Biochemical and Biophysical Research Communications 404 (2011) 407–412

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structure of the receptor binding domain of the botulinum C–D mosaic neurotoxin reveals potential roles of lysines 1118 and 1136 in membrane interactions

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ARTICLE INFO

Article history:
Received 18 November 2010
Available online 3 December 2010

Keywords:
Botulinum neurotoxin
C–D mosaic
Botulism
Phosphatidylethanolamine
Membrane recognition

ABSTRACT

The botulinum neurotoxins (BoNTs) produced by different strains of the bacterium *Clostridium botulinum* are responsible for the disease botulism and include a group of immunologically distinct serotypes (A, B, E, and F) that are considered to be the most lethal natural proteins known for humans. Two BoNT serotypes, C and D, while rarely associated with human infection, are responsible for deadly botulism outbreaks afflicting animals. Also associated with animal infections is the BoNT C–D mosaic protein (BoNT/CD), a BoNT subtype that is essentially a hybrid of the BoNT/C (~two-third) and BoNT/D (~one-third) serotypes. While the amino acid sequence of the heavy chain receptor binding (HCR) domain of BoNT/CD (BoNT/CD-HCR) is very similar to the corresponding amino acid sequence of BoNT/D, BoNT/CD-HCR binds synaptosome membranes better than BoNT/D-HCR. To obtain structural insights for the different membrane binding properties, the crystal structure of BoNT/CD-HCR (S867-E1280) was determined at 1.56 Å resolution and compared to previously reported structures for BoNT/D-HCR. Overall, the BoNT/CD-HCR structure is similar to the two sub-domain organization observed for other BoNT HCRs: an N-terminal jellyroll barrel motif and a C-terminal β-trefoil fold. Comparison of the structure of BoNT/CD-HCR with BoNT/D-HCR indicates that K1118 has a similar structural role as the equivalent residue, E1114, in BoNT/D-HCR, while K1136 has a structurally different role than the equivalent residue, G1132, in BoNT/D-HCR. Lysine-1118 forms a salt bridge with E1247 and may enhance membrane interactions by stabilizing the putative membrane binding loop (K1240-N1248). Lysine-1136 is observed on the surface of the protein. A sulfate ion bound to K1136 may mimic a natural interaction with the negatively changed phospholipid membrane surface. Liposome-binding experiments demonstrate that BoNT/CD-HCR binds phosphatidylethanolamine liposomes more tightly than BoNT/D-HCR.

1. Introduction

The anaerobic, gram-positive bacterium, *Clostridium botulinum*, is a NIAID category A pathogen responsible for botulism and produces the most toxic biological substances known, botulinum neurotoxins (BoNTs) [1]. The extreme potency of BoNTs is due to their neurospecific interactions with unmyelinated regions of nerve terminals where they inhibit the release of the neurotransmitter, acetylcholine, leading to flaccid muscular paralysis [2]. While the frequency of natural botulism is low, the high mortality rate of the untreated disease makes it an important human health issue and there is concern for the potential use of BoNTs as agents of bioterrorism [3]. Botulism is also extremely lethal to livestock and responsible for significant economic losses worldwide [4].

Seven BoNT serotypes (A–G) have been identified based on neutralization characteristics of antiserum. Serotypes A, B, E, and F are associated with human botulism while serotype C and D are predominately associated with animal botulism [5–7]. In addition to the seven basic serotypes, numerous sub-serotypes have also been identified. Two such sub-serotypes are BoNT mosaic proteins C–D and D–C isolated from *C. botulinum* strains causing animal botulinum in Africa and Japan [4,7], that are essentially hybrids of

Abbreviations: BoNT, botulinum neurotoxin; BoNT/CD-HCR, the HCR domain of BoNT C–D mosaic serotype; BoNT/D-HCR, the HCR domain of BoNT serotype D; Hc, BoNT C-terminal 100 kDa heavy chain; Hcc, the C-terminal sub-domain of HCR; Hcn, the N-terminal sub-domain of HCR; HCR, the receptor binding domain; Lc, BoNT N-terminal 50 kDa light chain; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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doi:10.1016/j.bbrc.2010.11.134

BoNT/C and BoNT/D. The C–D mosaic (BoNT/CD) is approximately two-third BoNT/C and one-third BoNT/D while the D-C mosaic is approximately two-third BoNT/D and one-third BoNT/C. All BoNTs are initially translated as ~150 kDa single chain polypeptides that are proteolytically activated into a re-associated molecule consisting of an N-terminal ~50 kDa light chain (Lc) and the C-terminal ~100 kDa heavy chain (Hc) held together with a single disulfide bond [8]. The Lc polypeptide is a zinc-dependent endopeptidase while the Hc polypeptide is divided into an N-terminal translocation domain and C-terminal receptor binding domain (HCR) of approximately equal length. The HCR domain may be further divided into two sub-domains: a C-terminal Hcc sub-domain involved in cell surface recognition and an N-terminal Hcn sub-domain with an unclear function, although it has been suggested that it may interact with phosphoinositides [9]. Following endocytosis into neuronal cells, a conformational change triggered by the acidic pH environment inside the endosomes releases the Lc domain to the cytosol [10,11] where it inhibits the function of acetylcholine by hydrolyzing SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptors) proteins.

The internalization of BoNTs into the host neuronal cells, via endocytosis after the HCR domain binds to the surface of neuronal cells, is crucial for the progression of the disease. Uptake of serotype A, B, E, F, and G occur via a dual receptor mechanism [12] involving a synaptic vesicle protein (Synaptotagmin or synaptic vesicle protein 2) and a ganglioside (reviewed by [13]). The mechanism by which BoNT serotypes C and D enter neuronal cells is not clear. Neurotoxicity is dependent upon ganglioside binding for both C and D [14,15], although a ganglioside binding motif SxWY does not exist in the BoNT/D amino acid sequence. Additionally, protein receptors have not been identified for either BoNT/C or BoNT/D serotype [16]. Furthermore, BoNT/CD-HCR bound to synaptosomes more tightly than BoNT/D-HCR [16,17], and K1118 and K1136 of BoNT/CD were identified as key residues critical for the binding activity [17]. These two lysine residues are absent in BoNT/D. To obtain insights into the entry mechanism for BoNT/D and BoNT/CD, the crystal structure for the BoNT/CD-HCR was determined at 1.56 Å resolution. The structure was compared to previously reported structures for BoNT/D-HCR [15,18,19] and potential roles of residues K1118 and K1136 of BoNT/CD in membrane interactions were analyzed.

2. Materials and methods

2.1. Protein expression and purification

The DNA sequence encoding the receptor binding domain of *C. botulinum* BoNT/CD (S867-E1280) was codon-optimized for expression in *Escherichia coli* with a C-terminal 6× his tag (DNA 2.0) and cloned into the expression vector pJexpress411 (see Fig. S1 for the sequence of the optimized cDNA). This recombinant vector was then transformed into the host *E. coli* bacterial strain BL21 (DE3). Greater than 98% pure BoNT/CD-HCR was obtained following the protocols previously described in detail for BoNT/D-HCR [18,29] using the antibiotic kanamycin during cell growth in LB media, isopropyl-β-D-1-thiogalactopyranoside induction at 12 °C, initial purification on a Ni–NTA agarose column (Qiagen), and final purification on a HiTrap Q ion exchange column (GE Healthcare).

2.2. Liposome-binding assay

Phosphatidylcholine (PC) (Avanti Polar Lipids) and a 1:1 mixture of PC and bovine brain phosphatidylethanolamine (PE) (Sigma, P7693), were first dissolved in chloroform and then dried under a stream of nitrogen. Following resuspension in TBS buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.0), the lipids were incubated at 30 °C for 1 h, vortexed for 5 min, and centrifuged at 20,000*g* (4 °C) for 10 min. One hundred μg liposome pellets were resuspended in 50 μl binding buffer (TBS with 2 mM $MgCl_2$). The binding reaction was then performed by adding 2 μg of purified BoNT/CD-HCR to the resuspended liposomes. Following incubation at 30 °C for 30 min, the solution was centrifuged at 20,000*g* (4 °C) and the distribution of BoNT/CD-HCR in the pellet and supernatant fractions analyzed by SDS–PAGE [18].

2.3. Crystallization and structure determination

Crystallization screening was performed using the hanging-drop vapor diffusion method. The best crystals grew with mother liquor composed of 20% (w/v) PEG 8000, 10% (w/v) isopropanol, 0.2 M $(NH_4)_2SO_4$, and 0.1 M Hepes, pH 7.5. Crystals were harvested by stepwise transfer into cryoprotectant solutions containing increasing concentrations of glycerol for cryoprotection. X-ray diffraction data was collected at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory on beamline X29A. Data was processed using *DENZO*, and integrated intensities scaled using the *SCALEPACK* from the *HKL-2000* program package [20]. Original phasing for the BoNT/CD-HCR crystal was obtained by molecular replacement using the *CCP4* suite of programs [21] and the crystal structure of the HCR domain of BoNT/D (PDB ID 3OGG) for the molecular search template [18]. The model was further refined using *Coot* [22] and *PHENIX* [23]. Structural validation was performed using *Molprobity* [24]. Coordinates and structure factors for BoNT/CD-HCR have been deposited in the Protein Data Bank (PDB) with Accession No. 3PME.

3. Results and discussion

3.1. Overall structure

The crystal structure of BoNT/CD-HCR (S867-E1280) at 1.56 Å resolution was solved using a molecular replacement method. The asymmetric unit of the crystal contained a single protein unit, two sulfate ions, a glycerol molecule, and 374 water molecules. The data collection and refinement statistics, summarized in Table S1, show that the final model was a quality representation of the BoNT/CD-HCR structure. Electron density could be modeled into the entire protein except for the final two histidine residues on the C-terminal poly-histidine tag and residues S867, N1218, and K1219. The overall structure of BoNT/CD-HCR, shown in Fig. 1, is similar with that of BoNT/D-HCR and the other serotype HCR domains. It contains two sub-domains, Hcn (S867-S1072) and Hcc (L1085-E1280), connected by a helix (N1073-I1084). The Hcn sub-domain folds into a jelly roll barrel motif and the Hcc sub-domain adopts a β-trefoil fold.

3.2. Structural comparison to BoNT/D-HCR

The amino acid sequence of BoNT/CD-HCR is highly similar with that of BoNT/D-HCR. While the overall tertiary structure for the two proteins is consequently very similar (Fig. 1A and B), the two structures contain some differences as the backbone RMSD of the ordered regions, when superimposed by the program SuperPose [25], is 1.05 Å. Many of these differences are due to minor variations in the length of corresponding β-strands and α-helices that are likely without functional consequences. However, a few of the structural differences located on the surface of the protein may be responsible for some of the different synaptosome binding properties observed between BoNT/CD-HCR and BoNT/D-HCR.