A Novel Subunit Structure of Clostridium botulinum Serotype D Toxin Complex with Three Extended Arms

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The botulinum neurotoxins (BoNTs) are the most potent toxins known in nature, causing the lethal disease known as botulism in humans and animals. The BoNTs act by inhibiting neurotransmitter release from cholinergic synapses. Clostridium botulinum strains produce large BoNTs toxin complexes, which include auxiliary non-toxic proteins that appear not only to protect BoNTs from the hostile environment of the digestive tract but also to assist BoNT translocation across the intestinal mucosal layer. In this study, we visualize for the first time a series of botulinum serotype D toxin complexes using negative stain transmission electron microscopy (TEM). The complexes consist of the 150-kDa BoNT, 130-kDa non-toxic non-hemagglutinin (NTNHA), and three kinds of hemagglutinin (HA) subcomponents: 70-kDa HA-70, 33-kDa HA-33, and 17-kDa HA-17. These components assemble sequentially to form the complex. A novel TEM image of the mature L-TC revealed an ellipsoidal-shaped structure with “three arms” attached. The “body” section was comprised of a single BoNT, a single NTNHA and three HA-70 molecules. The arm section consisted of a complex of HA-33 and HA-17 molecules. We determined the x-ray crystal structure of the complex formed by two HA-33 plus one HA-17. On the basis of the TEM image and biochemical results, we propose a novel 14-mer subunit model for the botulinum toxin complex. This unique model suggests how non-toxic components make up a “delivery vehicle” for BoNT.

Different strains of Clostridium botulinum produce seven distinct serotypes of neurotoxins (BoNTs), classified A through G. BoNT has attracted much interest in recent years due to extensive research on its biochemistry, determination of its crystal structure, and investigations into the pharmacology and applications of BoNTs as therapeutic agents for the treatment of human disease (1–3). After ingestion of BoNT, the BoNT is absorbed from intestinal epithelial cells into the bloodstream, after which it consequently reaches the neuromuscular junctions. BoNT enters nerve cells via receptor-mediated endocytosis, where it cleaves specific sites on target proteins, inhibiting release of neurotransmitters from peripheral cholinergic synapses through its zinc protease activity (4–6). This process causes muscular paralysis in humans and animals, leading to the disease botulism.

Toxins with serotypes A–D and G are encoded by two gene clusters in close proximity to each other; cluster 1 contains the bont and ntnha genes, and cluster 2 contains three genes: ha-70, ha-33, and ha-17 (7, 8). Therefore, botulinum TC consists of five components: BoNT, non-toxic non-hemagglutinin (NTNHA) and three hemagglutinin subcomponents (HA-70, HA-33, and HA-17). All serotypes of BoNT associate non-covalently with auxiliary non-toxic proteins, thereby forming large toxin complexes (TCs). Serotype A–D strains produce the M-TC (BoNT-NTNHA complex) and L-TC (BoNT-NTNHA-HAs complex) in the culture medium, while serotype E and F strains produce only M-TC. The major biological function of the non-toxic components appears to be protection of the BoNT against the hostile environment of the digestive tract (9, 10). Additionally, several lines of evidence have shown that the non-toxic proteins play a role in the uptake and transcytosis of L-TC into the intestinal epithelium (11–15). Recently, we have also demonstrated, using a cultured Caco-2 cell layer, that serotype D HA-33 plays a critical role in the permeation of TC species into the intestinal epithelium (16). It is therefore likely that the non-toxic components play a role in the trans-epithelial transport of BoNT across the intestinal barrier.

However, few studies have addressed the structure of the TC, because of the large sizes of the complexes consisting of multiple non-toxic components. These complexes vary from 300 kDa up to 900 kDa, depending upon the BoNT serotypes. C. botulinum serotype D strain 4947 (D-4947) produces TC species, which consist only of intact components, without any nicking. We previously proposed an assembly pathway, including the binding order of individual subunits, through in vitro reconstitution of the D-4947 L-TC (17): The M-TC is formed...
first by assembly of a single BoNT and a single NTNHA molecule, and is subsequently converted to the complete L-TC by forming complexes with HA-70 and HA-33-HA-17 molecules. Additionally, we found hemagglutination-negative TC species in which HA-33-HA-17 molecules were missing from the mature L-TC (18, 19). These TC species are considered to be intermediates in the 650-kDa L-TC assembly pathway (20).

Regarding the subunit structure of the botulinum TC, historically there has been only one model, our schematic one that was constructed based on biochemical results (18, 21). In this work, we propose for the first time a more detailed structure of a botulinum serotype D TC, and we describe a potential assembly process that specifies the binding order of individual subunits. This novel and unusual 14-mer model may explain how non-toxic components comprise a delivery vehicle for BoNT in food-borne botulism, how BoNT is protected in the digestive environment, and how BoNT is internalized into the bloodstream.

**EXPERIMENTAL PROCEDURES**

Production and Purification of Botulinum D-4947 TC Species—C. botulinum D-4947 was cultured using a dialysis method as previously described (22). The TC species were purified from the culture supernatants by SP-Toyopearl 650 S cation-exchange column chromatography as described previously (18). Each TC species fraction was collected separately, and the molecular mass was estimated by gel filtration. The M-TCHA-70 and 540-, 610-, and 650-kDa L-TC species were then purified to homogeneity, as determined by SDS- and native-PAGE. The TC species termed M-TC, M-TCHA-70, 540-kDa, 610-kDa, and 650-kDa L-TC were thus isolated for TEM and other experiments.

Isolation of the HA-33-HA-17 complex from 650-kDa L-TC—Isolation of the HA-33-HA-17 complex from 650-kDa L-TC was performed as described (17).

**PAGE and Densitometric Analyses**—PAGE under non-denaturing conditions (native-PAGE) was conducted at pH 8.8 using a 5–12.5% polyacrylamide linear gradient gel. SDS-PAGE was performed using a 13.6% polyacrylamide gel in the presence of 1% 2-mercaptoethanol. The Coomassie Brilliant Blue R-250 (CBB) staining intensities of the components were analyzed using ImageJ 1.30v. The protein concentration was determined using a BCA protein assay kit (Pierce, IL) with bovine serum albumin as the standard.

**Hemagglutination Assay and Titration of TC Species with the HA-33-HA-17 Complex**—A hemagglutination assay was performed by a microtitration method (18). To determine the binding capacities of the TC species, with isolated HA-33-HA-17 as titrant, the titration experiment was performed by adding HA-33-HA-17 to the TC species. The titration mixtures containing 0.7 μM TC species and several concentrations of HA-33-HA-17 were incubated for 1 h at room temperature, followed by a hemagglutination assay and native-PAGE.

**Analytical Ultracentrifugation**—The purified HA-33-HA-17 complex was dialyzed against 50 mM acetate buffer (pH 5.0) containing 0.15 M NaCl and adjusted to an appropriate protein concentration. Sedimentation equilibrium experiments were carried out using an Optima XL-I analytical ultracentrifuge (Beckman Coulter Inc.) with a 4-hole An60Ti rotor and two-channel charcoal-filled Epon cells at a rotor speed of 17,000 rpm. The data were analyzed using the software Ultrascan 6.01.

**Mass Spectrometric Analyses**—Nano-liquid chromatography/electrospray ionization time-of-flight mass spectrometry (nanoLC/ESI-TOF-MS) was carried out with a nanoLC system consisting of a Famos auto sampler and a Switchos automated switching valve (LC Packings), and a micrOTOF-focus orthogonal acceleration TOF instrument (Bruker Daltonics, Bremen, Germany) interfaced with a nano-ESI source. HA-33-HA-17 solution was prepared in 20 mM acetate buffer (pH 5.0) with a protein concentration of 462.8 μg/ml. 10 μl of sample was injected onto a C18 pre-column (0.3 mm inner diameter × 5 mm length). After connecting the pre-column to the nano-ESI source, the sample was eluted with a linear gradient of acetonitrile (0–50%). All experiments were carried out in positive mode.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out with an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics) in positive linear mode at an acceleration voltage of 25 kV. As the matrix solution, sinapic acid (SA) and 2,5-dihydroxyacetophenone (2,5-DHAP) were used to achieve acidic and neutral conditions, respectively. HA-33-HA-17 solution was prepared in 20 mM Tris-HCl (pH 7.8) at a protein concentration of 764.2 μg/ml.

**Transmission Electron Microscopy (TEM)**—Appropriate aliquots of the TC specimens (10 μg/ml) in 50 mM acetate buffer (pH 5.0) were distributed on carbon support film. The specimens were negatively stained with 2% uranyl acetate. Electron micrographs were recorded with the JEM-1011 and the JEM-1230 (JEOL, Tokyo, Japan) at a magnification of ×50,000 and an accelerating voltage of 100 kV.

**Crystallization of HA-33-HA-17 Complex**—For crystallization of the HA-33-HA-17 complex, the protein solution was concentrated by ultrafiltration with an Amicon YM10 membrane (Amicon, MA) and by centrifugation using Ultrafree-0.5 with a molecular weight cut-off (MWCO) of 10 K, followed by filtration through an Ultrafree-MC 0.2 μm filter unit (Millipore, MA). An expanded set of crystallization conditions was screened to obtain HA-33-HA-17 crystals suitable for x-ray analysis. Crystal Screen Kit and Crystal Screen Kit 2 (Hampton Research) were used as mother liquor for both hanging-drop, sitting-drop, and floating-drop (23) vapor-diffusion methods, at temperatures 4 and 20°C. The protein concentrations ranged from 3.4 to 20 mg/ml. The ratios of sample to crystallization solution were 2:6, 4:4 and 6:2 (v/v, total volume 8 μl).

**X-ray Data Collection**—Preliminary x-ray data were collected at 100 K on a Rigaku R-AXIS VII imaging-plate system, using CuKα radiation from a Rigaku FR-E rotating-anode generator with Rigaku MaxScreen optics. The crystals used for this in-house experiment diffracted up to 2.60Å resolution. To obtain a high resolution data set, x-ray diffraction data were collected from frozen crystals at 100 K using a Rigaku Jupiter 210 CCD detector installed on the RIKEN structural genomics beam line BL26B2 at the SPring-8 synchrotron facility (Harima, Hyogo, Japan). Diffraction data collected using the synchrotron
radiation source were up to 1.85 Å resolution. Data statistics are summarized in Table 1.

Structure Determination and Crystallographic Refinement—All x-ray diffraction data were integrated and scaled using the CrystalClear package (Rigaku, Tokyo, Japan). Subsequent data manipulation was carried out using the CCP4 program package (24). An initial phase set was calculated by molecular replacement using MOLREP (25). The search model for molecular replacement was the structure of the HA1 from serotype C (PDB code 1QXM), which showed the highest sequence identity (95.8%) to the D-4947 HA-33 subcomponent. Noncrystallographic averaging, solvent flattening, and histogram modification were carried out to modify the density using the program DM (26), prior to the calculation of SigmaA-weighted maps (27), which were then used for manual rebuilding of the model. The electron-density map (2Fo — Fo) was calculated using FFT. Several cycles of manual model fitting were carried out using the programs XtaLView 4.3 (28) and Refmac 5.0 (29). The geometry of the model was greatly improved using CNS 1.1 for rigid body refinement and simulated annealing refinement. After the model reached a reasonable R factor, water was added to the structure using ARP/wARP (30). The final model was analyzed by PROCHECK (31) to evaluate its stereochemical quality.

RESULTS AND DISCUSSION

The crude toxin fraction from the *C. botulinum* D-4947 culture supernatant was applied to an SP-Toyopearl 650 S column under acidic conditions (pH 4.0). After several consecutive chromatographic purification runs for each peak fraction, five highly purified TC species were obtained. They were tentatively termed 280-kDa M-TC (BoNT-NTNHA complex), 410-kDa M-TC-HA-70 complex, 540-kDa, 610-kDa, and 650-kDa L-TC, with the molecular weights determined by gel filtration, SDS-PAGE, and N-terminal amino acid sequence analysis. The 540-kDa and 610-kDa L-TCs showed a banding pattern on SDS-PAGE similar to that of the 650-kDa L-TC, but with lower staining intensities of the HA-33 and HA-17 bands than those for the 650-kDa L-TC (Fig. 1A), indicating that these bands corresponded to intermediates species in the pathway to 650-kDa L-TC assembly. The 650-kDa L-TC is apparently the complete L-TC complex, because no larger complex was purified from the culture supernatant.

All samples produced two bands on native-PAGE under alkaline conditions, where the complexed forms of non-toxic components (just NTNHA for M-TC) dissociate from the BoNT, as shown in Fig. 1B. The electrophoretic mobility of the non-toxic complex bands derived from the M-TC-HA-70, 540-kDa, 610-kDa, and 650-kDa L-TC revealed a ladder, with bands migrating at equal intervals according to their molecular sizes. Because these TC species, except for the 650-kDa L-TC, lack hemagglutination activity, we deduced that the HA-33 and HA-17 molecules must have been missing in the subunit composition of TC species other than the complete L-TC.

**Negative Stain TEM**—To further characterize the BoNT complex, the purified D-4947 TC species were visualized by negative stain TEM. Fig. 2A shows the representative shapes of TC species that were visualized after manual alignment of particles observed in the TEM images. Tracings showing the outlines of subunit components are presented in Fig. 2B. The M-TC, which is composed of BoNT and NTNHA, appears as an ~13-nm spherical (or ellipsoidal) particle (Fig. 2, A-1). The M-TC-HA-70 complex is an acorn-like particle,
**Subunit Structure of the Serotype D Botulinum Toxin Complex**

with the HA-70 “cone” lying on the M-TC (Fig. 2, A-2), suggesting blocks building up sequentially to form a complex. It is noteworthy that the HA-70 molecule appears to have no rigid configuration. This may be supported by the observation during differential scanning calorimetry, that the isolated HA-70 molecules exhibited no distinct melting transition through the range 10 to 100 °C (data not shown). Interestingly, subsequent L-TC species revealed unique “arm” attachments that appeared to be rod-like particle. We concluded that these were HA-33-HA-17 complexes that adhered to the M-TC-HA-70 complex via the HA-70 molecule, as shown in Fig. 2, A-3–5. The number of “arm” attachments with ~10 nm length increased with the size of the L-TC species; the average number of arms was: one (84.4%, n = 209), two (73.3%, n = 172), and three (55.6%, n = 135) for 540-kDa L-TC, 610-kDa L-TC, and 650-kDa L-TC, respectively. No TC image with four or more arms was observed in the field of view. This is therefore a novel observation regarding the structure of the botulinum TC. In an earlier study, based on negative stain electron microscopy of the serotype A BoNT and analysis of a separated non-toxic complex, Boroff et al. (32) designed a model for the complex that allowed the BoNT strand to fit within the coils of the HAs. Likewise, Burkard et al. (33) proposed a model for the serotype A 900-kDa LL-TC (dimer of L-TC) that was triangular in shape, with six lobes apparent in the electron crystallographic images that were obtained using a lipid-layer technique. However, both models provided neither the stoichiometry of the components nor their structural organization.

To our surprise, the number of HA-33-HA-17 molecules observed in all TC species consisted of one molecule less than the number predicted by our initial schematic model (21), which was derived from titration-based experiments. These initial experiments led to estimates of four HA-33-HA-17 molecules in the 650-kDa L-TC, three in the 610-kDa L-TC and two in the 540-kDa L-TC, respectively. This discrepancy arose because Superdex 200 pg gel filtration provided an estimate of 50 kDa for the HA-33-HA-17 complex, implying that the complex is composed of one molecule each of HA-33 and HA-17.

Our present results indicate that the L-TC species may have flexible characteristics, for instance the relative positions of the arms of the 650-kDa L-TC varied, and sometimes fewer arms were observed, probably because some HA-33-HA-17 molecules could adhere to the carbon-coated membrane and thus be hidden beneath the TC molecule itself. The HA-70 molecules also seemed to acquire a dramatically more rigid folded configuration upon binding of HA-33-HA-17 to form complexes. A black spot that is presumed to be a zinc atom or an active site cavity was observed in all TEM images of TC species; this could constitute a marker for the BoNT molecule. Such electron microscopic observations will undoubtedly result in a better understanding of the subunit structure of botulinum TC, which will be further clarified by x-ray crystallographic analysis of additional complexes in the near future.

**Molecular Composition of the HA-33-HA-17 Complex**—Because both TEM observation and PAGEn analyses suggested strongly that the arm section consists of HA-33 and HA-17, we attempted to character-

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**TABLE 1**

Summary of crystallographic parameters, data collection, and refinement statistics for D-4947 HA-33-HA-17 complex (PDB code 2E4M)

| Crystallography conditions | Method         | Hanging-drop vapor diffusion |
|----------------------------|----------------|------------------------------|
| Temperature (K)            | 293.0          |                              |
| Protein concentration      | 12.0 mg/ml in 50 mM HEPES (pH 7.0) + 0.1 mM NaCl | |
| Mother liquor              | 7% PEG 8000, 0.1 mM MES (pH 6.5), 0.1 mM MgCl2 | |

**Data collection**

| Wave length (Å) | 1.000          |
|-----------------|----------------|
| Space group     | H32            |
| Unit cell parameters (Å) | a = b = 239.132, c = 100.024 |
| Number of reflections | 92648         |
| Completeness (%) | 99.8           |
| Resolution (Å)  | 119.52–1.85    |

**Refinement statistics**

| Number of reflections | 87820          |
| Completeness (%)      | 99.8           |
| Resolution (Å)        | 49.81–1.85     |
| Rwct/Rfree (%)        | 19.4/22.9      |
| Number of waters      | 718            |
| Mean B value (Å)      | 26.86          |

**Ramachandran plot (%)**

| Most favored regions  | 86.0           |
| Additional allowed regions | 13.0          |
| Generally allowed regions | 0.7           |

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**FIGURE 3. Crystal structure of the HA-33-HA-17 trimer complex at 1.85 Å.**

A, structure of HA-33-HA-17 trimer complex isolated from serotype D-4947 L-TC as represented by a ribbon diagram. The putative carbohydrate-binding sites in the HA-33 molecules are indicated with arrows. The N and C termini of each subunit are labeled N and C and indicated with red open circles. B, surface representation of HA-33-HA-17 trimer complex: HA-33 molecules in blue and HA-17 molecule in cyan. Figures were prepared using MolFeat version 3.0 (FiatLux Corp.).
ize its molecular composition. The HA-33-HA-17 component complex, which could not be separated as a pure component during the isolation procedure, was isolated from L-TC using 4 M guanidine as a denaturant, as previously described (17). Ultracentrifugal analysis using sedimentation equilibrium yielded a calculated molecular weight of 77,400 Da. On the other hand, nanoLC/ESI-TOF-MS of HA-33-HA-17 yielded a mass of 84,118 Da, while MALDI-TOF-MS analysis indicated a mass of 83,623 Da (using the SA matrix) and 83,743 Da (using the 2,5-DHAP matrix). These estimates were reasonably close to the calculated mass, 84,239 Da, obtained by adding the masses of two HA-33 molecules and one HA-17 molecule.

**X-ray Crystallography of the HA-33-HA-17 Complex**—Crystals of the HA-33-HA-17 complex (~0.1 mm in size) were obtained for x-ray analysis using the hanging-drop method. We determined the x-ray crystal structure of the isolated HA-33-HA-17 complex from D-4947 L-TC at 1.85 Å resolution by molecular replacement using the serotype C HA-33 structure (34) as the search model. The hexagonal unit cell parameters were \( a = b = 239.13 \text{ Å} \) and \( c = 100.02 \text{ Å} \), and each cell contained one complex molecule in the asymmetric unit. The final model was refined to an \( R \) factor of 0.19, and included 713 amino acid residues (Ser2–Ile286 for HA-33 and Glu4–Leu146 for HA-17) (PDB code 2E4M). X-ray data collection and refinement statistics are summarized in Table 1. The Ramachandran plot showed that all residues were within allowed regions.

Fig. 3 shows the crystal structure of the HA-33-HA-17 complex represented as ribbon (Fig. 3A) and surface (Fig. 3B) models. The final model includes two HA-33 molecules and one HA-17 molecule in the asymmetric unit. This model is fairly consistent with the molecular composition of HA-33-HA-17 (2:1) complex as determined by molecular mass. Each HA-33 molecule consists of a single polypeptide chain of 285 residues folded into a dumbbell-like shape made up of N-terminal (residues 2–143) and C-terminal (residues 144–286) domains. As observed in the crystal structures of serotypes A HA-33 (35) and C HA-33 (34), D-4947 HA-33 is also composed of two \( \beta \)-trefoil domains, which consist of a single polypeptide chain of 285 residues folded into a dumbbell-like shape made up of N-terminal (residues 2–143) and C-terminal (residues 144–286) domains. As observed in the crystal structures of serotypes A HA-33 (35) and C HA-33 (34), D-4947 HA-33 is also composed of two \( \beta \)-trefoil domains, which consist of a single polypeptide chain of 143 residues (density was not seen for two amino acid residues at the N terminus) forms a base that supports two HA-33 molecules. A structural alignment with the secondary structure of HA-33 showed that the \( \beta \)-trefoil repeats were also found in the HA-17 structure, although one hairpin loop was not seen in the \( \beta \)-trefoil repeat, probably due to weak electron density. Additionally, no amino acid residues responsible for putative carbohydrate-binding were observed in the HA-17 molecule, suggesting that HA-17 may not bind carbohydrates.

As shown in Fig. 4A, two HA-33 molecules form contacts with each other at their N-terminal domains, with extensive interactions between tyrosine residues (Tyr92, Tyr99, Tyr106, and Tyr99).
The hemagglutination activity of the 650-kDa L-TC was determined by adding increasing amounts of HA-33/H18528 to form L-TC complexes. The number of HA-33/H18528 HA-17 complexes is required for hemagglutination activity. To define the molecular composition of incomplete TC species, we examined whether the hemagglutination activity of the M-TC/H18528 HA-70 molecule, forming an interface with one β-sheet region of HA-17 (Asn106, Thr108, Asp123, Pro125, Leu127, Leu129, and Pro130), as shown in Fig. 4B. Although little is known about the function of HA-17, its most likely function is to fix two molecules of HA-33 and tether the HA-70 molecule, as if by nuts and bolts, to form the HA-70-HA-17-HA-33 conjunction.

**Number of HA-33-HA-17 Complexes in the L-TC**—We performed in vitro reconstitution of the mature L-TC by adding increasing amounts of HA-33-HA-17 to form L-TC complexes. The hemagglutination activity of the 650-kDa L-TC was determined to be titer of $2^7$, while no hemagglutination titer could be detected for the other TC species. This implies that the full number of HA-33-HA-17 complexes is required for hemagglutination activity. To define the molecular composition of incomplete TC species, therefore, we examined whether the hemagglutination activity of the M-TC/HA-70, 540-kDa L-TC, and 610-kDa L-TC arises by binding of HA-33-HA-17 to unoccupied sites. As the HA-33-HA-17 (with calculated mass 84,239 Da) was titrated, every TC species exhibited a similar titer to that of L-TC, as shown in Fig. 5. At the end points of the titration, the M-TC/HA-70, 540-kDa and 610-kDa TC species (0.7 μM each) required 2.1, 1.4, and 0.7 μM HA-33-HA-17, respectively, to exert full hemagglutination activity. Concomitantly, every TC species exhibited an electrophoretic pattern on native-PAGE similar to that of the 650-kDa L-TC, as seen in Fig. 1 (data not shown). Accordingly, the molar ratio of HA-33-HA-17 complexes was estimated as 3:2:1 against M-TC/HA-70, 540-kDa and 610-kDa TC. This leads to a model in which three HA-33-HA-17 complexes occupy surface positions in the subunit structure of L-TC. Such observations, including the TEM images, are consistent with the hemagglutination activity of the botulinum TC. Our model suggests that six HA-33 molecules can be exposed at the L-TC surface, where they play a role in anchoring the complex at the epithelial cell surface.

**Determination of the Number of HA-70 Subunits**—Because the number of HA-70 subunits in the L-TC has been unclear, we tried to obtain the molar ratios of HA-70 subunits by densitometric analysis of CB8 staining bands on SDS-PAGE gels. Based on several experiments, all of which were repeated, we conclude that the molar ratio of HA-70 was ~3.0 (mean 2.92, S.E. = 0.17, n = 47), i.e. it consists of three subunits. This ratio was calculated by dividing the density of the BoNT bands by each molecular mass. In light of the number of HA-33-HA17 molecules in the complex, this value is quite reasonable.

**Model of the Arrangement of Botulinum L-TC**—We now show that virtually all TC species from the culture medium appear to form oligomeric subunits, which predominantly comprise L-TC. The results described here were combined to construct images of the total botulinum serotype D TC, allowing us to draw conclusions as to the extent of oligomerization and the numbers of each component protein present in native botulinum D-4947 L-TC. Fig. 6 shows our proposed 14-mer model (based on the sum of calculated masses of subunits, 749.43 kDa), which proposes an arrangement of the individual subunits in the mature L-TC. The assembled complex includes a single BoNT, a single NNNHA, three HA-70, six HA-33, and three HA-17. This hypothetical model of the D-4947 BoNT structure was generated using the homology modeling server. We employed serotype A (36) and B BoNTs as templates. Shapes and relative sizes of NTNHA and HA-70 molecules are based on TEM images and molecular masses. The BoNT is highlighted in red, the NTNHA in green, three HA-70 in yellow, six HA-33 in blue, and three HA-17 in cyan. The catalytic zinc ion in BoNT is indicated by the orange circle and the arrow.
relative sizes of NTNHA and HA-70 molecules were derived from the TEM images and their molecular masses. This model differs from our previous 12-mer subunit model (18, 21), which was based on biochemical results. The new experimental data described here allow us to propose a more realistic model having novel features. Further verification of this structure will require higher resolution x-ray crystallography and a three-dimensional reconstruction using the resulting models.

The three-dimensional structures of NTNHA and HA-70 proteins have not yet been determined; therefore, our model must be subject to a few caveats. Nevertheless, we believe that this model can explain important features of botulinum TC as a "delivery vehicle", in which HA subcomponents may increase the internalization of BoNT into the bloodstream via binding to intestinal membranes. In addition, the model explains how BoNT is protected from protease digestion. Our ultimate goal is to define the unique subunit structure of botulinum TC by combining TEM observations of the various assembly states of the botulinum TC with x-ray crystallographic analysis of the individual components. Thus, this model represents a first step toward elucidating the complete three-dimensional structure of the entire botulinum toxin complex.

Acknowledgments—We thank M. Sakai (Institute for Protein Research, Osaka University) and Drs. E. Arai and H. Kashimori (Beckman Coulter Inc.) for ultracentrifugation analysis of the HA-33-HA-17 complex, and D. Higo (Bruker Daltonics K. K.) for nLC/ESI-TOF-MS of the HA-33-HA-17 complex. We thank K. Sawaguchi, Y. Ikeda, S. Kurosawa, D. Takenaka, Y. Ishikawa, S. Mizuuchi, Y. Shimomukai, H. Hosaka, S. Kunii, and A. Mikami for assistance in preparing the botulinum TC species.

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J. Biol. Chem. 2007, 282:24777-24783.
doi: 10.1074/jbc.M703446200 originally published online June 20, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M703446200

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