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Sugar-induced conformational change found in the HA-33/HA-17 trimer of the botulinum toxin complex

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ABSTRACT

Large-sized botulinum toxin complex (L-TC) is formed by conjugation of neurotoxin, nontoxic nonhemagglutinin and hemagglutinin (HA) complex. The HA complex is formed by association of three HA-70 molecules and three HA-33/HA-17 trimers, comprised of a single HA-17 and two HA-33 proteins. The HA-33/HA-17 trimer isolated from serotype D L-TC has the ability to bind to and penetrate through the intestinal epithelial cell monolayer in a sialic acid-dependent manner, and thus it plays an important role in toxin delivery through the intestinal cell wall. In this study, we determined the solution structure of the HA-33/HA-17 trimer by using small-angle X-ray scattering (SAXS). The SAXS image of HA-33/HA-17 exhibited broadly similar appearance to the crystal image of the complex. On the other hand, in the presence of *N*-acetylneuraminic acid, glucose and galactose, the solution structure of the HA-33/HA-17 trimer was drastically altered compared to the structure in the absence of the sugars. Sugar-induced structural change of the HA-33/HA-17 trimer may contribute to cell binding and subsequent transport across the intestinal cell layer.

1. Introduction

Botulinum toxin complex (TC), which is produced by the anaerobic gram-positive bacterium *Clostridium botulinum*, is the causative agent of food-borne botulism. The TC is composed of the botulinum neurotoxin (BoNT; 150 kDa) and auxiliary nontoxic proteins. *C. botulinum* is classified into seven serotypes, A–G, based on the antigenicity of the BoNT. Human botulism is caused predominantly by serotypes A, B, E and F, while animal and avian botulism is due to the serotypes C and D [7,10]. Orally ingested TC passes through the digestive tract and enters the blood stream via the intestinal wall. Once in the blood stream BoNT dissociates from the TC then reaches and penetrates into the nerve cells of the neuromuscular junction, where it cleaves a specific site on the protein involved in neurotransmitter release resulting in paralysis of the muscle.

The auxiliary nontoxic proteins of the TC include nontoxic nonhemagglutinin (NTNHA; 130 kDa) and three types of hemagglutinin (HA) components with molecular masses of 70, 33 and 17 kDa (HA-70, HA-33 and HA-17, respectively). The TC assembly includes association of a single BoNT and a single NTNHA to form M-TC, which is amazingly stable in the acidic and proteolytic conditions of the digestive tract [9]. Further binding of three HA-70 proteins onto the M-TC yields M-TC/HA-70. Finally, attachment of three HA-33/HA-17 trimers, each of which are comprised of a single HA-17 and two HA-33 proteins, to M-TC/HA-70 via binding of HA-70 and HA-17 results in forming the 14-mer mature L-TC (see Fig. 1A). In addition, there are intermediate L-TC species having fewer HA-33/HA-17 trimers in the culture supernatant of *C. botulinum* [11].

Generally, the physical barrier presented by the intestinal wall prevents the entrance of macromolecules such as proteins into the body. All the TCs, however, produced by the serotype D *C. botulinum* strain 4947 (D-4947), including pure BoNT, M-TC, M-TC/HA-70 and L-TC, can be transported across a rat intestinal epithelial cell (IEC-6) monolayer [4]. Additionally, transport of the toxins is enhanced depending on the number of HA-33/HA-17 trimers.

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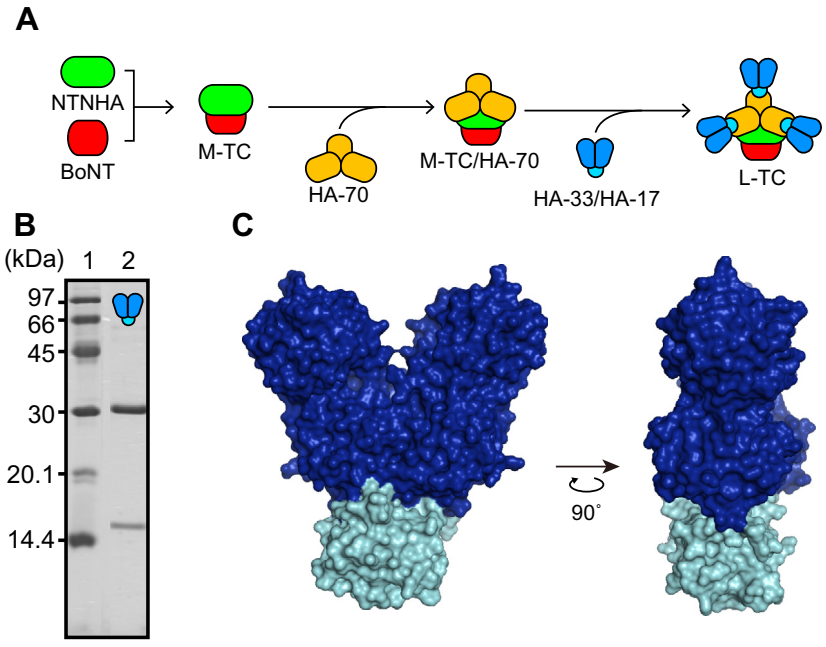

Fig. 1. Assembly pathway of the botulinum toxin complex (TC) and characterization of the HA-33/HA-17 trimer used in this study. (A) Assembly pathway of the botulinum TC. Binding of BoNT and NTNHA yields M-TC. Attachment of three HA-70 components to the M-TC forms M-TC/HA-70, finally three HA-33/HA-17 trimers consisting of a single HA-17 and two HA-33 proteins are conjugated to the M-TC/HA-70, resulting in the mature L-TC. (B) SDS–PAGE banding pattern of purified HA-33/HA-17 trimer. After purification from L-TC, HA-33/HA-17 trimer was analyzed by SDS–PAGE using a 15% polyacrylamide gel. The proteins in the gel were stained with Coomassie Brilliant Blue. The molecular masses of the protein standards (lane 1) are labeled on the left in kDa. The HA-33/HA-17 complex (lane 2) displayed two bands with molecular masses of 33 and 17 kDa. (C) Surface representation of the D-4947 HA-33/HA-17 trimer revealed in a previous study [2]. The HA-33 and HA-17 molecules are indicated in blue and light blue, respectively. The left image is rotated 90° clockwise around the y-axis compared to the right image. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

Similar to the TC species, the HA-33/HA-17 trimer isolated from the D-4947 L-TC can also be transported across the IEC-6 cell layer [14]. Therefore, the HA-33/HA-17 complex plays an important role in the effective transport of toxin across the intestinal wall. The transport of the L-TC and HA-33/HA-17 trimer across the intestinal epithelial cells was inhibited by the presence of the sialic acid, and thus it appears to be mediated by a sialic acid-dependent receptor on the cell surface. In this study, we determined the solution structure of the HA-33/HA-17 trimer in the absence and presence of the sugars *N*-acetylneuraminic acid (Neu5Ac), glucose and galactose, based on small-angle X-ray scattering (SAXS) analysis. The results indicate that sugars induce a conformational change of the HA-33/HA-17 trimer. Sugar-induced conformational change has also been observed in other carbohydrate-binding proteins [1,13].

2. Materials and methods

2.1. Production and purification of botulinum TC

C. botulinum serotype D strain 4947 (D-4947) was cultured using a dialysis method as described previously [8]. The TC in the culture supernatant was precipitated with 60% saturation of ammonium sulfate. The resultant precipitate was dissolved and dialyzed against 50 mM acetate buffer, pH 4.0, containing 0.2 M NaCl and applied to a TOYOPEARL SP-650S (Tosoh, Tokyo, Japan) cation-exchange column (1.6 × 40 cm) equilibrated with dialysis buffer. Bound protein was eluted with a linear gradient of NaCl (0.2–0.8 M). The peak fraction containing the L-TC, judged by SDS- and native-PAGE, was collected, concentrated and further purified with a HiLoad 16/60 Superdex 200 pg (GE Healthcare, Little Chalfont, UK) equilibrated with 50 mM acetate buffer (pH 5.0) containing 0.15 M NaCl. The peak fraction containing the L-TC was precipitated with 80% saturation of ammonium sulfate.

2.2. Isolation of the HA-33/HA-17 trimer from the L-TC

Isolation of the HA-33/HA-17 trimer from the L-TC was performed as previously reported [5]. The concentrated L-TC, in a 250-mg precipitate pellet, was dissolved in 0.7 ml of 20 mM Tris–HCl (pH 7.8) containing 4 M guanidine hydrochloride (Gdn buffer) and incubated at 21 °C for 4 h. Treated sample was applied to a HiLoad Superdex 200 pg 16/60 gel-filtration column equilibrated with the Gdn buffer. The fraction containing HA-33/HA-17 trimer was collected, diluted to 0.1 absorbance at 280 nm with Gdn buffer, and then dialyzed against 20 mM Tris–HCl, pH 7.8, at 4 °C for 15 h to remove the guanidine hydrochloride.

2.3. PAGE analysis

SDS–PAGE was performed as described by Laemmli [6] using a 13.6% polyacrylamide gel in the presence of 2-mercaptoethanol. Native PAGE was carried out using the method of Davis et al. at pH 8.8 using a 5–12.5% polyacrylamide linear gradient gel. The separated peptides were stained with Coomassie Brilliant Blue R-250.

2.4. Small-angle X-ray scattering analysis

Small-angle X-ray scattering (SAXS) measurements of the HA-33/HA-17 trimer in 20 mM Tris–HCl, pH 7.8 were performed on a Rigaku BioSAXS-1000 using 10–20 μl of protein solution. A total of eight datasets were collected after 120 min exposure (15 min per data set). Raw data were analyzed using the SAXSLab software package (Rigaku, Tokyo, Japan). SAXS curves were generated after subtracting the scattering due to the solvent in the protein solution, using the program PRIMUS from the ATSAS package.

3. Results

HA-33/HA-17 trimer was purified from L-TC produced by *C. botulinum* D-4947 in the presence of guanidine hydrochloride. The purified HA-33/HA-17 trimer displayed two bands in SDS–PAGE with molecular masses of 33 and 17 kDa (Fig. 1B). The crystal structure of the D-4947 HA-33/HA-17 trimer was previously determined and revealed an isosceles triangle-like structure in which one end of each of two ellipsoidal HA-33 molecules is attached to a sphere-shaped HA-17 molecule (Fig. 1C) [2]. After removal of the guanidine hydrochloride, the HA-33/HA-17 trimer was subjected to SAXS analysis and the dummy atom model (DAM) was generated (Fig. 2 and Supplementary video 1). The SAXS-derived DAM image also indicated that the HA-33/HA-17 trimer forms an isosceles triangle-like structure but kinked at the middle of the triangle. Further, the SAXS DAM image is slightly larger than that seen in the crystallographic image as shown in the right panel of Fig. 2. Therefore the junction between the single HA-17 and dual HA-33 molecules would be flexible allowing iterative hinge-like movement of the trimer from flat to a kinked triangle as shown in the middle panel of Fig. 2.

Fig. 3 and Supplementary video 2 show the SAXS DAM images of the HA-33/HA-17 trimer in the presence of 1 mM of Neu5Ac, glucose and galactose. All the images in the presence of sugars displayed similar appearance, with a more elongated shape than that seen in the absence of the sugars. As shown in the rightmost panel of Fig. 3, a single HA-17 and two HA-33 molecules were superimposed into the SAXS DAM image manually, so that all molecules were fitted into the image. The second panel from the right of Fig. 3 contains the best-fit model of the HA-33/HA-17 trimer in the presence of the sugars. In the absence of the sugars, the dual HA-33 molecules are attached onto the single HA-17 molecule in juxtaposition (Figs. 1C and 2). In contrast, the rearranged model in the presence of the sugars indicates that the two HA-33 molecules are not juxtaposed, instead they are attached to HA-17 at a drastically increased angle of ~120°.

4. Discussion

In a previous report [15], we revealed the crystallographic and SAXS images of the NTNHA protein, another nontoxic component of the botulinum TC. By comparing the images, a part of the SAXS image of the NTNHA displayed a more extended appearance than that seen in the crystallographic image. The discrepancy observed in the crystallographic and SAXS images implied a high flexibility of the C-terminal region of the NTNHA molecule. SAXS analysis therefore would be useful to clarify the structural dynamics of protein in solution. In this study we revealed a sugar-induced structural shift of the HA-33/HA-17 trimer in solution.

Sugar binding proteins sometimes demonstrate a conformational change induced by the presence of sugars, such as D-Glucose/D-Galactose-binding protein [1] and lactose permease [13]. Previously, Nakamura et al. [12] identified the two sugar-binding sites of the serotype C HA1 (our HA-33) designated as site I and II. Site I is involved in binding with the sugars Neu5Ac, *N*-acetylgalactosamine, and galactose, whereas site II binds only with galactose. Additionally, X-ray analysis of the HA-33/HA-17 trimer crystal indicated that there is a region with low electron density in the HA-17 molecule [2], implying its structural flexibility. Therefore, binding of the sugars to these sites may induce a structural shift. However, the glucose-binding site in the HA-33 molecule has not been identified.

Another possibility to explain the difference observed in the trimer structure in the presence and absence of sugars, is that one of

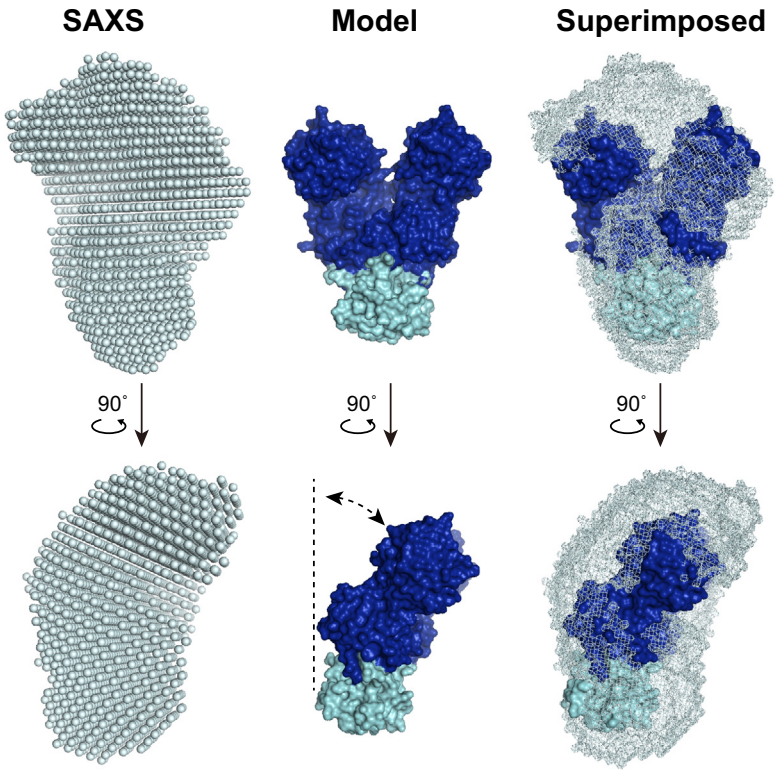

Fig. 2. Solution structure of the HA-33/HA-17 trimer revealed by SAXS analysis. Solution structure of the HA-33/HA-17 trimer is represented by the dummy atom model (DAM) (left). A model of the solution structure of the HA-33/HA-17 trimer (center) is illustrated based on the superimposition of the SAXS image and surface representation image of the crystal (right). Dotted line and arrow indicate the original position of the dual HA-33 molecules revealed by the crystallographic image (see Fig. 1B) and iterative movement of the molecules presumed by the SAXS DAM image, respectively. The HA-33 molecules in the trimer were displaced so that the crystal structure fits into the SAXS image.

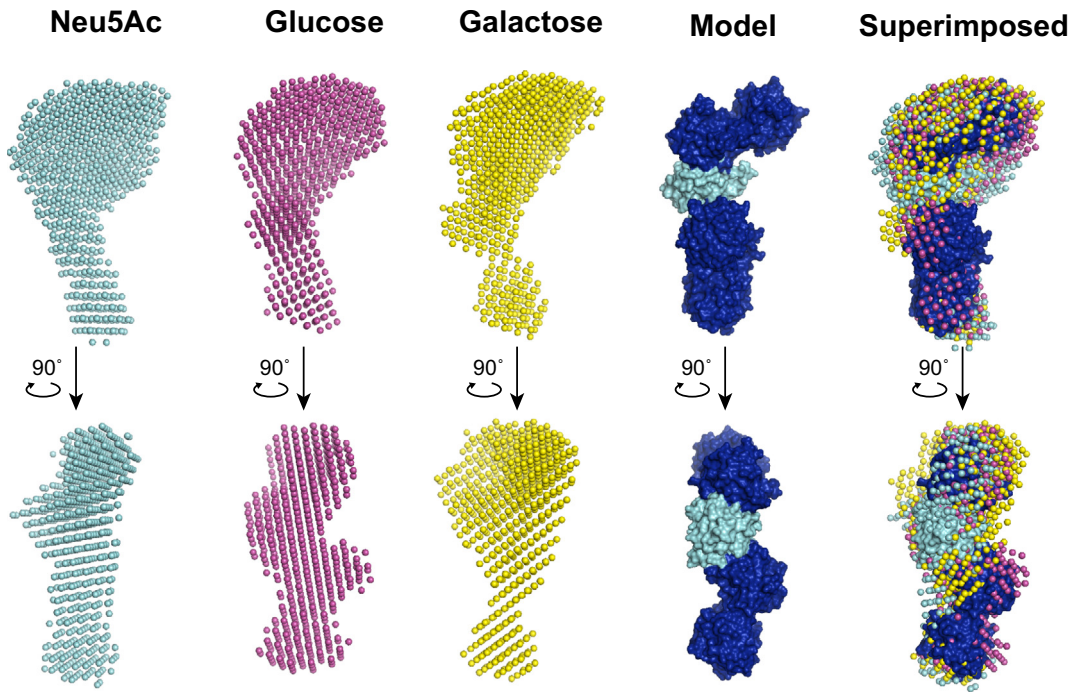

Fig. 3. Solution structure of the HA-33/HA17 trimer in the presence of various sugars. SAXS images of the HA-33/HA-17 trimer in the presence of Neu5Ac, glucose and galactose are represented in DAM. A model of the solution structure of the trimer in the presence of the sugars (second panel from right) is illustrated based on the superimposition of the SAXS image and surface representation image of the crystal (rightmost panels). The HA-33 molecules in the trimer were displaced so that the crystal structure fits into the SAXS image as shown in the rightmost panels.

the HA-33 molecules could transfer to an alternate binding site on HA-17 in the presence of sugars. RADAR (http://www.ebi.ac.uk/Tools/pfa/radar) [3], a program used to detect repeats in protein sequences, revealed two pairs of repeat-sequences, DYGFIYLSSSSNNSLWNPI (72–90) and DYAWTIYDNNNNITDQPI (109–126), and NGNYKIKSLFSDSLYLTY (10–27) and NGFRFSNVAEPNKYLAY (54–70), in the HA-17 molecule. The crystal structure of the HA-33/HA-17 trimer indicated that a set of Asp123, Pro125 and Leu127 residues in HA-17 is responsible for the interaction with one of the two HA-33 molecules, whereas the set of Asn106, Thr108, and Pro130 is involved in the binding to the other HA-33 molecule. Most of the residues in the first set are included in Asp109–Ile126, which is one-half of the first pair of internal repeat sequences. Therefore the site in the other half of the pair, Asp72–Ile90, of HA-17 would be a candidate for the binding site for the HA-33, which detached from the original position of the HA-17 molecule.

In this study, we found a sugar-induced structural shift of the HA-33/HA-17 trimer based on SAXS analysis. However, the physiological meaning of the structural shift remains unclear. Transport of botulinum TC through the intestinal epithelial cell layer is enhanced by the HA-33/HA-17 arm of the TC in a sialic-acid dependent manner [4]. The structural shift of the HA-33/HA-17 arm of the TC may be induced by the binding of HA-33 to the sugar chain on the epithelial cell surface. This could result in enhancement of penetration by the TC across the cell layer. On the other hand, in the food-poisoning process the toxins transit the digestive tract with dietary macromolecules including polysaccharides. During transit, the polysaccharides would be degraded into monosaccharides, and such monosaccharides may affect transport of the botulinum toxin across the intestinal wall via the sugar-induced structural shift of the HA-33/HA-17 arms on the TC. In future studies, the effect of sugars on the transport of the TC across the intestinal epithelial cell layer should be examined to clarify the physiological meaning of the structural shift of the HA-33/HA-17 trimer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.112.

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