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Building-block architecture of botulinum toxin complex: Conformational changes provide insights into the hemagglutination ability of the complex

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ABSTRACT

Clostridium botulinum produces the botulinum neurotoxin (BoNT). Previously, we provided evidence for the "building-block" model of botulinum toxin complex (TC). In this model, a single BoNT is associated with a single nontoxic nonhemagglutinin (NTNHA), yielding M-TC; three HA-70 molecules are attached and form M-TC/HA-70, and one to three "arms" of the HA-33/HA-17 trimer (two HA-33 and one HA-17) further bind to M-TC/HA-70 via HA-17 and HA-70 binding, yielding one-, two-, and three-arm L-TC. Of all TCs, only the three-arm L-TC caused hemagglutination. In this study, we determined the solution structures for the botulinum TCs using small-angle X-ray scattering (SAXS). The mature three-arm L-TC exhibited the shape of a "bird spreading its wings", in contrast to the model having three "arms", as revealed by transmission electron microscopy. SAXS images indicated that one of the three arms of the HA-33/HA-17 trimer bound to both HA-70 and BoNT. Taken together, these findings regarding the conformational changes in the building-block architecture of TC may explain why only three-arm L-TC exhibited hemagglutination.

1. Introduction

Clostridium botulinum produces botulinum neurotoxin (BoNT), the most potent toxin in nature. This neurotoxin is a causative agent of human and animal botulism disease and can be classified into seven serotypes (A–G) [1]. Serotypes A, B, E, and F are associated with human botulism, whereas serotypes C and D predominantly cause animal and avian disease. BoNT is a metalloendopeptidase having a zinc ion in its molecule [2]. BoNT targets nerve endings at the neuromuscular junction and enters into nerve cells via endocytosis, after which BoNT cleaves the specific site in soluble NSF attachment protein receptors (SNAREs) associated with neurotransmitter release [3–5]. This process results in muscle paralysis and may cause death in both humans and animals.

In culture fluids and contaminated foods, BoNT is present as a part of a toxin complex (TC) through its associations with auxiliary nontoxic proteins, i.e., nontoxic nonhemagglutinin (NTNHA) and three types of hemagglutinin (HA; HA-70, HA-33, and HA-17). BoNT is susceptible to digestion in the gastrointestinal tract of humans and animals; therefore, its toxicity is decreased when it is exposed to digestive juices. However, when part of the TC, BoNT is stable, even in the presence of digestive juices [6]. Thus, nontoxic proteins play a role in protection of BoNT against digestive conditions. Furthermore, the HA protein may facilitate transepithelial transport at the intestinal wall [7]. Recent studies have shown that the serotype A TC disrupts the E-cadherin-mediated intercellular barrier of the intestinal epithelia and facilitates the paracellular absorption of the TC [8].

In the TCs produced by serotype C and D strains, there are two types of TCs: hemagglutination-negative and hemagglutination-positive TCs [9,10]. Hemagglutination-negative TCs are referred to as M-TCs and include BoNT and NTNHA, whereas hemagglutination-positive TCs are referred to as L-TCs and include M-TC and HA proteins. Therefore, hemagglutination is thought to be caused by HA proteins [9]. However, we have previously identified an intermediate TC species [11]. M-TC is composed of a single molecule of BoNT and a single molecule of NTNHA. The HA-70 trimer is attached to the M-TC molecule via an interaction between NTNHA and HA-70, yielding the M-TC/HA-70 complex. Additionally, three "arms" of the HA-33/HA-17

Abbreviations: BoNT, botulinum neurotoxin; TC, toxin complex; NTNHA, nontoxic nonhemagglutinin; HA, hemagglutinin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SAXS, small-angle X-ray scattering; QCM, quartz crystal microbalance; DAM, dummy atom model; TEM, transmission electron microscopy

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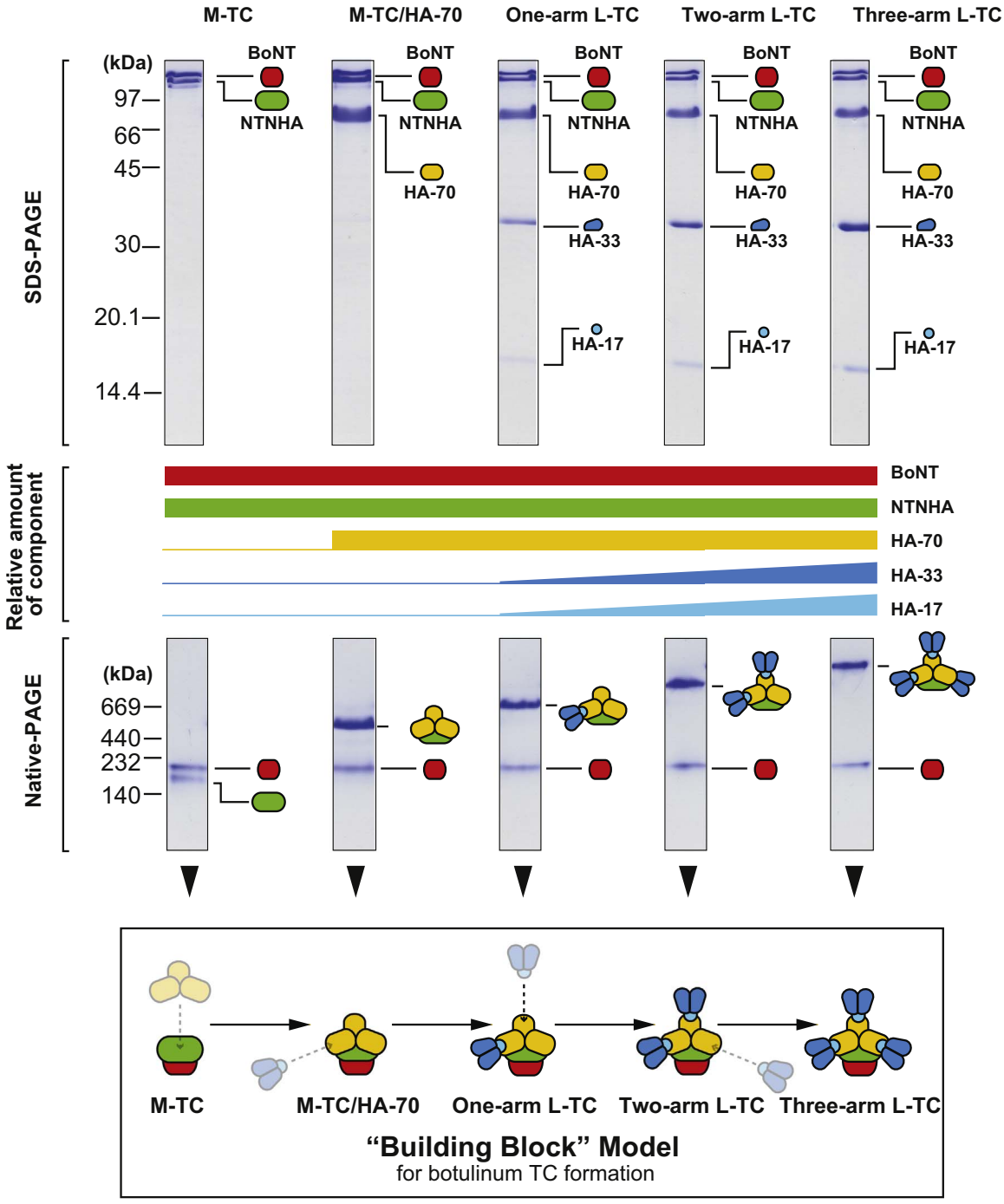

Fig. 1. Building-block architecture model for the botulinum toxin complex (TC). All TC species were developed on the SDS-PAGE and native PAGE as shown in supplementary fig. 1. Relative amounts of each component in the TC species are represented by the width of the ribbon. As shown in the "building-block" model in the bottom of the figure, the M-TC matured into the three-arm L-TC via intermediate TC species, including M-TC/HA-70, one-arm L-TC, and two-arm L-TC.

trimer (a complex of two HA-33 proteins and one HA-17 protein) are bound to the M-TC/HA-70, forming mature three-arm L-TC [12]. As intermediates between the M-TC/HA-70 to three-arm L-TC, one- and two-arm L-TCs, which have only one or two "arms" of the HA-33/HA-17 trimer, respectively (see Fig. 1), have also been observed. The botulinum TC is not formed by random association of the component proteins, but is constructed through a specific mechanism; thus the construction of botulinum TC is a sort of "building-block architecture". Interestingly, hemagglutination occurs only by the three-arm L-TC, but not by intermediate TC species, although these species also contain HA proteins [11].

In this study, we aimed to determine the solution structure of the "building-block" TC species, including the M-TC, M-TC/HA-70, one-arm L-TC, two-arm L-TC, and three-arm L-TC. Furthermore, we discussed the relationship between the molecular conformation of the TC species and hemagglutination.

2. Materials and methods

2.1. Production and purification of TC species

C. botulinum serotype D strain 4947 was cultured anaerobically for 5 days using dialysis [13]. Crude TC was precipitated with 60% (w/v) saturated ammonium sulfate, dialyzed against 50 mM acetate buffer

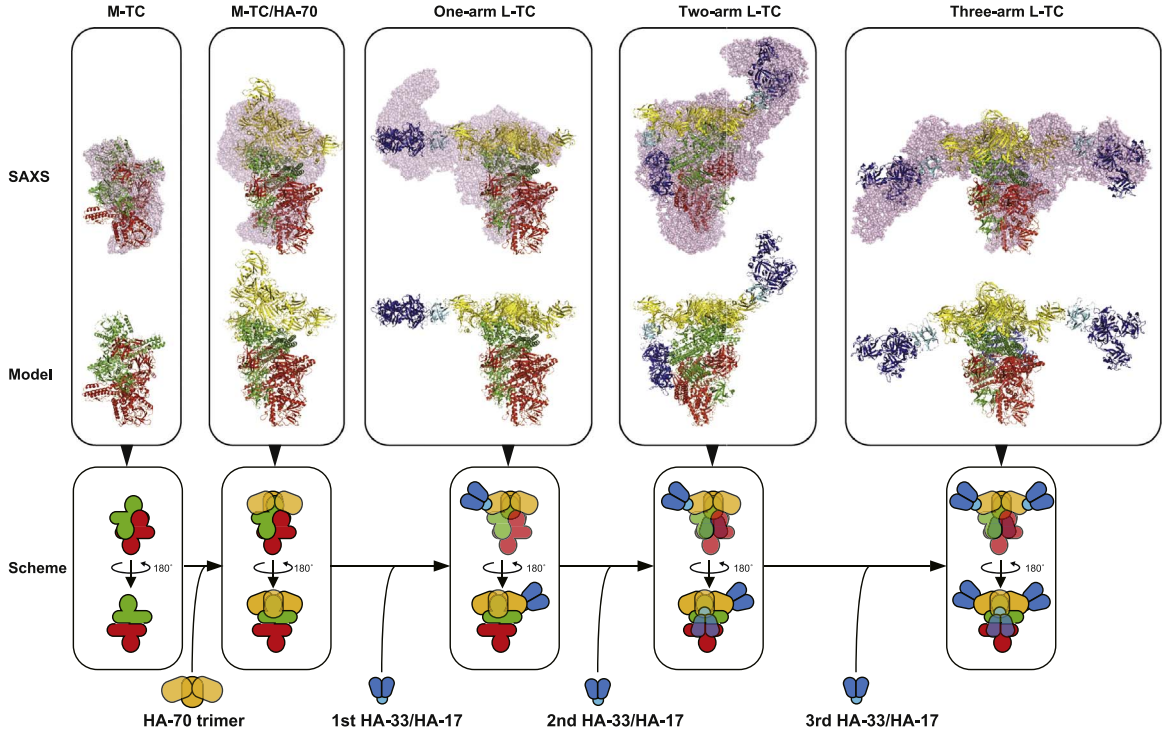

Fig. 2. Solution structures of the botulinum TC species revealed by SAXS analyses. The upper row of images shows SAXS images of each TC species with crystal structures for a single molecule of serotype A M-TC, a single molecule of serotype C HA-70 trimer, and one to three molecules of serotype D HA-33/HA-17 trimers, which were manually superimposed on the SAXS image such that all molecules were fitted into the image (see Supplementary Movie 1). The second row indicates the model of each molecule represented as cartoon images of the crystal structures. The lowest row indicates the scheme of each molecule with the assembly pathway of the HA proteins to the M-TC molecule.

(pH 4.0) containing 0.2 M NaCl, and applied to a TOYOPEARL SP-650S (Tosoh, Tokyo, Japan) cation-exchange column (1.6 cm×40 cm) equilibrated with dialysis buffer. TC species bound to the resin were eluted with a linear gradient of NaCl (0.2–0.8 M). Each fraction (M-TC, M-TC/HA-70, one-arm L-TC, two-arm L-TC, and three-arm L-TC) was pooled separately, concentrated, and further purified with a HiLoad 16/60 Superdex 200 pg gel-filtration column (GE Healthcare, Little Chalfont, UK 1.6 cm×60 cm) equilibrated with 50 mM acetate buffer (pH 5.0) containing 0.15 M NaCl. The TC fraction was then applied to a Mono S HR5/5 cation-exchange column (GE Healthcare UK; 0.5 cm×5 cm) equilibrated with 50 mM acetate buffer (pH 5.0) and eluted using a linear gradient of NaCl (0–0.5 M). The purities of the M-TC, M-TC/HA-70, one-arm L-TC, two-arm L-TC, and three-arm L-TC were evaluated by native PAGE and SDS-PAGE.

2.2. PAGE

PAGE under nondenaturing conditions (native PAGE) was carried out at pH 8.8 using 5–12.5% polyacrylamide linear gradient gels. SDS-PAGE was performed as described by Laemmli [14] using 13.6% polyacrylamide gels in the presence of 2-mercaptoethanol. The separated protein bands were detected with Coomassie Brilliant Blue R-250.

2.3. Small-angle X-ray scattering analysis (SAXS)

SAXS measurements of the botulinum TC species purified from strain D 4947 (5 mg/mL) in 10–20 μL of 50 mM acetate buffer (pH 4.0) were performed on a Rigaku BioSAXS-1000 (Rigaku, Tokyo, Japan). In total, eight datasets were collected after 120 min of exposure (15 min per data set). Raw data were analyzed using the SAXSLab software package (Rigaku). SAXS curves were generated after subtracting solvent scattering using the program PRIMUS from the ATSAS package.

2.4. Measurement of the association constant using a quartz crystal microbalance

To measure the association constant (K_a) between the HA-33/HA-17 trimer and TC species, including the M-TC/HA-70, one-arm L-TC, and two-arm L-TC, 27-MHz quartz crystal microbalance (QCM) analysis using AffinixQ4 (Initium, Chigasaki, Japan) was performed. After rinsing with 1% SDS and Piranha solution, the electrode was exposed to 100 μL of 3,3′-dithiodipropionic acid (210 μg/mL) for 60 min and exposed to 100 μL of a 1:1 mixture of *N*-hydroxysuccinimide (NHS; 20 mg/mL) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (20 mg/mL) for 15 min. The electrode was covered with 450 μL of TC species (100 μg/mL) in 10 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl and incubated for 30 min. Thereafter, the electrode was covered with 100 μL of 1 M 2-aminoethanol (pH 8.5) and incubated for 30 min to deactivate the free NHS residues on the electrode. Measurement of the frequency change was performed in the presence of various concentrations (10–200 nM) of HA-33/HA-17 trimer in 10 mM phosphate buffer (pH 6.0) containing 0.15 M NaCl and blocking reagent for AffinixQ (Initium). The K_a values were calculated using AQUA software (Initium).

3. Results and discussion

In this study, we isolated and purified several TC species, including the M-TC, M-TC/HA-70, one-arm L-TC, two-arm L-TC, and three-arm L-TC, from the culture supernatants of the *C. botulinum* serotype D 4947. Components of the purified TC species were developed on denaturing SDS-PAGE, as shown in Fig. 1 (original gel picture is shown in supplementary figure 1). The M-TC and M-TC/HA-70 were composed of BoNT/NTNHA and BoNT/NTNHA/HA-70, respectively. In contrast, the one- to three-arm L-TCs were complexes of BoNT/NTNHA/HA-70/HA-17/HA-33. Staining intensities for the protein bands representing BoNT, NTNHA, and HA-70 were similar for all TC species. However, the intensities of the protein bands representing

Table 1
Association constants (K_a) for the binding between HA-33/HA-17 and TC species based on the QCM analysis.

Guest: Host	K_a (nM^{-1})
HA-33/HA-17: M-TC/HA-70	0.004
HA-33/HA-17: 1-arm L-TC	0.16
HA-33/HA-17: 2-arm L-TC	0.016

HA-33 and HA-17 were increased as the number of "arms" increased for L-TCs.

Each of the purified TC species was subjected to SAXS analysis, and the dummy atom model (DAM) was generated as shown in Fig. 2. The maximum diameters of the M-TC, M-TC/HA-70, one-arm L-TC, two-arm L-TC, and three-arm L-TC were found to be 150, 177, 234, 277, and 400 Å, respectively. Previously, Hasegawa et al. [12] demonstrated that mature L-TC from serotype D displayed an ellipsoidal-shaped structure with three "arms" attached, based on transmission electron microscopy (TEM) images. Similar structures were also identified in serotypes A and B botulinum TCs [15], based on a combination of TEM and crystallographic analyses. Hasegawa et al. [12] also showed that the one or two "arms" are attached structures for the one-arm and two-arm L-TCs, respectively. However, the SAXS image displayed only one "arm"-like structure for both the one-arm L-TC and two-arm L-TC. In the SAXS image for the three-arm L-TC, only two "arms" were extended, and the appearance of the three-arm L-TC exhibited a shape resembling a "bird spreading its wings". As shown in Fig. 2, we constructed a model of the coordination of each component in the TC species based on the superimposition of the SAXS image and representation of the crystal structure. In this model, the discrepancy between the SAXS image showing only two "wings" and the TEM images showing three "arms" could be explained by coordination of the "second arm", which was composed of a HA-33/HA-17 trimer attached during formation of the two-arm L-TC. The HA-33/HA-17 trimer could interact with the M-TC/HA-70 via binding with HA-17/HA-70 and HA-33/BoNT. Therefore, the first and third "arms" of the HA-33/HA-17 trimer could explain the appearance of "spreading wings" in the SAXS "bird" model.

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.bbrep.2016.11.008.

Previously, Benefield et al. [16] suggested that the "arms" of the HA-33/HA-17 trimer in the 16S PC (corresponding to our three-arm L-TC) are flexible, and the trimer in 90% of the TC displayed a "flat" orientation containing an ovoid body with only two well-defined arms. Thus, these data suggest that one of the three "arms" of the HA-33/HA-17 trimer is attached to the ovoid body. Sharma and Singh [17] reported that the HA-33 molecule interacts with BoNT via strong protein-protein interactions. These reports also supported the additional interaction between HA-33 and BoNT molecules in the TC. To detect the unique interaction in the second "arm" of the HA-33/HA-17 trimer with the one-arm L-TC, the binding kinetics of the HA-33/HA-17 trimer to the M-TC/HA-70, one-arm L-TC, and two-arm L-TC were analyzed using a QCM-based method. As shown in Table 1, the K_a values for binding of the HA-33/HA-17 trimer with the M-TC/HA-70, one-arm L-TC, and two-arm L-TC were found to be 0.004, 0.16, and 0.016, respectively. Thus, binding of the HA-33/HA-17 trimer with the one-arm L-TC was the strongest interaction among the combinations tested.

As shown in Fig. 3, our model for the solution structure of botulinum TC species would explain why only the three-arm L-TC exhibited hemagglutination. The membrane of the erythrocyte is negatively charged due to the sialic acid on the sugar chain attached to the cell surface. If the erythrocytes are suspended in an ionic medium, such as saline, which is usually employed in hemagglutination assays, cations arrange themselves around each cell to form an ionic cloud. The ionic cloud keeps erythrocytes from adhering to each other in the peripheral blood. Thus, the potential providing the force that repels erythrocytes from each other, which is created by the ionic cloud, is the zeta potential [18]. In solution, the zeta potential kept the erythrocytes about 250 Å apart (Fig. 3A). Therefore, to exhibit hemagglutination, a protein must have at least two cell surface binding sites, and the distance between the two sites must be greater than 250 Å. As shown in Fig. 3B, the M-TC/HA-70, one-arm L-TC, and two-arm L-TC possessed multiple cell binding sites. However, the distances between the binding sites were estimated to be approximately 130, 160, and 200 Å, respectively, based on the arrangements of the components in

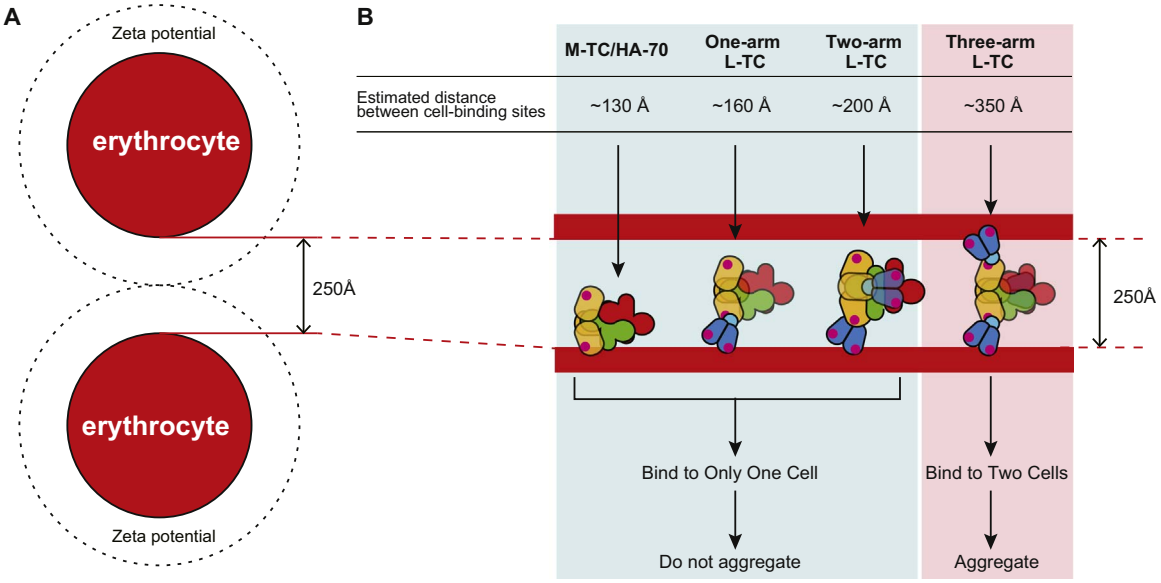

Fig. 3. Schematic diagram of erythrocytes in the ionic solution and the botulinum TC species binding to the surface of the cells. **A.** In the ionic solution, erythrocytes were positioned approximately 250 Å apart due to the zeta potential created by the cationic cloud surrounding the negatively charged erythrocytes, resulting from the presence of sialic acid on the cell surface. **B.** Schematic indicating the size of the gap between two erythrocytes and the botulinum TC species. Magenta closed-circles indicate the cell-binding sites on the HA-33 and HA-70 molecules suggested by Sugawara et al. [21]. In all TC species except for the three-arm L-TC, TC could bind to a single erythrocyte, but could not bind to two cells because of the proximity of the two cell-binding sites. Thus, the erythrocytes did not aggregate. In contrast, the three-arm L-TC could bind concomitantly to two cells because two cell-binding sites on the HA-33 protein could allow a sufficient distance to be maintained between the two cells.

the TC and the Dmax of each TC. In contrast, in the three-arm L-TC, the distance between the cell-binding sites in two HA-33 molecules in the "two wings" was estimated as ~350 Å and was therefore longer than 250 Å. Thus, only the three-arm L-TC exhibited hemagglutination. The maximum diameter of the three-arm L-TC was determined to be 400 Å using SAXS analysis. The arrangement of components in the SAXS image indicated that this diameter represented the width of the HA complex (HA-70/HA-17/HA-33 complex). However, the width of the serotype-A HA complex was determined to be approximately 260 Å, based on the TEM analysis [15]. The discrepancy in the size of the complex may be explained by the structural flexibility of the HA proteins, as shown in previous reports describing SAXS analyses [19,20].

In summary, these findings suggested that the HA complex plays an important role in the transepithelial transport of the toxin, which may be associated with the flexibility of the complex. The assembly pathway of the TC also would be dynamic. Although the structure of the botulinum TC has been gradually elucidated, current information is limited to the static structure. Thus, dynamic analyses including SAXS-based methods will help to clarify the pathogenic mechanisms of the botulinum TC.

Transparency document. Supplementary document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.11.008.

Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.11.008.

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