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Structural analysis of the receptor binding domain of botulinum neurotoxin serotype D

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Abstract

Botulinum neurotoxins (BoNTs) are the most toxic proteins known. The mechanism for entry into neuronal cells for serotypes A, B, E, F, and G involves a well understood dual receptor (protein and ganglioside) process, however, the mechanism of entry for serotypes C and D remains unclear. To provide structural insights into how BoNT/D enters neuronal cells, the crystal structure of the receptor binding domain (S863-E1276) for this serotype (BoNT/D-HCR) was determined at 1.65 Å resolution. While BoNT/D-HCR adopts an overall fold similar to that observed in other known BoNT HCRs, several major structural differences are present. These structural differences are located at, or near, putative receptor binding sites and may be responsible for BoNT/D host preferences. Two loops, S1195-I1204 and K1236-N1244, located on both sides of the putative protein receptor binding pocket, are displaced >10 Å relative to the corresponding residues in the crystal structures of BoNT/B and G. Obvious clashes were observed in the putative protein receptor binding site when the BoNT/B protein receptor synaptotagmin II was modeled into the BoNT/D-HCR structure. Although a ganglioside binding site has never been unambiguously identified in BoNT/D-HCR, a shallow cavity in an analogous location to the other BoNT serotypes HCR domains is observed in BoNT/D-HCR that has features compatible with membrane binding. A portion of a loop near the putative receptor binding site, K1236-N1244, is hydrophobic and solvent-exposed and may directly bind membrane lipids. Liposome-binding experiments with BoNT/D-HCR demonstrate that this membrane lipid may be phosphatidylethanolamine.

Keywords

botulinum neurotoxin D; crystal structure; botulism; phosphatidylethanolamine; gangliosides

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1. Introduction

Botulinum neurotoxins (BoNTs) produced by the bacterium *Clostridium botulinum*, are highly toxic proteins with an extremely low LD₅₀ of 1 ng/kg [1]. These neurotoxins damage the nervous system by inhibiting the release of a neurotransmitter, acetylcholine, resulting in flaccid paralysis [2]. Among seven serotypes (A-G) of BoNTs, A, B, E, F, and G are responsible for human botulism, while C and D predominately cause avian and animal botulism [3].

Botulinum neurotoxins are translated as a 150 kDa single chain protein that is subsequently processed into two components by proteases. The two components then re-link via the formation of a disulfide bond between the N-terminal 50 kDa light chain and the C-terminal 100 kDa heavy chain [4]. The heavy chain can be further divided into the 50 kDa N-terminal translocation domain and the 50 kDa C-terminal receptor binding domain (HCR). The three domains form a tri-molecular architecture that controls the mechanism of cellular intoxication [5]. The light chain possesses a Zn²⁺ dependant endoprotease activity and cleaves proteins that are essential for neurotransmitter release such as SNAP-25 (synaptosome associated protein of 25 kDa) and synaptobrevin. While different serotypes recognize different substrates each enzymatic mechanism is similar. BoNT/A, C, and E cleave SNAP-25 and serotypes B, D, F, and G cleave synaptobrevin [6,7,8,9]. The translocation domain is involved in the transportation of BoNTs into cytosol, while the HCR domain is responsible for interactions with neuronal membranes. The HCR domain contains two sub-domains: Hcn and Hcc. While the Hcc sub-domain is involved in cell surface recognition, no function for the Hcn sub-domain has been identified.

The initial step of BoNT intoxication, entering the host cells, occurs via a “dual receptor” mechanism [10,11] that involves binding with a protein and ganglioside on neuronal cell surfaces. Binding to the protein receptor with high affinity is required for membrane recognition. Two major types of botulinum neurotoxin membrane protein receptors have been identified: synaptotagmin (Syt) I and II, and synaptic vesicle protein 2 (SV2). The B and G serotypes bind Syt I or II [12,13], and serotypes A, E, and F bind SV2 [14,15,16,17]. To date, a protein receptor for BoNT/C and D has not been identified. In addition to binding a protein receptor, all the serotypes, except for BoNT/D, bind a second ganglioside receptor. An SxWY ganglioside binding motif has been identified in all the serotypes, except BoNT/D, and it appears that this ganglioside binding motif prefers to interact with ganglioside possessing sialic acid moieties [5]. In contrast, the C-termini of the HCR domain from serotypes C/D mosaic and D were reported to interact with phosphatidylethanolamine (PE) [18,19].

Crystal structures are available for the A, B, and E holotoxins, all the serotype light chains, and the HCR domains of BoNT/F and G [20,21,22]. In addition, co-crystal structures of BoNT/B and BoNT/A-HCR in complex with a Syt II peptide and with the GT1b analog, respectively, have also been determined [23,24,25]. The latter two complex structures revealed detailed structural information on receptor and co-receptor binding that is likely to extend to all the BoNT serotypes. In order to examine the receptor binding domain of BoNT/D, we have determined the crystal structure of the HCR domain of BoNT/D at a resolution of 1.65 Å. By comparing the structure of BoNT/D-HCR with the other serotypes HCR structures, putative receptor binding sites on the surface of BoNT/D were identified. Further, we have demonstrated that BoNT/D-HCR binds PE using a liposome-binding assay.

2. Materials and Methods

2.1. Protein expression, purification, and crystallization

Details of the DNA cloning, protein expression and purification, and crystallization of BoNT/D-HCR are described elsewhere (Y Zhang, X Gao, L Qin, GW Buchko, H Robinson, and SM Varnum, manuscript submitted). Briefly, the BoNT/D-HCR protein was expressed using a codon-optimized cDNA in *Escherichia coli*, purified using Ni-NTA, ion exchange, and size exclusion chromatography, and crystallized using a hanging drop vapor diffusion method.

2.2. Liposome-binding assay

The lipids, phosphatidylcholine (PC) (Avanti Polar Lipids) and a 1:1 mixture of PC and phosphatidylethanolamine (PE) (Sigma), were dissolved in chloroform and dried under a nitrogen stream. The phospholipids were then resuspended in TBS buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.0) and incubated at 30 °C for 1 h. The solution was vortexed for 5 min and centrifuged at 20,000×g at 4 °C. Pellets containing 100 µg liposomes were resuspended in 50 µl TBS containing 2 mM MgCl₂. The binding reaction was performed by adding 2 µg of purified BoNT/D-HCR to the resuspended liposomes and incubating at 30 °C for 30 min. The solution was then centrifuged at 20,000×g at 4 °C and the distribution of BoNT/D-HCR in the pellet and supernatant fractions determined by SDS-PAGE.

2.3. Structural determination and refinement

Original phases for the BoNT/D-HCR crystals were obtained by molecular replacement using the program *BALBES* in the automated crystal structure determination platform *Auto-Rickshaw* [26]. The crystal structure of the HCR domain of BoNT/B (PDB ID 1EPW) was used for the molecular search template [27]. The model was further refined using *Coot* [28] and *PHENIX* [29] (Table S1). Structural validation was performed using *Molprobit* [30]. Coordinates and structure factors for BoNT/D-HCR have been deposited in the Protein Data Bank (PDB) with accession numbers 3OGG.

3. Results and Discussion

3.1. Overall structure

The 1.65 Å resolution crystal structure of BoNT/D-HCR (S863-E1276) was solved using molecular replacement with the structure of BoNT/B as the original molecular search template (PDB entry 1EPW) [27]. One protein molecule was observed in the asymmetric unit and the absence of significant intermolecular contacts with other protein molecules in the crystal lattice suggests that BoNT/D-HCR is a monomer in solution. The final model contained 404 amino acid residues plus 508 water molecules. Electron density for residues Y1176 through Q1185 was poor, and consequently, these residues could not be reliably modeled into the final structure. The structure refinement statistics, summarized in Table S1, indicate that the final model was a quality representation of the structure of BoNT/D-HCR. The overall structure of BoNT/D-HCR contains two distinct sub-domains, Hcn (S863-S1068) and Hcc (L1081-E1276), connected by a helix, N1069-I1080 (Fig. 1A). Sixteen anti-parallel β-strands in the Hcn sub-domain fold into a jelly roll barrel motif and form the core of the Hcn sub-domain. The Hcc sub-domain has 12 β-strands forming six two-strand anti-parallel β-sheets that are organized into a β-trefoil fold.

3.2. Comparison to other HCR domains

Amino acid sequence comparisons show that BoNT/D-HCR has a low sequence identity with HCRs from other serotypes (40% to C-HCR, 33% to B-HCR, ~30% to A, E, F, and G-

HCRs). However, the overall tertiary structure of BoNT/D-HCR is similar to those of the other BoNT HCRs, as the R.M.S.D (root-mean-square deviations) values from pair-wise structure comparison are on average 2.2 Å (Table S2).

The overall structure of the Hcn sub-domain is largely conserved with the Hcn sub-domains of the other serotypes, although a few minor shifts and length differences occur in some of the β -strands. Perhaps the most noteworthy differences relative to the Hcn sub-domain of the other serotypes are with two inter β -strand regions, N917-S931 and I939-Y950. The N917-S931 region is a loop that is longer and protrudes to the outside of the molecule in comparison to the same region in the other serotypes that are shorter and fold towards the interior of the molecule (Fig. S1). This protrusion may provide opportunities to interact with other regions of the full-length protein or other molecular partners. In contrast, the I939-Y950 region is shorter and indented towards the interior of the molecule with a six-residue helix in the middle of the region (Fig. S1). A similar helix appears in the same region of serotypes B, F, and G but not in A and E, all of which are longer and wider. The I939-Y950 region has a shape that is similar to a β -barrel lid and might act to exclude other molecules from the interior of BoNT/D-HCR. Because the function of the Hcn sub-domain has not been identified, it is difficult to determine the significance of these structural differences.

The structural analysis of the Hcc sub-domain identifies a number of differences between BoNT/D-HCR and the HCR domains from other BoNT serotypes, especially between the analogous regions of the putative receptor and co-receptor binding sites. The largest structural differences are the S1195-I1204 and the K1236-N1244 loops, which both shift >10 Å (Fig. 1B, S1). In addition, the V1251-F1264 region moves towards the interior of the protein approximately 3~4 Å and contains two short 3_{10} -helices (3-4 residues each) on both ends (Fig. 1B, S2). The similar regions of the other BoNT serotypes form a helix containing approximately eight residues in the middle of the region (except for BoNT/A-HCR in complex with the GT1b analog). Because of the importance of these regions for receptor and co-receptor binding, they will be discussed in more detail below.

A loop, Q1122-I1135, near the connection between the Hcn and Hcc sub-domains, is also different between the BoNT/D-HCR domain and the analogous region in the other serotypes (Fig. S1). In the other serotypes this same region is longer and forms either a loop (BoNT/B, E) or anti-parallel β -sheets (BoNT/A, F, G). In both cases, the result is that the Hcc and Hcn sub-domains are further apart in the BoNT/D serotype than in the other serotypes. The increased proximity of the Hcc and Hcn sub-domains in the other BoNT serotypes may result in a uniform rotational correlation time for the HCR domain while the two sub-domains of BoNT/D-HCR may tumble more independently.

3.3. Analysis of potential protein receptor binding site

To obtain insights into how BoNT/D-HCR interacts with a protein receptor, the structure of BoNT/D-HCR was compared to the co-crystal structure of BoNT/B in complex with the Syt II recognition domain peptide [23,24]. The BoNT/B-Syt II crystal structure identified a hydrophobic groove on the surface of the receptor binding domain critical for Syt II binding. Likewise, the BoNT/D-HCR crystal structure contains an open groove in the analogous location, however, in BoNT/D-HCR this region shows several large structural differences relative to the BoNT/B Syt II binding site (Fig. 2). First, the K1236-N1244 loop, which is present in BoNT/B and G, but not in BoNT/A, E, and F, shifts approximately 13 Å into the groove. When the Syt II peptide from the co-structure of BoNT/B-Syt II was modeled into the BoNT/D-HCR structure, an obvious clash was observed between the tip of the K1236-N1244 loop and the C-terminus of Syt II (Fig. 2A). Such a clash suggests that it is highly unlikely that BoNT/D interacts with Syt II in this groove. Second, the S1195-I1204 loop on the opposite side of the groove rotates approximately 10 Å in the same direction as the

K1236-N1244 loop, but, towards the outside of the groove (Fig. 2A). The parallel movement of the two loops between the groove keeps the groove “open” as observed in the other serotypes. Third, while this groove is large enough to accommodate a protein receptor, the amino acid composition of the groove may prohibit interaction with a Syt II-like protein receptor (Fig. 2B). From the structural analysis of the BoNT/B-Syt II co-structure, it was observed that hydrophobic contacts between aromatic residues in BoNT/B and Syt II were critical for receptor binding [23,24]. Although there are two phenylalanines and a tryptophan present in the K1236-N1244 loop, there is not an adequate aromatic residue cluster inside the putative protein receptor binding groove of BoNT/D-HCR that is capable of providing the π -stacking interactions required for binding with an Syt II-like peptide.

No co-crystal structures are available for BoNT with SV2, however, the co-crystal structure of BoNT/B with Syt II and mutagenesis analyses indicate that the other BoNT serotypes may bind their protein receptor at an analogous location as observed in the Syt II binding site of BoNT/B [23,24]. As shown for Syt II, the K1236-N1244 loop of BoNT/D-HCR may also sterically prevent BoNT/D from binding SV2. Instead, the positively charged and hydrophobic residues in the K1236-N1244 loop may be responsible for binding to a protein receptor markedly different than Syt II or to a lipid molecule.

3.4. PE liposome-binding experiments and potential BoNT/D interactions with PE

Using thin layer chromatography, the BoNT/D-HCR domain was reported to bind PE [18]. Since liposome vesicles are a better approximation of the *in vivo* lipid membrane structure and environment than immobilized lipids, a liposome-binding assay was used to confirm the interaction of BoNT/D-HCR with PE. Liposomes containing PC and PE were incubated with purified BoNT/D-HCR followed by centrifugation. Proteins that bind to lipids will be spun down with the liposomes and found in the pellet. BoNT/D-HCR was recovered in the pellet using liposomes composed of PE and PC but not PC alone (Fig. 3A), indicating that BoNT/D-HCR interacts with PE in a membrane environment.

Without a co-crystal structure of PE bound to BoNT/D-HCR, it is difficult to determine the amino acids that interact with the head groups of the lipid. However, evidence from site-directed mutagenesis experiments indicated that K1117 and K1135 of the BoNT/CD mosaic serotype are important for HCR binding to rat brain synaptosomes, and this binding is probably mediated by PE [19]. Although BoNT/D-HCR does not possess these two lysine residues, there are other lysine residues near one of the corresponding residues, E1114, which might be capable of binding PE. One potential candidate is K1243, a residue in the K1236-N1244 loop that straddles the putative protein receptor binding groove (Fig. 3B). Lysine-1243 is oriented in the same direction as E1114 but is displaced ~ 4 Å away from it. The entire K1236-N1244 loop itself is also a good candidate, not only because of K1243 and the loop's proximity to the putative protein receptor binding site, but also, because this loop contains both positively charged and aromatic residues (Fig. 2B) to balance the hydrophobicity of PE's fatty acid chains. The second residue corresponding to one of the two PE binding lysines, G1132, is located near the linker between the Hcn and Hcc sub-domains (Fig. 3B) and does not have any basic neighbors within reasonable distance to directly bind PE. Therefore, it appears likely that the K1236-N1244 loop has the capability of interacting with PE but unlikely that residue G1132 can interact with PE.

3.5. Analogous region of co-receptor binding site

As discussed above, all the BoNT serotypes and TeNT, except BoNT/D, have been shown to bind gangliosides as co-receptors. Other than PE, it is not known what, if any, co-receptor BoNT/D utilizes to enhance membrane recognition. For the BoNT serotypes that bind gangliosides, these interactions are facilitated by a SxWY motif located in the C-terminus of

the HCR domain [5] (Fig. S3). BoNT/D lacks an SxWY motif. Nevertheless, the C-terminal region of BoNT/D-HCR was compared with the corresponding region responsible for binding co-receptor from the co-structure of BoNT/A-HCR in complex with a ganglioside analog. Surprisingly, other than the lack of the SxWY motif, the three-dimensional structure of this region in BoNT/D is fairly conserved. A shallow cavity is identified that is similar in size and shape with the gangliosides binding site of BoNT/A (Fig. 4A). The V1251-F1264 region and the N1103-D1108 loop move approximately 3~4 Å towards the interior of the molecule (Fig. 4A). However, these movements are in parallel, and consequently, do not significantly change the overall size and shape of the cavity. Two short 3_{10} -helices (E1255-L1258 and T1261-S1263) in the V1251-F1264 region of BoNT/D-HCR are separated by residues L1259 and S1260. These two 3_{10} -helices correspond to one continuous α -helix in the BoNT/B, E, F, and G serotypes, however, these differences are unlikely to significantly impact the overall cavity structure.

While the overall three-dimensional structure of the cavity in BoNT/D-HCR is quite similar to those observed in the other serotypes, the amino acid composition inside the cavity of BoNT/D-HCR differs markedly from the cavity in the other serotypes. In BoNT/D-HCR, there are no aromatic amino acid clusters to provide a hydrophobic environment for accommodating the sugar rings of a ganglioside molecule (Fig. 4B). Only a single aromatic residue, Y1106, is within the cavity in a position and orientation similar to the equivalent residue, Y1117, in BoNT/A. Tyrosine-1254 is in the same position as the equivalent residue, Y1267 in BoNT/A, but, the side chain is oriented in the opposite direction. Therefore, even if the cavity is capable of binding a ganglioside, the detailed molecular interactions will be different from all the other BoNT serotypes. It is possible that this cavity is involved in recognizing a membrane molecule other than a ganglioside. Further biochemical experiments are needed to clarify the function of the BoNT/D HCR domain.

Addendum

During the preparation of this manuscript, two other groups pre-published the structure of the BoNT/D-HCR online [31,32]. However, we were unable to directly compare the structure of BoNT/D-HCR described here (PDB entry 3OGG) to the two structures because the coordinates have not been released by the RCSB PDB (3OBR and 3N7J) at the time of submission.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BoNT botulinum neurotoxin

Hcc	the C-terminal sub-domain of HCR
Hcn	the N-terminal sub-domain of HCR
HCR	the receptor binding domain
PC	phosphatidylcholine
PE	Phosphatidylethanolamine
SV	synaptic vesicle
Syt	synaptotagmin
TeNT	tetanus neurotoxin

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Fig. 1. BoNT/D-HCR structure and comparison to BoNT/B-HCR. (A) Cartoon representation of BoNT/D-HCR colored spectrally (violet to red) from the N-terminus. (B) Superposition of

BoNT/D-HCR (green) and BoNT/B-HCR-Syt II (2NM1: BoNT/B-HCR, red; Syt II, blue). Also shown is the position of the ganglioside co-receptor when BoNT/D-HCR is superposed on the structure of the BoNT/A-HCR-GT1b analog complex (2VU9, spherical representation). Labels for residue numbers in all figures are according to BoNT/D-HCR sequence unless specifically stated.

Fig. 2.

Putative protein receptor binding site. (A) Detailed superposition of BoNT/D-HCR (green) with BoNT/B-HCR (red, 2NM1) and Syt II peptides (blue). The K1236-N1244 loop moves about 13 Å and would potentially clash with the modeled Syt II peptide. The S1195-I1204 loop moves about 10 Å to the same direction of K1236-N1244 loop and make the putative receptor binding site similar in size to that of BoNT/B. (B) Putative receptor binding site of BoNT/D-HCR. Important loops and regions are indicated and residues on the loops are shown as grey sticks. The orientation is similar to (A).

Fig. 3. Analysis of PE binding by BoNT/D-HCR. (A) SDS-PAGE gel showing the results of liposome-binding assay. MW, protein molecular weight standard (in kDa); PC, 100% phosphatidylcholine; PC+PE, 50%:50%; SN, supernatant fraction showing non-bound protein; P, pellet fraction showing bound protein. (B) Putative residues important for PE

binding. The side chains of E1114 and K1243, and the main chain of G1132 are shown as grey sticks. The orientation of the protein is the same as in 2B.

Fig. 4. Detailed structure of BoNT/D-HCR in the region analogous to ganglioside binding site. (A) Superimposition of BoNT/D-HCR (green) with BoNT/A-HCR (purple, 2VU9) and GT1b analog (grey sticks). The V1251-F1264 and N1103-D1108 regions shift about 3~4 Å towards the protein's core. (B) Detailed residues (grey sticks) pointing to the pocket.