Characterization of the interaction between subunits of the botulinum toxin complex produced by serotype D through tryptic susceptibility of the isolated components and complex forms

Tomonori Suzuki,1 Toshihiro Watanabe,1 Shingo Mutoh,1 Kimiko Hasegawa,1 Hirokazu Kouguchi,2 Yoshimasa Sagane,3 Yukako Fujinaga,4 Keiji Oguma5 and Tohru Ohyama1

1Department of Food Science and Technology, Faculty of Bioindustry, Tokyo University of Agriculture, 196 Yasaka, Abashiri 099-2493, Japan
2Hokkaido Institute of Public Health, N19, W12, Kita-Ku, Sapporo 060-0819, Japan
3The Sars International Centre for Marine Molecular Biology, Thormøhlensgt 55, N-5008 Bergen, Norway
4Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita 565-0871, Japan
5Department of Bacteriology, Okayama University, Medical School, 2-5-1 Shikata-Cho, Okayama 700-8558, Japan

Received 1 December 2004
Revised 28 January 2005
Accepted 1 February 2005

The 650 kDa large toxin complex (L-TC) produced by Clostridium botulinum serotype D strain 4947 (D-4947) has a subunit structure composed of unnicked components, i.e. neurotoxin (NT), non-toxic non-haemagglutinin (NTNHA) and three haemagglutinin subcomponents (HA-70, HA-33 and HA-17). In this study, subunit interactions were investigated through the susceptibilities of the toxin components to limited trypsin proteolysis. Additionally, complex forms were reconstituted in vitro by various combinations of individual components. Trypsin treatment of intact D-4947 L-TC led to the formation of mature L-TC with nicks at specific sites of each component, which is usually observed in other strains of serotype D. NT, NTNHA and HA-17 were cleaved at their specific sites in either the single or complex forms, but HA-33 showed no sign of proteolysis. Unlike the other components, HA-70 was digested into random fragments as a single form, but it was cleaved into two fragments in the complex form. Based on the relative position of exposed or hidden regions of the individual components in the complex derived from their tryptic susceptibilities, an assembly model is proposed for the arrangement of individual subunits in the botulinum L-TC.

INTRODUCTION

The anaerobic bacterium Clostridium botulinum produces seven neurotoxin (NT; 150 kDa) serotypes, which are classified as A to G (Sugiyama, 1980). All NT serotypes are absorbed by intestinal epithelial cells into the bloodstream, reaching the neuromuscular junction. NT inhibits cholinergic vesicle docking at the neuromuscular junction through its endopeptidase activity, leading to flaccid paralysis (Montecucco & Schiavo, 1993). Although they all share sequence and possibly structural similarity, each NT has exclusive substrate specificity and scissile bond selectivity (Schiavo et al., 2000). In addition, these toxins differ in their complex forms, in post-translational modification (nicking and deletion), receptor-binding specificity, duration of toxic action and target specificity (Simpson, 2000).

In culture, NT is part of a large complex (toxin complex; TC) through association with non-toxic components by non-covalent binding, such as the non-toxic non-haemagglutinin (NTNHA; 130 kDa) and haemagglutinin (HA) components. HA, which has haemagglutination activity, is composed of three subcomponents, HA-70, -33 and -17 (respectively 70, 33 and 17 kDa). C. botulinum strains produce different forms of TC, the 280 kDa M-TC (a complex of NT and NTNHA), the 650 kDa L-TC (a
complex of NT, NTNHA and HAs) and the LL-TC (dimer of L-TC) (Sakaguchi, 1982). Thus, both L-TC (serotypes A–D and G) and LL-TC (serotype A) are haemagglutination-positive, while M-TC (serotypes A–F) is haemagglutination-negative. Recently, haemagglutination-negative TC species (410, 540 and 610 kDa TC), which have a smaller number of HA-33/HA-17 molecules than those of L-TC, have also been found in the culture supernatant of serotype D strains (Mutoh et al., 2003).

The NT, NTNHA and HA-70 subunits of the TC species produced by serotype A, C and D strains have always been found to be nicked at specific sites due to bacterial proteases, yielding more protein bands on SDS-PAGE than the expected. Fortunately, we found that the botulinum toxin of the serotype D unique strain 4947 (D-4947) can be purified from the culture supernatant as an L-TC and an M-TC, which consisted only of unnicked components (Kouguchi et al., 2002). Using the isolated components of the D-4947 TC, we have demonstrated in vitro reconstitution of the 650 kDa L-TC with properties that are indistinguishable from those of the parent L-TC (Kouguchi et al., 2002). In addition, we have shown that the complete subunit composition of D-4947 L-TC is a dodecamer composed of a single NT, a single NTNHA, two HA-70, four HA-33 and four HA-17 molecules (Mutoh et al., 2003).

However, little is known about the relative configuration of individual components in the botulinum TC subunit structure or the interaction between subunit components. In this study, the interaction domains between subunit components of the D-4947 TC were examined. Assays for individual toxin component susceptibilities to limited proteolysis with trypsin using single components and complex forms, which were reconstituted in vitro by various combinations of individual components, were performed. Based on these results, we propose a dodecamer model of the subunit structure of the D-4947 TC. The model enhances understanding of the three-dimensional subunit structure of botulinum TC, which will be explored with X-ray crystallographic analysis in the future and may explain how the non-toxic components protect the NT from proteolytic attack.

METHODS

Production and purification of the botulinum TCs. The production and purification of TC species (M-TC and L-TC) of C. botulinum D-4947 were performed as described previously (Mutoh et al., 2003). The culture supernatant was fractionated with ammonium sulfate at 60% saturation and the resulting precipitate was dialysed against 50 mM acetic acid buffer (pH 4-0) containing 0-2 M NaCl. The sample solution was applied to an SP-Toyopearl 650S (Tosoh) column (1×6 cm) equilibrated with dialysis buffer and was eluted with a linear gradient of NaCl (0-2–0-8 M). Four distinct peaks were eluted, with the second and the fourth peaks containing M-TC and L-TC, respectively, as assessed by SDS-PAGE. Each fraction was pooled separately, concentrated and further purified by a HiLoad 16/60 Superdex 200 pg (Amersham Bioscience) gel-filtration column (1×60 cm) equilibrated with 50 mM phosphate buffer (pH 6-0) containing 0-15 M NaCl. The L-TC fraction was further applied to a Mono S HR5/5 (Amersham Bioscience) cation-exchange column. The purity of both TCs was examined by native-PAGE and SDS-PAGE.

PAGE and N-terminal amino acid sequence analysis. PAGE under non-denaturing conditions (native PAGE) was carried out using the method of Davis (1964) at pH 8-8 using a 5–12.5% polyacrylamide linear gradient gel. SDS-PAGE was performed as described by Laemmli (1970) using a 15% polyacrylamide gel in the presence of 2-mercaptoethanol (2-ME). The molecular size markers were phosphorylase b (97 kDa), BSA (66 kDa), egg ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20-1 kDa) and α-lactalbumin (14-4 kDa). The separated protein bands were detected with Coo massie brilliant blue R-250 (CBB). The CBB-staining intensities of the bands were analysed with ImageJ 1.30v (http://rsb.info.nih.gov/ij/). The bands separated by SDS-PAGE were transferred onto a PVDF membrane according to a previously described method (Hirano & Watanabe, 1990) and the amino acid sequences of the components were determined using an automated protein sequence analyser (model 492HT; Applied Biosystems).

Isolation of individual components from D-4947 TCs. Isolation of HA-70 and HA-33/HA-17 from D-4947 TCs was performed according to the method described by Kouguchi et al. (2002). The purified L-TC (250 mg) dissolved in 0-7 ml 20 mM Tris/HCl (pH 7-8), containing 4 M guanidine hydrochloride, was incubated at 21°C for 4 h. The sample was then applied to a HiLoad 16/60 Superdex 200 pg gel-filtration column equilibrated with guanidine hydrochloride buffer. Two peak fractions containing HA-70 and HA-33/HA-17 were eluted and pooled separately. After removal of guanidine hydrochloride buffer by dialysis against 20 mM Tris/HCl (pH 7-8) at 4°C for 15 h, each component was concentrated using appropriate ultrafiltration membranes.

The separation of NT and NTNHA/HAs (NTNHA/HA-70/HA-33/HA-17) from L-TC was achieved by the method developed by Hasegawa et al. (2004) using a HiLoad 16/60 Superdex 200 pg gel-filtration column equilibrated with 20 M Tris/HCl (pH 8-8) containing 0-4 M NaCl. Each separated sample was applied to a Mono Q HR5/5 anion-exchange column equilibrated with 20 mM Tris/HCl (pH 7-8) and eluted by a linear gradient of NaCl (0–1 M). NTNHA was also separated from M-TC using the method described above.

Reconstitution of TC-related complexes by various combinations of the components. Reconstitution of the HA-70/HA-33/HA-17 complex was achieved by mixing isolated HA-70 and HA-33/HA-17 at a protein ratio of 1:1 in 20 mM Tris/HCl (pH 7-8), followed by a 30 min incubation at room temperature. For reconstitution of the M-TC/HA-70 complex, M-TC and isolated HA-70 were mixed at a protein ratio of 1:3 and then transferred into reconstitution buffer with a final concentration of 5 mM sodium phosphate (pH 6-0), 350 mM KCl, 20 mM MgCl2, 6 mM 2-ME and 0-5 mM PMSF. After incubation at 27°C for 21 h, the M-TC/HA-70 complex was separated from uncomplexed components using a HiLoad 16/60 Superdex 200 pg gel-filtration column. NTNHA/HA-70 was derived by separating NT from the reconstituted M-TC/HA-70 complex. M-TC/HA-70 was dialysed against 20 mM Tris/HCl (pH 7-8) and applied to a Mono Q HR5/5 column equilibrated with dialysis buffer. The target protein was eluted by a linear gradient of NaCl (0–1 M).
Analytical gel filtration. A Superdex 200 HR 10/30 (Amersham Bioscience) gel-filtration column (1·0×30 cm) was equilibrated with 50 mM phosphate buffer (pH 6·0) that contained 0·15 M NaCl. The molecular masses of the isolated components and complexes were estimated by calculations with standard proteins thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), egg ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and RNase A (13·7 kDa). The molecular masses were estimated from the means of three experiments.

Limited proteolysis with trypsin. Complex forms (L-TC, NTNHA/HA-70, M-TC/HA-70, NTNHA/HA-70, M-TC, HA-70/HA-33/HA-17 and HA-33/HA-17) and the single forms (NT, NTNHA and HA-70) were incubated with TPCK-trypsin (0·06 U mg⁻¹) using N-α-benzoyl-DL-arginine-p-nitroanilide as substrate) (Sigma) in 50 mM phosphate buffer (pH 6·0) containing 0·15 M NaCl at 37°C. For each experiment, 1:100 protein ratio of trypsin against each preparation was used. The reaction was terminated by the removal of aliquots from the mixture at 10, 30, 60, 120 and 360 min, which were then subjected to SDS-PAGE.

Far-UV circular dichroism (CD) spectroscopy. The secondary structure of HA-70 was analysed using a far-UV CD spectrophotometer (model J-720W; JASCO). CD spectra were recorded using a 10 mm path length cuvette at room temperature. The sample was dialysed against 50 mM phosphate buffer (pH 6·0) containing 0·15 M NaCl and 0·32 mg protein ml⁻¹. The secondary structure content was estimated by the method of Yang et al. (1986) and Manavalan & Johnson (1987) using software provided with the instrument.

RESULTS AND DISCUSSION

Molecular composition of the components and complexes used in this study

*C. botulinum* serotype A–D toxins are encoded by five genes, *nt, ntnha, ha-70, ha-33* and *ha-17*. According to the gene organization, it is expected that botulinum TC would consist of five components, NT, NTNHA, HA-70, HA-33 and HA-17. Nevertheless, *C. botulinum* serotype A–D strains produce two TC species of different sizes, M and L, which separate as distinct peaks by cation-exchange column chromatography and can be further purified individually by gel-filtration chromatography. Purified L-TC shows five bands on SDS-PAGE, of 150, 130, 70, 33 and 17 kDa (Fig. 1b, lane 1). The N-terminal amino acid sequences of the bands were identical to those of the deduced sequences from the D-4947 progenitor toxin gene (accession no. AB037920). The 150 and 130 kDa bands correspond to the NT and the NTNHA, respectively, and the remaining 70, 33 and 17 kDa bands correspond to the HA-70, HA-33 and HA-17 subcomponents of HA. In contrast, purified M toxin had just two bands, of 150 and 130 kDa on SDS-PAGE (Fig. 1b, lane 5), and their N-terminal amino acid sequences...
corresponded to those of NT and NTNHA deduced from the D-4947 gene.

Unlike most purified L-TC preparations from serotypes A–D, the serotype D unique strain D-4947 produces intact TC species without any nicking in the components of the complex (Kouguchi et al., 2002; Hasegawa et al., 2004). Using four isolated subunit components from D-4947 L-TC or M-TC, we attempted to construct possible complex forms by utilizing various combinations of individual components. Since separation of HA-33 and HA-17 from the complex failed because of irreversible precipitation during dialysis to remove the denaturant, the HA-33/HA-17 complex was used in reconstitution experiments. In addition, the assembly pathway of serotype D botulinum TC has been clarified (Kouguchi et al., 2002; Mutoh et al., 2003); the M-TC forms first by the association of one molecule of NT with one molecule of NTNHA and one molecule of M-TC and two molecules of HA-70 then form the L-TC (dodecamer) by the assembly of the four molecules of HA-33 via four molecules of HA-17.

To characterize the molecular composition of various reconstituted complexes, each component responsible for the reconstituted complexes was purified by gel filtration (Fig. 1a). After mixing the components in various combinations of the appropriate molar ratios, products were analysed by SDS-PAGE (Fig. 1b). The molecular composition of single components or complexes was estimated based on the determined molecular masses by gel filtration and calculated from the deduced sequences from D-4947 TC, as summarized in Table 1. However, the molecular masses of isolated NTNHA/HAs and NTNHA/HA-70 that were estimated based on their elution volumes were 30–40% greater than the expected molecular masses. In contrast, the estimated molecular mass of the isolated NT was much lower than the expected 150 kDa (Hasegawa et al., 2004), probably due to hydrophobic interactions of the high-ionic-strength buffer employed between unique D-4947 NT and the Superdex 200 gel matrix, which is composed of highly cross-linked agarose and dextran (Lee & Whitaker, 2004).

### Tryptic susceptibility of single and complex forms of NT

Since complexes containing NT easily dissociate into NT and non-toxic complexes under alkaline conditions (pH greater than 7.5), in this study, every tryptic digestion experiment was performed at pH 6.0. When a single form of 150 kDa NT was treated with trypsin, it was nicked to produce the dichain structure, consisting of the 50 kDa light chain (L-chain) and the 100 kDa heavy chain (H-chain) linked by a disulfide bond (Fig. 2a, lane 2). However, after more than 6 h incubation, the band intensity corresponding to the H-chain decreased by half, and two additional bands, of 90 and 10 kDa, appeared, as shown in Fig. 2(a) (lane 3). The N-terminal amino acid sequence of the 90 kDa band was identical to that of the H-chain beginning at D445, while the 10 kDa band had the N-terminal sequence N1184, indicating it was derived from the C-terminal region of the H-chain. Thus, NT has two trypsin-susceptible sites with different preferences depending on the conditions employed. After treatment for 360 min, the NT was subjected to gel filtration and two distinct peaks were eluted (Fig. 2b, lane 3). SDS-PAGE of the peak fraction in the presence of 2-ME resulted in three bands, 90 kDa H-chain, 10 kDa H-chain and 50 kDa L-chain (Fig. 2c, left), while SDS-PAGE in the absence of 2-ME yielded a 10 kDa band and a 140 kDa band, consisting of the 90 kDa H-chain and the 50 kDa L-chain linked via a disulfide bond (Fig. 2c, right). This suggests that the 90 and

<table>
<thead>
<tr>
<th>Component/complex</th>
<th>Determined (kDa)</th>
<th>Calculated (Da)</th>
<th>Estimated molecular composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NT</td>
</tr>
<tr>
<td>NT</td>
<td>21*</td>
<td>147 341</td>
<td>1</td>
</tr>
<tr>
<td>NTNHA</td>
<td>150</td>
<td>138 444</td>
<td>1</td>
</tr>
<tr>
<td>HA-70</td>
<td>77</td>
<td>70 306</td>
<td>1</td>
</tr>
<tr>
<td>HA-33/HA-17</td>
<td>49</td>
<td>50 471</td>
<td>1</td>
</tr>
<tr>
<td>L-TC</td>
<td>690</td>
<td>628 286</td>
<td>1</td>
</tr>
<tr>
<td>NTNHA/HAs</td>
<td>630</td>
<td>480 944</td>
<td>1</td>
</tr>
<tr>
<td>M-TC/HA-70†</td>
<td>470</td>
<td>426 399</td>
<td>1</td>
</tr>
<tr>
<td>NTNHA/HA-70†</td>
<td>390</td>
<td>279 056</td>
<td>1</td>
</tr>
<tr>
<td>M-TC</td>
<td>260</td>
<td>285 785</td>
<td>1</td>
</tr>
<tr>
<td>HA-70/HA-33/HA-17†</td>
<td>190</td>
<td>171 249</td>
<td>1</td>
</tr>
</tbody>
</table>

*Discrepancy is probably due to a specific interaction with the gel resin, as discussed in the text.
†Reconstituted complexes.
10 kDa fragments of the H-chain are still connected through a non-covalent bond but not a disulfide bond, despite the existence of four Cys residues in the H-chain amino acid sequence, one of which (C1213) is present within the 10 kDa fragment. Similarly, serotype A NT was also found to be nicked by trypsin at specific sites of the C-terminal region of the H-chain; however, the subsequent two fragments were linked with C1234 via C1279 (Shone et al., 1985; Krieglstein et al., 1994).

To characterize the exposed domain of NT through trypsin susceptibility of NT complex forms with other non-toxic components, various complexes, including NT, were prepared. In M-TC (NT/NTNHA), NT was nicked at one specific site at a disulfide loop region that split into the H-chain and L-chain as observed in the NT single form, and an additional cleavage of H-chain still occurred after 6 h incubation as seen in the NT single form. NTNHA was also nicked at a specific site located 135 amino acids downstream.

---

**Fig. 2.** SDS-PAGE in the presence and absence of 2-ME, the N-terminal amino acid sequences of the single form of NT after trypsin treatment and the gel filtration profile. (a) The trypsin reaction was terminated by the removal of aliquots from the mixture at 0, 10 and 360 min (as indicated below the gel), directly followed by SDS-PAGE of these aliquots in the presence of 2-ME. The N-terminal amino acid sequences are shown adjacent to the respective band. (b) Superdex 200 HR 10/30 gel-filtration profiles of NT after trypsin treatment. Each lane corresponds to those in (a). (c) SDS-PAGE banding patterns of NT corresponding to the peaks indicated by arrows in the presence [2-ME (+)] and absence [2-ME (−)] of 2-ME.

**Fig. 3.** SDS-PAGE analysis of complexes containing NT molecules in the presence of 2-ME after trypsin treatment. SDS-PAGE was performed on digests that were terminated by the removal of aliquots from the mixture at 0, 60 and 360 min, which is indicated below the gel in each panel. The N-terminal amino acid sequences of the bands generated are shown. The proteolysis conditions are described in Methods. Susceptibilities of M-TC (NT/NTNHA complex) (a) M-TC/HA-70 (NT/NTNHA/HA-70 complex) (b) and L-TC (NT/NTNHA/HA-70/HA-33/HA-17 complex) (c) to trypsin are shown.
from the N terminus, thus generating 15 kDa N-terminal and 115 kDa C-terminal fragments (Fig. 3a). These results suggest that (i) NTNHA may not protect the C-terminal region of H-chain from proteolytic attack and (ii) the disulfide loop region of NT connecting the L-chain and the H-chain is still exposed on the M-TC complex form. The gel-filtration profiles of the trypsin-treated sample showed a single peak, suggesting strongly that M-TC still maintains a complex form with nicks in its individual components.

In the case of M-TC/HA-70 (NT/NTNHA/HA-70) and L-TC (NT/NTNHA/HA-70/HA-33/HA-17) complex forms, NT has only one site susceptible to trypsin (in the loop region), and nicking of the C-terminal side of H-chain was no longer observed, as shown in Fig. 3(b, c). This observation implies that the C-terminal side of NT can be covered by HA-70, since both complexes contained HA-70. Additionally, it suggests that the loop region of NT in every complex form is always exposed, even in the L-TC. On the other hand, NTNHA in both complex forms containing HA-70 showed no sign of tryptic cleavage at specific sites (Fig. 3b, c). This indicates that HA-70 may also interact specifically and cover particular susceptible sites of NTNHA in such a way that it remains inaccessible to trypsin attack.

**Tryptic susceptibility of single and complex forms of HA subcomponents**

Since it was predicted that the HA-70 subcomponent plays a key role as an adherent protein linking M-TC and HA-33/HA-17, leading to the formation of L-TC, the trypptic characteristics of the HA-70 molecule were examined. Interestingly, unlike other components, an isolated form of HA-70 was highly sensitive to trypsin digestion, yielding many fragments within a 10 min incubation, as shown in Fig. 4(a). Similarly, in complexes containing one molecule of HA-70 such as HA-70/HA-33/HA-17, HA-70 was still degraded into many fragments by trypsin digestion (Fig. 4b). On the other hand, when two molecules of HA-70 formed complexes with NTNHA, such as NTNHA/HA-70 and NTNHA/HA-70/HA-33/HA-17 (see Table 1), HA-70 protein in both complexes was protected from random cleavage with trypsin, although it was cleaved at specific nicking sites, leading to 22 to 23 kDa N-terminal and 55 kDa C-terminal fragments, as shown in Fig. 5(a, b). Similarly, in the M-TC/HA-70 (NT/NTNHA/HA-70) and L-TC (NT/NTNHA/HA-70/HA-33/HA-17) complexes, HA-70 protein was also cleaved at specific sites (Fig. 3b, c). This suggests that two HA-70 molecules are buried in the interior of L-TC, adhering to NTNHA and HA-33/HA-17, and expose their specific cleavage sites on the surface of L-TC.

Generally, trypsin has a strong preference for cleavage of amide substrates following Arg or Lys residues when proteins are unfolded or denatured. To characterize the molecular properties of HA-70, the IR-based secondary structure was examined (Fig. 6). Unexpectedly, far-UV CD analysis of the HA-70 molecule as a monomer in aqueous solution showed a β-sheet-rich structure (79.3% β-sheet, 19.5% random coil structures and 1.2% α-helix), in spite of the suggestion that a β-sheet-rich structure may be a common feature essential for trypsin resistance (Fu et al., 1998). Therefore, when the unfolded HA-70 monomer in guanidine hydrochloride buffer was diluted and dialysed, a partially folded intermediate of HA-70 may be formed that is unlike the native HA-70 structure found in the complex, therefore resulting in susceptibility to trypsin digestion.

In the remaining HA subcomponents, when the HA-33/HA-17 complex was treated with trypsin, HA-33 showed no sign of tryptic digestion, and only HA-17 was observed to have a cleavage site at the N-terminal region (R4/T5), as

---

**Fig. 4.** SDS-PAGE of HA subcomponents and their complex forms in the presence of 2-ME after trypsin treatment. Digestion was terminated by the removal of aliquots from the mixture at 0, 10 and 360 min as indicated, followed by SDS-PAGE of these aliquots. The N-terminal amino acid sequences of the bands generated are shown. Susceptibilities of the HA-70 single form (a), the HA-70/HA-33/HA-17 complex (b) and the HA-33/HA-17 complex (c) to trypsin are shown.
shown in Fig. 4(c). However, no cleavage was observed in HA-17 in all the complex forms, except the HA-33/HA-17 complex. According to our previous report on the assembly pathway of D-4947 L-TC (Mutoh et al., 2003), HA-17 is required for the formation of the linkage between HA-70 and HA-33. It is therefore suggested that the N-terminal region of HA-17 may be covered by HA-70 or bind directly to HA-70. Additionally, since HA-33 was found to be absolutely resistant to trypsin digestion, it is speculated that HA-33 is exposed on the surface of TC, leading to the complete configuration in which four HA-33 molecules occupy surface positions in the subunit structure of L-TC.

Similarly, the secondary structure of serotype C strain 6814 HA-33, which closely resembles D-4947 HA-33, with 96% amino acid sequence identity, showed a predominantly \( \beta \)-sheet (70–71%) and random coil (29–30%) structure as estimated from the CD spectrum (Kouguchi et al., 2001). A similar \( \beta \)-sheet-rich structure was observed for serotype A HA-33, which was also absolutely resistant to trypsin digestion (Sharma et al., 1999). Recently, Inoue et al. (2003) demonstrated that HA-33 from serotype C is composed of two \( \beta \)-trefoil domains linked by an \( \alpha \)-helix, according to crystal structure analysis.

**Representation of a possible arrangement of the subunit structure of the botulinum TC**

Based on the combined results of the experiments described here and the subunit composition of L-TC (a single NT, a single NTNHA, two HA-70, four HA-33 and four HA-17) in our previous report (Mutoh et al., 2003), we propose the model for the arrangement of the individual components in the botulinum L-TC shown in Fig. 7. The model was deduced from the following experimental observations.

The specific cleavable sites in the loop region of NT (50 kDa L-chain and 100 kDa H-chain) are always exposed in either its single or complex forms. The C-terminal region of NT is covered by HA-70.

HA-70 seems to adhere to NTNHA with the other HA-70 molecule, leading to the protection of the specific cleavage site of NTNHA (15 kDa N-terminal and 115 kDa C-terminal fragments) by HA-70. Simultaneously, HA-70 adhering to NTNHA also escapes from random degradation by trypsin.

HA-17, with its N-terminal region covered by HA-70, is interposed between HA-70 and HA-33 molecules.

Four HA-33 molecules, which are absolutely resistant to proteolysis, are exposed on the surface of TC.

Previously, a partial model for the botulinum NT complex was proposed by Chen et al. (1997) based on antibody mapping to the domain of type A NT in complexed and
uncomplexed forms. However, their model demonstrated merely the interaction between the NT and non-toxic portion in the complex, since they failed to distinguish individual non-toxic components such as NTNHA and the HAs. X-ray crystallography had indicated the most probable set of three-dimensional coordinates for nearly all of the atoms of serotype A and B NT proteins (Lacy et al., 1998; Swaminathan & Eswaranmooorthy, 2000) and serotype C HA-33 (Inoue et al., 2003). Currently, the three-dimensional structures of NTNHA, HA-70 and HA-17 proteins are not available; thus, it is not possible to know their structural features. Therefore, our model may be subject to a few caveats. However, results on the subunit interaction of the botulinum TC raise the possibility that the botulinum non-toxic complex could be used as a novel drug delivery vehicle in the future, according to the current understanding that HA subcomponents may increase the internalization of the NT into the bloodstream via binding to intestinal membrane (Fujinaga et al., 1997, 2000, 2004), in addition to protecting the structural integrity of NT from protease digestion.

ACKNOWLEDGEMENTS

The authors are grateful to Yozo Nakazawa for his CD spectra measurements and Norio Kumagai, Hideaki Kikuchi, Rei Saitoh, Hiroaki Fukuda and Kana Sunohara for technical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research (C), no. 15570103, from the Japan Society for the Promotion of Science.

REFERENCES


