Molecular Architecture of Botulinum Neurotoxin E Revealed by Single Particle Electron Microscopy*

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Clostridial botulinum neurotoxin (BoNT) causes a neuropa- lytic condition recognized as botulism by arresting synaptic vesicle exocytosis. Although the crystal structures of full- length BoNT/A and BoNT/B holotoxins are known, the molecular architecture of the five other serotypes remains elusive. Here, we present the structures of BoNT/A and BoNT/E using single particle electron microscopy. Labeling of the particles with three different monoclonal antibodies raised against BoNT/E revealed the positions of their epitopes in the electron microscopy structure, thereby identifying the three hallmark domains of BoNT (protease, translocation, and receptor binding). Correspondingly, these antibodies selectively inhibit BoNT translocation activity as detected using a single molecule assay. The global structure of BoNT/E is strikingly different from that of BoNT/A despite strong sequence similarity. We postulate that the unique architecture of functionally conserved modules underlies the distinguishing attributes of BoNT/E and contributes to differences with BoNT/A.

Botulinum neurotoxin (BoNT),4 considered the most potent toxin known, causes botulism (1) by selectively inhibiting synaptic vesicle exocytosis (2). This conspicuously specific activity has transformed BoNT into the first bacterial toxin approved by the FDA for treatment of a number of diseases characterized by abnormal muscle contraction and as a blockbuster cosmeceutical and a most feared bioweapon (1, 3). Clostridium botulinum cells produce seven BoNT isoforms designated serotypes A to G (2). All BoNT serotypes are synthesized as a single polypeptide chain with molecular mass ~150 kDa. This precur- sor protein is cleaved by a clostridial protease into two polypeptides that remain linked by a disulfide bridge. The mature di- chain toxin consists of a 50-kDa light chain (LC) Zn2+ metalloprotease and a 100-kDa heavy chain (HC). The HC encompasses the translocation domain (TD) (the N-terminal half) and the receptor-binding domain (RBD) (the C-terminal half). BoNT/E is atypical in that it is not activated by proteolytic cleavage in the clostridial cells, thereby requiring unidentified proteases in the host cells to cleave the LC from the HC to achieve full toxicity (4, 5).

BoNTs exert their neuroparalytic effect by a multistep mecha- nism (2, 6). RBD-mediated binding to protein and lipid recep- tors on the cell surface of peripheral nerve endings (7–11) trig- gers receptor-mediated endocytosis and traffic to the endosomes. The acidic pH of endosomes induces a confor- mational change of the toxin; the HC inserts into the lipid bilayer and forms a protein-conducting channel (12, 13). The HC channel then translocates the protease domain into the cyto- plasm (13), colocalizing with its substrate SNARE (soluble NSF attachment protein receptor) (14–16). Because the SNARE core complex is essential for synaptic vesicle fusion with the presynaptic membrane (14–16), BoNTs efficiently block synaptic vesicle exocytosis.

In contrast to BoNT/A (17), little is known about the molec- ular architecture of BoNT/E, making it a novel target for structural analysis. Here we report the three-dimensional structure of BoNT/E holotoxin at ~30 Å resolution as determined by single particle electron microscopy (EM). Domains of BoNT/E were assigned to the globular features observed in the structure by labeling the toxin with functionally relevant monoclonal antibodies (mAbs). Although the individual domains of BoNT/E are similar to those of BoNT/A, their spatial arrange- ment within the global fold is unique. Analysis of the BoNT/E structure and structure-function correlation studies with mAbs bound to BoNT/A and BoNT/E define previously unrecognized biophysical characteristics that differ between these two BoNT isoforms.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich. Purified native BoNT sero- types A and E holotoxins were from Metabiologics. Di-chain BoNT/E holotoxin was generated by cleavage with trypsin: BoNT/E holotoxin (0.5 mg/ml) was incubated with 0.15 mg/ml trypsin in 20 mM HEPES, pH 7.0, for 30 min at 37 °C. Thereaf-
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ter, trypsin was inactivated with 0.25 mg/ml trypsin soybean inhibitor for 15 min at 20 °C. Trypsin and trypsin inhibitor were removed by centrifugal filtration on Millipore filters (molecular mass cutoff 50 kDa).

Cell Culture and Patch Clamp Recordings—Excised patches from Neuro 2A cells in the inside-out configuration were used as described (18). Current recordings were obtained under voltage clamp conditions. All experiments were conducted at 22 ± 2 °C.

Data Analysis—Analysis was performed on single bursts of each experimental record. Single channel conductance (γ) was calculated from Gaussian fits to current amplitude histograms (18). The total number of opening events (N) analyzed was 84,443. Time course of single channel conductance change for each experiment was calculated from γ of each record, where t = 0 s corresponds to onset of channel activity and average time course was constructed from the set of individual experiments for a single condition. n denotes the number of different experiments.

Monoclonal Antibody Generation and K_D Measurements—Human IgG1/κ mAbs were isolated from a single chain Fv library generated from a human volunteer immunized with pentavalent BoNT toxoid and displayed on yeast (19).5 IgG expression cassettes were constructed from the immunoglobulin heavy and light chain variable region genes of the scFv and used to generate stable Chinese hamster ovary cell lines from which the IgG were expressed and purified, exactly as previously described (20). Binding K_D,s were determined using flow fluorimetry in a KinExA (19). The epitopes of each mAb were determined using yeast-displayed BoNT/A or BoNT/E domains as previously described for BoNT/A mAbs. The epitopes and binding K_D,s of these mAbs are summarized in Table 1.

Fab Generation—The Pierce ImmunoPure Fab Preparation kit (44885) was used to generate Fab's. BoNT Fabs were quality checked using SDS-PAGE prior to the single molecule assays.

Protein Analysis—SDS-PAGE gels (12%) were used to visualize BoNT/A and BoNT/E holotoxins (21). If indicated, loading buffer was supplemented with 100 mM dithiothreitol prior to sample addition.

Specimen Preparation and Electron Microscopy—BoNT/A and BoNT/E holotoxins in 0.05% (w/v) dodecylphosphocholine and 20 mM HEPES, pH 7.0, were negatively stained with 0.75% (w/v) uranyl formate as described (22). Dodecylphosphocholine was used to increase the propensity to generate uniform populations of single particles; in the EM samples, its final concentration is >10^6 lower than the critical micelle concentration, and at best <5% of the BoNT molecule is decorated with dodecylphosphocholine molecules (13). BoNT-Fab complexes were prepared by incubating Fab fragments with BoNT holotoxin (2:1 molar ratio) in 0.05% dodecylphosphocholine and 20 mM HEPES, pH 7.0, for 24 h at 4 °C prior to negative stain. Images were recorded using a FEI Sphera electron microscope equipped with a LaB6 filament operated at an acceleration voltage of 200 KeV. Images were taken at a magnification of ×50,000; defocus value = −1.5 to −1.8 μm. Specimens were imaged at 0° and 60° tilt for three-dimensional reconstruction; the defocus value for 0° = −1.5 ~ −1.8 μm and 60° tilt = −2.0 ~ −2.2 μm. All images were recorded using SO-163 film with a Kodak D-19 developer at full strength for 12 min at 20 °C.

Image Processing—Electron micrographs were digitized with a CoolScan 9000 (Nikon) using a step size 6.35 μm, and 3 × 3 pixels were binned so the specimen level pixel size used was 3.75 Å. Projection averages were calculated from windowed small images of 74 × 74 pixels over 10 cycles of K-means classification and multireference alignment specifying 50 or 100 classes (two-dimensional only). For three-dimensional reconstructions of BoNT/E, a total of 7,090 particle pairs were interactively selected using WEB display program for SPIDER (23), windowed, and averaged into 50 classes as before. Images of the tilted specimens for each class were used to calculate initial three-dimensional reconstructions of individual classes by back-projection, back-projection refinement, and angular refinement. The final volume obtained by angular refinement with SPIDER was used as the input model for FREALIGN (24); this was used for refinement of orientation parameters of individual particles and for individual image contrast transfer function correction based upon the defocus value. The defocus of each particle was deduced from its position on the micrograph and tilt angles and defocus values determined with CTFiTILT (25). Particles selected from tilted and untilted specimens were used for FREALIGN refinement. The final three-dimensional reconstruction was in good agreement with the raw data as the particle images were compared with reprojections from the reconstruction. UCSF Chimera was used to render BoNT structures using Protein Data Bank accession codes 3BTA (17) and 1T3A (26) for BoNT/A holotoxin and BoNT/E LC.

RESULTS

Modular Organization of BoNT/A and BoNT/E Holotoxins—BoNT/A and BoNT/E exhibit high sequence similarity at each of their functional domains, with more conservation at the RBD and TD as compared with the LC (Fig. 1A) (27). Because an endogenous clostridial protease cleaves BoNT/A holotoxin between the LC and the HC, purified BoNT/A appears as two bands in SDS-PAGE after reduction with dithiothreitol (Fig. 1, A and B). In contrast, purified BoNT/E migrates in SDS-PAGE as a single band even after chemical reduction, consistent with an uncleaved full-length single chain protein (Fig. 1, A and B).

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Highly purified preparations (≥95%) of both BoNT/A and BoNT/E holotoxin proteins were used to conduct further experiments.

**Different Molecular Shapes of BoNT/A and BoNT/E Holotoxin**—The molecular mass of BoNT is ~150 kDa, a size relatively small to conduct single particle analyses. Therefore, we first examined whether BoNT/A, whose crystal structure is known (17), could be visualized by EM after negative staining. BoNT/A particles were monodispersed and homogeneous in size and shape (Fig. 2A). These particle images were subjected to image classification and alignment using multivariate statistics and multireference alignment. Representative class averages show a butterfly-like particle with dimensions 130 × 120 Å. The particle displays a central elongated domain (30 × 120 Å) bounded by a globular domain on either side (Fig. 2B): a double-lobed domain with dimensions 40 × 80 Å and a spherical domain with dimensions of 60 × 60 Å. Under our imaging conditions, BoNT/A adopted a single conformation similar to the known crystal structure (17) determined at pH 7.0 (Fig. 2C). The density of TD, consisting of a packed bundle of four α-helices (17, 28), emerged as a conspicuous feature in the class averages appearing as the central elongated domain. The overlay of the projection structure obtained by EM (Fig. 2C, left; scale bar, 5 nm) and the known crystal structure (Fig. 2C, middle) provides an unambiguous correspondence of the overall structure (Fig. 2C, right): the elongated TD flanked by the double-lobed RBD and the globular LC. Collectively, these results validate that molecular features of BoNT can be studied by single particle EM.

Negative stained BoNT/E molecules reveal monomeric particles with shapes distinct from BoNT/A (Fig. 2D). BoNT/E particles appear monodispersed and uniform in size. The particles, however, exhibit a heterogeneous population as demonstrated in the representative class averages obtained from 6,370 particles (Fig. 2E). All class averages represent a global bi-lobed structure of BoNT/E with small variation, which we classify into three categories. Type 1 (top panel) is the most prevalent shape (35%) as compared with type 2 (middle, 24%) and type 3 (bottom, 21%). The relative angle of the two global lobes is different between type 1 and type 2. The small lobe of the type 3 particle is smaller than the small lobes in type 1 and type 2 particles. The similarities in structural details between the class averages reveal the overall BoNT/E structure to consist of two lobes of different sizes connected together (Fig. 2, D and E).

**Identification and Biophysical Characterization of BoNT Domain-specific Monoclonal Antibodies**—To gain molecular insights into the EM structure of BoNTs, we explored functionally relevant molecular probes that can be used to label the individual domains. We identified four antibodies that bind with high affinity to individual modules of BoNT/A or BoNT/E (Table 1). First, we tested whether Fab fragments derived from these antibodies have any functional effect on the toxins, specifically on the translocation activity of BoNTs across neuronal membranes. Previous work led to the development of a single molecule assay that electrophysiologically monitors translocation of BoNT LC by the BoNT HC channel in excised membrane patches from Neuro 2A neuroblastoma cells (18) (Fig. 3). BoNT LC translocation requires conditions that emulate the pH and redox gradient across endosomes (18). Initially, the protein-conducting channel is occupied by the partially unfolded LC and by Na"+ present in the solutions bathing the membrane. The LC partially occludes the permeation pathway for the measured conductive species (Na"+), detected as a low γ. After translocation is complete the channel is unoccluded, attaining a higher, constant γ. The entire process is recorded in the control
experiments; representative single channel currents of BoNT/A and BoNT/E obtained at the beginning (10 s) and the end (1000 s) of LC translocation are displayed in the top two panels of Fig. 3A. The characteristic fast transitions between the closed and open states are clearly discernible. The amplitude of $\gamma$ is determined from the fluctuations between the closed and open states. The average time course of change of $\gamma$ for nine experiments is shown in Fig. 3B. BoNT channel activity is initially measured at $\gamma = 12$ pS (Fig. 3A, middle panels, and 3B), characteristic of both BoNT/A and BoNT/E unoccluded HC channels (18) (Fig. 3B).

We analyzed the effects of mAbs by preincubating BoNTs with Fabs for 1 h at pH 7 prior to the translocation assay. As illustrated in a representative experiment, a Fab specific for BoNT/A LC (Table 1, ING2) induces persistent block of the HC channel (Fig. 3A). Analysis of five experiments shows that the Fab ING2 allows channel formation, detected within minutes of patch formation at $\gamma = 21$ pS (Fig. 3, A and B). Thus, ING2 arrests translocation after initiation of LC entry into the HC channel (18).

Next, we asked whether mAb 3E6.1 or mAb 4E17.1, selective for BoNT/E TD (Table 1), affects the translocation step. The epitope of 3E6.1 is located in the region demarcated by residues 573–579 which, by sequence similarity to BoNT/A, maps to an extended region parallel to the helical bundle of the TD (17). In contrast, the epitope of 4E17.1 has been mapped to the loop region at one end of the BoNT/E TD encompassing residues 752–759. Preincubation of BoNT/E with 3E6.1 or 4E17.1 results in no channel activity monitored over the minimum experimental time of 30 min ($n = 24$ experiments for each condition) (Fig. 3, A and B). Thus, these Fab fragments bind to the BoNT/E TD with subnanomolar affinity (Table 1), precluding its insertion into the membrane and selectively disrupting the BoNT translocation activity.

### TABLE 1

| Antibody | BoNT | Epitope (residue number) | $K_D$ (pM) |
|----------|------|--------------------------|------------|
| ING2     | A    | LC                       | 9.6        |
| 4E16.1   | E    | LC (around 142)          | 3.4        |
| 4E17.1   | E    | TD (572–579)             | 240        |
| 3E6.1    | E    | TD (573–579)             | 40         |

BoNT Domain Labeling by Monoclonal Antibodies—A complex formed of BoNT/A holotoxin and ING2 Fab was imaged by negative stain EM and further analyzed by image classification and averaging. Representative class averages of the BoNT/A-ING2 complex are shown in Fig. 4A. By comparing the structure of the complex (Fig. 4A, middle panels) with BoNT/A alone (Fig. 4A, left panel), we infer that the extra density appearing on the BoNT/A-Fab complex structure corresponds to the bound Fab. A schematic diagram is shown in the right panel of Fig. 4A that depicts the profile of BoNT/A in white (designated by $\gamma$) and the Fab outline in gray (indicated with an arrow). At the resolution of our imaging, the binding of the Fab to BoNT/A shows minimal distortion of the native conformation of the toxin. The recognizable silhouette of the Fab fragment in the complex allows us to unambiguously assign the location of the LC in the holotoxin structure.

Next, we labeled BoNT/E with the Fabs derived from anti-BoNT/E antibodies (Table 1). The epitope of mAb 4E16.1 is centered on the surface-exposed residue 142 in the LC of BoNT/E (Table 1). When compared with the structure of BoNT/E alone, the particles of BoNT/E-4E16.1 Fab complex (Fig. 4B, middle panels) exhibit an extra density attached to the intermediate protrusion of the large lobe of BoNT/E (Fig. 4B, left panel, also see scheme in right panel). Similar to ING2 Fab, 4E16.1 Fab binds to the particle without creating any distortion to the overall structure, thus unambiguously assigning the location of the LC epitope (residue 142) in BoNT/E structure.

Unique identification of individual domains of the BoNT/E holotoxin molecule was further ensured by using TD-specific mAbs. In the structure of the BoNT/E-Fab 4E17.1 complex (Fig. 4C), the BoNT/E and the Fab are easily discerned. The extra density attached to the tip of the large lobe on the BoNT/E-Fab complex structure corresponds to the bound Fab, as shown in the schematic of Fig. 4C. Although 4E17.1 Fab blocks the translocation activity of the toxin, the overall structure of BoNT/E was not affected by its binding. The particles of the BoNT/E-Fab 3E6.1 complex display a drastically different profile from that of BoNT/E (Fig. 4D, left panel), as reflected in the representative particle averages shown in Fig. 4D, middle pan-
The three-dimensional structure of BoNT/E holotoxin—The three-dimensional structure of BoNT/E particle was calculated by random conical tilt reconstruction. For this purpose, negative stained particle images of BoNT/E were taken as tilt pairs (0° and 60°). Untilted particle images were subjected to multivariate statistical analysis, multireference alignment, and classification into 50 classes. A well defined class average from type 1 particles was chosen to further calculate the three-dimensional structure using the corresponding tilted particle images. The three-dimensional structure of BoNT/E after FREALIGN refinement is shown in Fig. 5A. The structure of BoNT/E holotoxin was strikingly different from that of BoNT/A. The small lobe and the large lobe are mostly separated except at the junction point. Within the three-dimensional structure of BoNT/E, the central elongated domain representing the unique feature of TD in the BoNT/A was less defined. Thus, in BoNT/E the TD is closely associated to either the LC or the RBD.

The three-dimensional reconstruction of different class averages (types 1–3) produced similar three-dimensional structures (data not shown). We conjecture that the subtle conformational variability represented in the class averages reflects the conformational flexibility of the BoNT/E; this interpretation is consistent with the widely recognized difficulty in generating quality crystals of BoNT/E. A more interesting inference is that it reflects the conformational plasticity of BoNT/E, exposing different conformational states related to its function. Based upon the Fourier shell correlation (FSC), the resolution of the final density map was estimated to be 24 Å with the FSC = 0.143 criterion and 30 Å with the more conservative FSC = 0.5 criterion (29).

The domain assignment derived from the analysis of BoNT/E in complex with domain-specific mAbs allows further interpretation of the three-dimensional structure. The crystal structures of the LC of BoNT/E (26) and of the RBD of BoNT/A (17) were placed into the EM density map of BoNT/E (Fig. 5, B and C). The large lobe consists of LC and TD; within the large lobe, the location of LC is restricted to the proximity of the binding site of 4E16.1 Fab. The crystal structure of LC (blue) was therefore placed such that the epitope of 4E16.1 aligns with the region on the density map corresponding to the Fab binding site. Because the 4E17.1 Fab recognizes the TD and binds to the tip of the large lobe, we inferred that the small lobe represents the RBD and placed the crystal structure of RBD of BoNT/A accordingly. The size and shape of the crystal structure of the RBD (red) is consistent with the density of the small lobe and reveals that the RBD for BoNT/E is well defined as a separate module in the tri-modular arrangement of the holotoxin. A large unaccounted density was identified that includes the tip of the large lobe where the 4E17.1 Fab binds. We interpret that this unaccounted density corresponds to the TD, apparently bent at its central region where it presumably accommodates the LC.

**DISCUSSION**

Our data highlight for the first time the global structure of BoNT/E. The three-dimensional reconstruction and the manual docking of the crystal structures of the isolated LC and RBD domains outline the novel architecture of BoNT/E and the different arrangement of domains within the tri-modular holotoxin with respect to BoNT/A (17) and BoNT/B (28). These
Three-dimensional Structure of BoNT/E

FIGURE 5. Three-dimensional reconstruction of single chain BoNT/E holotoxin and placement of crystal structure into the density map. A, three-dimensional map of BoNT/E holotoxin shown from three different angles. RBD is the small lobe; the large lobe consists of LC and TD. Positions of the epitopes of the monoclonal antibodies used for labeling experiments are indicated by arrows. The precise residues of the epitopes are indicated as numbers (Table 1). B, placement of known crystal structures into the EM density map. The Protein Data Bank accession codes for the crystal structures used are: RBD of BoNT/A (3BTA; red) and LC of BoNT/E (1T3A; blue). C, view from a different angle.

The BoNT/E and BoNT/A structures provide insights into the translocation function of the toxin. The channel activity of BoNT/E and BoNT/A is very similar (Fig. 3) (18), suggesting that the channel entity acts as a universal transporter of proteins (49). The strong sequence similarity within the TD between the two serotypes contrasted with the ambiguous structural alignment highlights the inherent flexibility of the TD domain. Notable is the tight correlation between the mAbs used to identify domains on the structure (Fig. 4) and their selective disruption of BoNT translocation activity in the electrophysiological assay (Fig. 3). The mAbs that recognize unique sequences on their SNARE subunits contribute to this discrepant effect. The two serotypes cleave the SNARE component SNAP-25 (synaptosome-associated protein of 25 kDa) at different sites near the C terminus (47), producing SNAP-25 fragments of different size that inhibit exocytosis by competing for SNARE complex assembly (44–46). In addition, the subcellular distribution of the two toxins is known to be different. BoNT/A LC co-localizes with the truncated SNAP-25 product at the plasma membrane, whereas BoNT/E appears to be diffusely distributed in the cytoplasm (48). Differences in the intricacies of intracellular trafficking, modification, and degradation of BoNTs have been reported (42, 43) that may be accounted for in part by the fact that individual modules in BoNT/A are more segregated rather than bundled as resolved in BoNT/E. These functional and structural differences between BoNT/A and BoNT/E highlight the elegance of BoNT molecular design, which appears to exploit the host cellular systems for its own function at each step of the intoxication process.

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Interesting inferences about the functional disparities between BoNT/A and BoNT/E can be drawn from our analysis. Intriguingly, cleavage of the same substrate by BoNT/A and BoNT/E produces the longest (several months) and the shortest (few days) duration of neurotransmitter blockade. Several factors contribute to this discrepant effect. The two serotypes cleave the SNARE component SNAP-25 (synaptosome-associated protein of 25 kDa) at different sites near the C terminus (47), producing SNAP-25 fragments of different size that inhibit exocytosis by competing for SNARE complex assembly (44–46). In addition, the subcellular distribution of the two toxins is known to be different. BoNT/A LC co-localizes with the truncated SNAP-25 product at the plasma membrane, whereas BoNT/E appears to be diffusely distributed in the cytoplasm.
powerful tools to gain insights into the mechanism of LC translocation. It will be interesting to visualize structural changes of the interacting HC channel with the LC cargo under conditions that imitate those across endosomes by pursuing the strategy outlined here in combination with a set of serotype-specific (BoNT/A or BoNT/E) and domain-specific (LC or TD) (Figs. 2 and 4) antibodies.

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