primer design, polymerase chain reaction and gene construction

2.1.1 DNA preparation

Genomic DNA was prepared as described previously (Singh et al., 1996). *Clostridium botulinum* type A (strain Hall), was inoculated in 10 ml of toxin production media (2%

N-Z amine, 0.5% yeast extract, 0.6% yeast paste for type A *C. botulinum*) and incubated at 37°C for 24 h. High molecular weight total cellular DNA was isolated according to a previously reported method (Szabo et al., 1993). Briefly, DNA was recovered from alkaline cell lysis by centrifuging vegetative cells, washing twice with 1 × SSC (sodium citrate, sodium chloride), resuspending in 1 M NaOH and heating at 65°C for 5 min. Following centrifugation, the supernatants were collected and DNA was precipitated with isopropanol and 0.3 M sodium acetate, pH 7.0. The DNA was pelleted, resuspended in water and purified using a DNA binding matrix (Prep-a-Gene, BioRad, Hercules, CA) equal to 25 ml binding matrix/20 mg DNA. A quantity of binding buffer (6 M sodium perchlorate, 50 mM Tris-HC1, pH 8.0, 10 mM EDTA) equal to three times the combined volumes of Prep-a-Gene matrix suspension and DNA solution was incubated at room temperature for 10 min and centrifuged. The pellet was rinsed twice with binding buffer. After centrifugation the matrix-DNA was washed three times with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 50% ethanol. The bound DNA was eluted in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, by incubating at 37°C to 50°C for 5 min followed by centrifugation. The DNA containing supernatant was stored at 4°C.

2.1.2 Gene construction

Polymerase chain reaction (PCR) was performed with the primers 5'-GGGCCCCCATAT GGCATTAAATGATTTATGTATC-3' and 5'-GGGCCCCGGATCCTTACAGTGGCC TTTCTTCCCA-3' to generate a gene encoding the entire HC from BoNT/A according to an established procedure (Li and Singh, 1999). Chromosomal DNA (50–100 ng) from *C. botulinum* (strain Hall) was used as template. An *NdeI* and a *BamHI* restriction site was incorporated into the 5' end of the forward sequence and reverse sequence primers, respectively. PCR reactions were carried out in a total volume of 50 µl containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 0.1% BSA, 100 µM concentration of each dNTP, 10mM β -mercaptoethanol (β-Me), 25 pmol of each primer, 2.5 units of DNA polymerase (New England Biolabs). The reaction mixtures were denatured for 2 min at 93°C and then subjected to 30 consecutive cycles consisting of denaturation (1 min at 90°C), annealing (2 min at 50°C) and polymerisation (3 min at 73°C). After amplification, the DNA fragments were cleaved with *NdeI* and *BamHI* and then electrophoresed on a 1.0% agarose gel. The *NdeI-BamHI* digested fragment was purified from the agarose gel using QIAGEN columns (QIAGEN Inc. Valencia, CA) and then ligated to the *NdeI*/*BamHI* digested vector pET-15b. A typical ligation reaction mixture contained 40 ng vector and 200 ng insert in a volume of 20 µl of water. 3 µl of 10× ligase buffer and 1 µl of T4 ligase was used. After overnight incubation at 16°C, the ligation product was transformed into competent *E. coli* strain XL1-Blu and grew in Luria broth supplemented with ampicillin (100 µg/ml).

Clones were screened by digestion of a miniprep (Promega Corp., Madison, WI) plasmid DNA with *BamHI* and *NdeI*. Confirmation that the amplified fragment encoded BAHC was obtained by DNA sequencing of a representative plasmid recombinant (designated pET-HCA). DNA sequencing was performed by the dideoxy chain termination method.

2.2 Expression, solubilisation and purification of the recombinant BoNT/A HC

2.2.1 Expression

Five millilitres of minimal medium for cell growth (MDG) medium containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol was inoculated with the freezer stock of BL21 (DE3) cells containing pET-HCA. After overnight growth at 37°C, 500 μl of this culture was used to inoculate 500 ml of ZYP 5052 [auto induction medium (Studier, 2005)] with ampicillin and chloramphenicol in a 2 L flask and the cells were grown at 37°C with shaking at 300 rpm until the A_{600} reached 0.8. At this point, the cells were incubated at 20°C for an additional 18 h shaking at 200 rpm. The induced cells were harvested by centrifugation at 6,000 rpm, at 4°C for 15 min.

The cell pellet from a 3 L culture was resuspended in 240 ml lysis buffer. The lysis buffer containing 50 mM Tris-Hcl, pH 8.5, 300 mM NaCl, 5 mM benzamidine, 0.1 mM PMSF, 1 μg/ml pepstatin A and 1 mM β-Me was supplemented with five tablets of protease inhibitor cocktail (Roche, Nutley, NJ) and 0.5 mg/ml lysozyme (Sigma, St. Loius, MO), 24 ml Bugbuster (Novagen, San Diego, CA), and 6 mM iodoacetamide. Forty micro litres of the Benzonase (Novagen, San Diego, CA) was added to lysis buffer to digest the DNA and reduce the viscosity of the lysate.

2.2.2 Solubilisation

The bacterial suspension was incubated for 30 min at room temperature and centrifuged at 20,000 rpm for 30 min. Since almost all of the BAHC goes into inclusion bodies, the supernatant was discarded. In preliminary experiments, several detergents and sulfobetaine were tried to solubilise the target protein (Vuillard et al., 1998). However, only LSN appeared to be useful (Table 1, Figure 1). In view of that the pellet was resolubilised with 1% LSN (Fluka, USA) in buffer (50 mM Tris-HCl, pH 8.5, 0.3 M NaCl and 1mM β-Me) and the suspension was kept at room temperature for 2 h and centrifuged at 20,000 rpm for 30 min to remove the insoluble material. The supernatant obtained from the above step was allowed to mix with 5 ml Ni-NTA agarose, pre-equilibrated with Tris-HCl buffer (50 mM Tris, pH 8.5, 300 mM NaCl, 0.2% LSN and 1 mM β-Me) at 4°C for an hour.

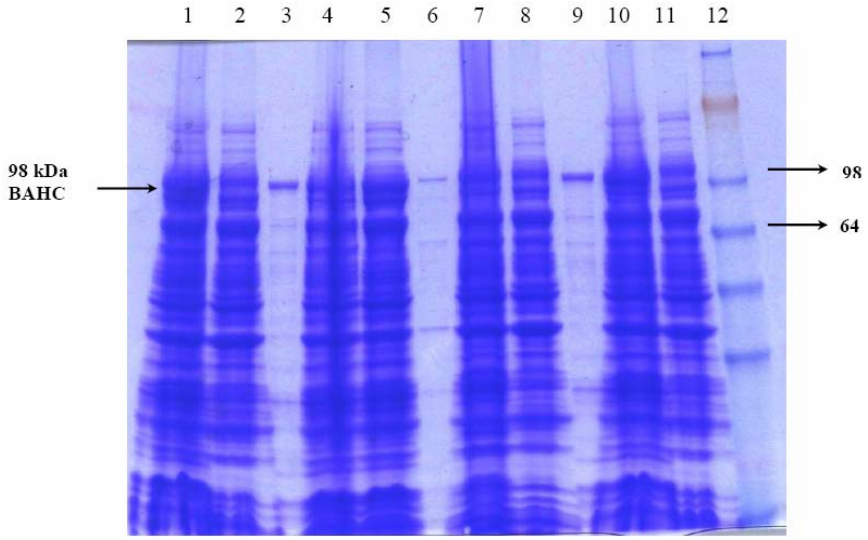
2.2.3 Purification

The mixture of Ni-NTA agarose and supernatant was poured into a glass column and the flow-through of the soluble fraction was collected. The column was washed with 100 ml of Tris buffer + 0.1% LSN followed by 100 ml wash with buffer containing 5, 10 and 20 mM imidazole. Strongly bound protein was eluted with 10 ml of 250 mM (thrice) and 10 ml of 500 mM (twice) imidazole (pH 8.5). Aliquots of all the above fractions were analysed by electrophoresis on 4% to 20% Tris-Glycine SDS-PAGE gel followed by staining with Coomassie blue. A 98 kDa band corresponding to BAHC reproducibly eluted in 250 mM imidazole + 0.1% LSN fractions was obtained (Figure 2).

Table 1 Detergents and non-detergent sulfobetamine tried for solubilisation

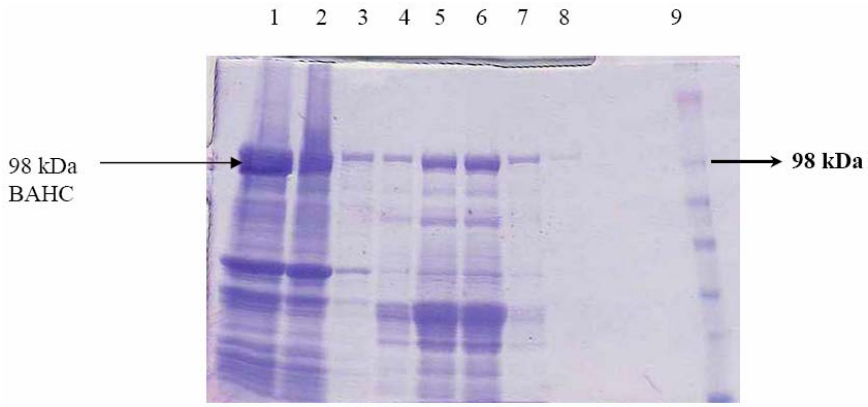
Detergent/sulfobetaine	*% used*	*Solubilisation success (yes/no)*
Deoxycholate	1	No
Nonaethylene glycol monoethyl ether	1	No
Octyl β-D-glucopyranoside (OG)	1	No
N-Lauroylsarcosine Sodium salt (LSN)	1	Yes
Lauryldimethylamine-oxide (LDAO)	1	No
N,N-Dimethyldecylamine-N-oxide (DDAO)	1	No
Myristyltrimethylammonium bromide (MTAB)	1	No
n-dodecyl-beta-D-maltoside (DDM)	1	No
Dimethylethyl-(3-Sulfopropyl)-ammonium	15	No
Dimethyl(2-hydroxyethyl)ammonium propane sulfonate	15	No
3-(N-Phenylmethyl-N,N-dimethylammonio) propanesulfonate	15	No
1-(3-Sulfopropyl)pyridinium betain	15	No
3-(1-Methylpyridinium)-1-propane sulfonate	15	No

Figure 1 Detergent solubilisation of BAHC



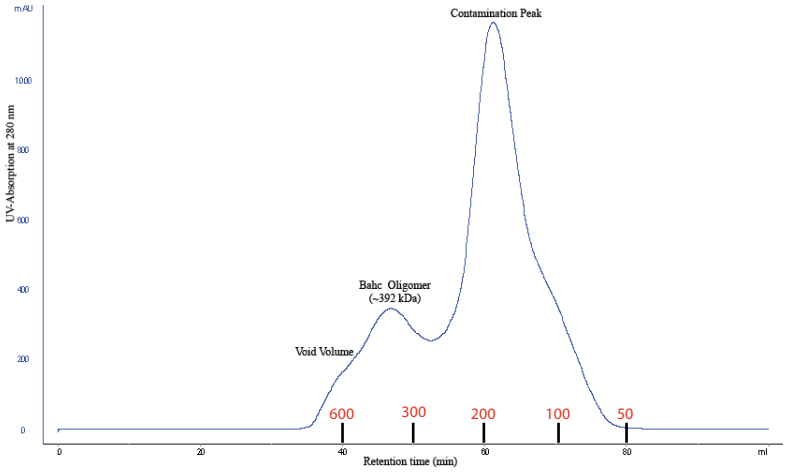
Notes: The samples were run on a 4% to 20% Tris-glycine SDS-PAGE gel. Lanes 1–3 (solubilisation with DDM), total lysate, supernatant and pellet; similarly lanes 4–6 (solubilisation with LSN), lanes 7–9 (solubilisation with DDAO), lanes 10 and 11 (solubilisation with OG), and lane 12 molecular weight marker. Though many detergents were tried, only four of them are shown here. While a portion of the protein still remains in pellet (lanes 3 and 9), at least most of it appears in supernatant with LSN (lane 6). The pellet of OG solubilised is not shown. Compared to other detergents and non-detergent sulfobetamine, LSN works the best.

Figure 2 Overexpression and Ni-NTA purification of BAHC



Notes: The samples were run on a 4% to 20% Tris-glycine SDS-PAGE gel. Lane 1. Supernatant after cell lysis of overnight auto-induced cells; lane 2. flow through from Ni-NTA column; lanes 3–8: 20, 50, 250, 250, 250, 500 mM imidazole elute fractions from Ni-NTA column; lane 9: molecular weight marker.

Figure 3 The size exclusion profile of the BAHC

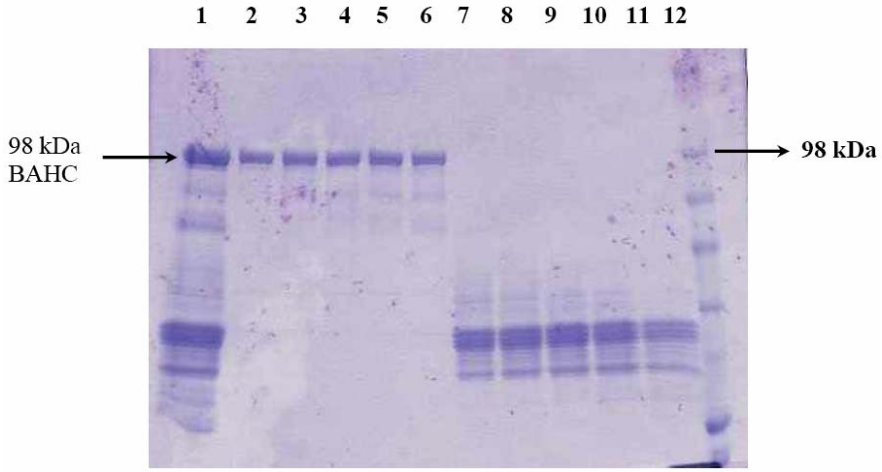


Notes: 5ml of Ni-affinity purified sample was loaded into a preparative S300 column. Protein sizes are shown in red colour along the X-axis. BAHC-LSN oligomer has an apparent molecular mass of ~392 kDa corresponding to a tetramer of BAHC (98 kDa).

The protein was further purified by a size-exclusion (Sephacryl-300) column that had previously been equilibrated with 50 mM Tris, pH 8.5, 300 mM NaCl, 0.1% LSN and 1 mM β-Me. The elution profile, shown in Figure 3 indicates that the protein eluted as tetramer. Highly purified protein obtained after the size exclusion column is shown in SDS-PAGE (Figure 4). Three litres of auto-induction medium yielded about 2 mg protein

with > 90% purity. Western blot using polyclonal rabbit IgG of BoNT/A confirmed the protein is BAHC.

Figure 4 Gel-filtration chromatography of BAHC



Notes: The samples were run on a 4% to 20% Tris-glycine SDS-PAGE gel. Lane 1. pooled fractions of Ni-NTA column before size-exclusion chromatography, lanes 2–6, fractions of BAHC, lanes 7–11, fractions of contamination, lane 12 molecular weight marker.

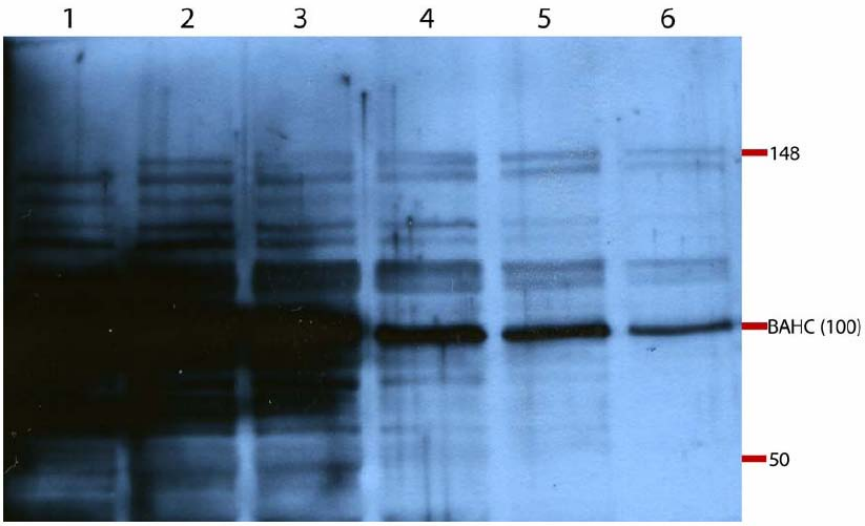
2.2.4 Western blot analysis

Extracted BAHC proteins after his-tag purification were separated by SDS-PAGE on 4% to 20% gradient polyacrylamide gels and electrophoretically transferred onto Nitrocellulose membranes (Figure 5). The membranes were probed with BAHC primary antibody raised against rabbit, followed by incubation with horseradish peroxidase-coupled secondary antibody. Detection was performed with a chemiluminescence-based detection kit.

2.3 Crystallisation and preliminary crystallographic data analysis

Crystals were grown at room temperature with 20% PEG 3350, 0.2M Tri-sodium citrate as precipitant (1 µl reservoir solution plus 1 µl protein at 5 mg ml^{-1} in 50 mM Tris-HCl pH 8.5, 300 mM NaCl, 0.1% LSN and 1 mM β-Me) using the sitting drop vapour diffusion technique. Crystals appeared within 15 days. Crystals were very briefly soaked in the mother liquor containing 15% glycerol added as cryoprotectant and immediately flash frozen in liquid nitrogen. X-ray diffraction data were collected at beamline X29 of NSLS (National Synchrotron Light Source, Brookhaven National Laboratory, New York) and were processed using HKL2000 (Otwinowski and Minor, 1997). The diffraction quality was both poor (maximum resolution ~6 Å) and anisotropic.

Figure 5 Western blot analysis of BAHC after Ni-NTA affinity chromatography



Note: Lanes 1 to 6 corresponds to 20 mM, 50 mM, 100 mM elution 1, 100 mM elution 2, 250 mM and 500 mM concentration of imidazole respectively.

3 Results and discussion

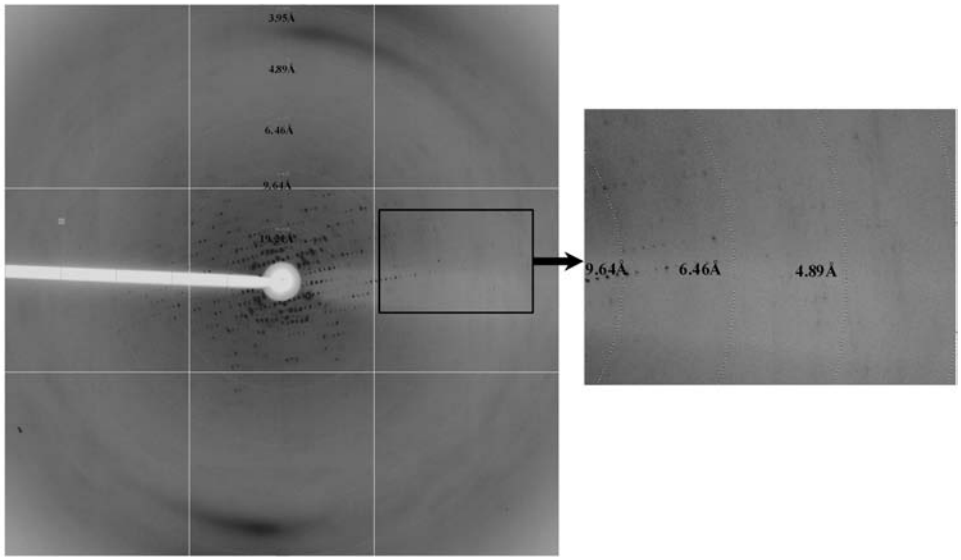
The BAHC contains residues from Ala448 to Leu1295 with a C-terminal 6X-His tag to enable purification by metal affinity chromatography. However, in the absence of a detergent, it forms inclusion bodies presumably due to the hydrophobic nature of the translocation domain. Similar results were obtained when auto induction was carried out at 20 and 37°C. Variation in cell growth incubation temperature, time and shaker speed did not yield the soluble protein. Expression of BAHC using conventional IPTG induction method did not help either. Initial screening of solubilsation was performed with 13 different detergents/sulfobetaines that include mild non-ionic detergents such as n-Dodecyl-β-D-maltoside (DDM) and n-Octyl-β-D-glucopyronoside (OG) (Table 1). Only LSN gave soluble protein and accordingly 0.1% LSN was used in buffers throughout BAHC purification process including the initial step of Ni-NTA affinity chromatography. Size exclusion chromatography shows that BAHC is eluted from the NTA resin as tetramer according to elution profile relative to known mol. wt. standards (Figure 3).

Different attempts were made to reduce the detergent concentration from the sample buffer prior to crystallisation but ended up in precipitation. Therefore, BAHC fractions collected from the size exclusion chromatography is used as such in the same LSN concentration for further crystallisation trials. The purified BAHC was crystallised by the sitting drop vapour diffusion method (Figure 6). The denaturation followed by refolding experiment on BAHC was not successful in terms of getting crystals and hence it is not discussed here. Crystals of BAHC diffracted only up to ~6 Å, belong to the C-centred orthorhombic space group and contain four molecules in the asymmetric unit. A representative diffraction pattern is shown in Figure 7 and data statistics are given in

Table 2. A self-rotation Patterson map was computed using MOLREP (Ccp4, 1994) which confirmed the presence of a non-crystallographic four-fold symmetry (Figure 8) consistent with tetrameric association seen in the sizing profile.

Figure 6 Representative crystals of the BAHC

Figure 7 Diffraction pattern from the recombinant BAHC crystal

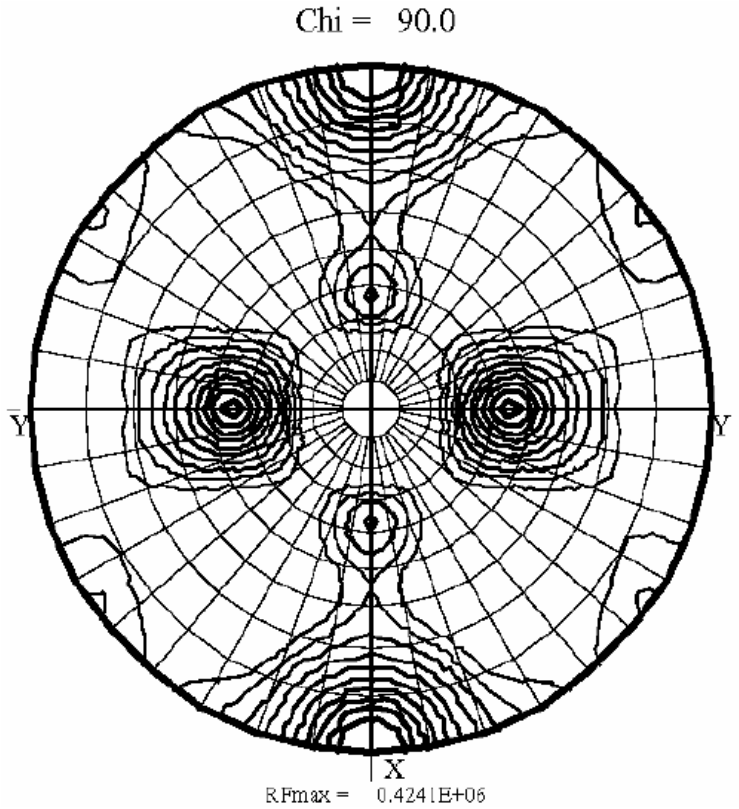


Note: The crystals diffract to better than 6.0 Å resolution. The inset shows the extent of the resolution limit.

Table 2 Data collection statistics of recombinant BAHC

Cell parameters	a = 176.33, b = 225.35 and c = 218.20 Å
Space group	C222
Resolution	50-6.0Å
No. of reflection	32,674
I/σ(I)	6.4
R_{merge}	0.08 (0.41)
Redundancy	3.0

Figure 8 $\chi = 90°$ section of the stereographic projection of Patterson self-rotation function calculated with reflections in the resolution range 50-6.0Å



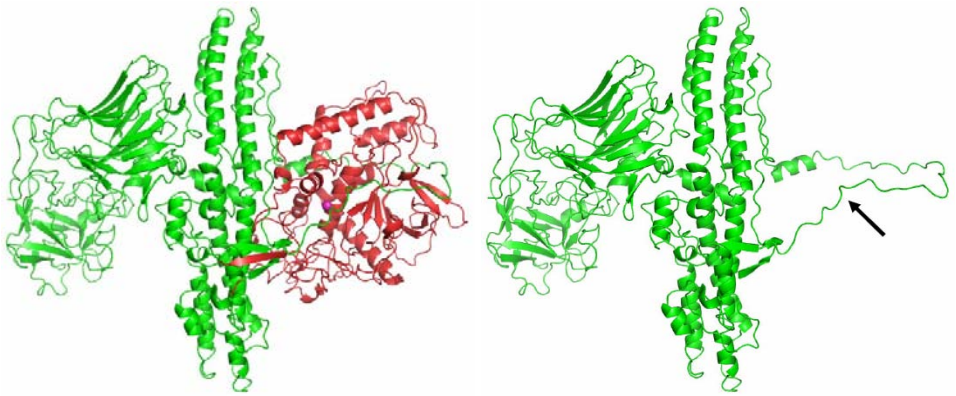
Note: The non-crystallographic four-fold axis is seen at $\theta = 45°$ and $\phi = 90°$.

BoNT HC channels in lipid bilayers have been studied for the last three decades (Blaustein et al., 1987; Donovan and Middlebrook, 1986; Hoch et al., 1985; Lebeda and Singh, 1999). Electron microscopy studies in the presence of phospholipid bilayers suggest that BoNT oligomerises to a tetrameric state to form a LC conducting channel (Schmid et al., 1993). Similarly, BoNT/C also forms a channel with two or more monomers (Donovan and Middlebrook, 1986). The initial and the final steps of the translocation process are known but the actual translocation process remains unexplained.

Crystal structures of intact BoNT/A, BoNT/B and BoNT/E are available. BoNT/E differs in domain organisation from BoNT/A and BoNT/B though all of them share significant sequence homology. BoNT/E has inherent translocation-competent domain organisation which causes faster action compared to BoNT/A (Kumaran et al., 2009). BoNT/A or /B has to be reoriented in the vesicle about the binding domain for it to attain a translocation competent conformation (Fu et al., 2009). It is also suggested that the translocation and catalytic domains might rotate about a flexible hinge region between the binding and translocation domains (Fu et al., 2009; Kumaran et al., 2009) to bring the transmembrane region of the translocation domain close to the vesicle membrane. Hence, there is a possibility for the presence of intrinsic domain flexibility which might be an impediment for the formation of good diffracting quality crystals of the HC domain

alone. This idea is consistent with electron microscopy studies on intact BoNT/E which show the presence of at least three types of domain organisation (Fu et al., 2009). A ribbons representation of BoNT/A is shown in Figure 9(a). The belt region which is part of the translocation domain wraps around the LC and acts as an intramolecular chaperone (Brunger et al., 2007) and without this belt the translocation domain has higher tendency to aggregate (Galloux et al., 2008). When the LC is deleted from the construct the belt region and one side of the translocation domain lose the hydrophobic contacts provided by the LC [Figure 9(b)]. Also, the belt region forming a long loop becomes flexible and can take various conformations. Because of this and the exposure of hydrophobic core, the protein tends to aggregate and probably is the reason for it going into inclusion bodies. The translocation domain of BoNT/A binds to anionic membrane and then permeates the bilayer without any detectable structural change observed by vesicle permeabilisation assays and also the belt region limits protein-membrane interaction (Galloux et al., 2008). In the absence of the LC, the hydrophobic regions of the translocation domain including the belt region are exposed causing aggregation and the use of detergents helps to avoid this problem. The flexibility of the belt region also may be partially compensated by the HC oligomerisation.

Figure 9 (a) Ribbons representation of BoNT/A molecule* (b) BAHC alone is shown**



(a) (b)

Notes: *This figure was created using PDB id: 3BTA and was drawn with Pymol (Delano, 2002). The HC, BAHC, is shown in green and the LC is shown in red. Zinc ion at the active site is shown as magenta sphere. The belt region wrapping around the LC is obvious in this figure. **When the LC is removed the belt region (shown by arrow mark) becomes very flexible since it loses all its interaction with the LC.

While crystallising the HC alone, the inherent flexibility of binding domain and the belt region might cause disorder in the crystal lattice; the presence of detergent mimicking a membrane environment may also be responsible for poor diffraction quality crystals. BoNT/A protein is suggested to make a channel immediately after the membrane insertion (Fischer and Montal, 2007b). Accordingly, the channel or oligomerisation state of BAHC might have occurred because of the use of detergent from the initial step of protein extraction.

4 Conclusions

The HC of BoNT/A was solubilised with detergent LSN and its gel filtration column chromatography showed that the oligomeric state is a tetramer. A self-rotation plot from the X-ray diffraction data shows a four-fold symmetry in the crystal structure. This is the first crystallographic study showing support for tetrameric pore formation by the BAHC for LC translocation. In this study, the oligomeric state was obtained in the presence of detergent that partly mimics the channel formation at endosomal membrane although at different pH. Further high resolution crystal structure might reveal the pore formation and help in understanding the translocation mechanism.

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