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Botulinum neurotoxin is shielded by NTNHA in an interlocked complex

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Abstract

Botulinum neurotoxins (BoNTs) are highly poisonous substances that are also effective medicines. Accidental BoNT poisoning often occurs through ingestion of *Clostridium botulinum*-contaminated food. Here, we present the crystal structure of a BoNT in complex with a clostridial non-toxic non-hemagglutinin (NTNHA) protein at 2.7 angstrom. Biochemical and functional studies show that NTNHA provides large and multivalent binding interfaces to protect BoNT from gastrointestinal degradation. Moreover, the structure highlights key residues in BoNT that regulate complex assembly in a pH-dependent manner. Collectively, our findings define the molecular mechanisms by which NTNHA shields BoNT in the hostile gastrointestinal environment and releases it upon entry into the circulation. These results will assist in the design of small molecules for inhibiting oral BoNT intoxication, and of delivery vehicles for oral administration of biologics.

The seven serotypes of BoNT (named A–G) are sequence-specific endopeptidases. They invade nerve cells at neuromuscular junctions, where they inhibit the release of the neurotransmitter acetylcholine by cleaving SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) proteins, and subsequently paralyze the affected muscles (1). Although BoNT poisoning often occurs through oral ingestion of tainted food products, the molecular mechanism by which BoNTs avoid destruction in the hostile environment of the gastrointestinal (GI) tract is unknown.

Naturally occurring BoNTs are protected within progenitor toxin complexes (PTC), which are high molecular weight multi-protein complexes composed of the BoNT and several non-toxic neurotoxin-associated proteins (NAPs) (2). The NAPs include the 140 kDa NTNHA, which together with BoNT forms the minimally functional PTC (M-PTC), and three hemagglutinin (HA) proteins, which assemble with the M-PTC and have been suggested to facilitate BoNT transcytosis across the intestinal barrier (1, 3). The protecting function of the M-PTC is sufficiently effective that it reduces the oral LD₅₀ of BoNT 10–20-fold compared to free BoNT (4, 5). Intriguingly, the protected BoNT is released from the PTC upon transition from acidic to neutral pH, as occurs during absorption from the intestine into the

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bloodstream (6). Once in the circulation, free BoNT travels to neuromuscular junctions where it invades neurons. The molecular architecture of PTC is largely unknown, with the exception of two low-resolution structures revealed by electron crystallography (30 Å) and electron microscopy (7, 8).

We focused our study on the M-PTC of serotype BoNT/A1 because it is a major cause of human botulism and is a concern for bioterrorism (9). We established a robust recombinant system in *E. coli* to produce a genetically modified, catalytically inactive BoNT/A (~150 kDa) that carries three mutations (E224Q/R363A/Y366F) in the catalytic site (10), and the corresponding full-length NTNHA-A1 (~140 kDa, referred to as NTNHA-A) (11). BoNT/A^{E224Q/R363A/Y366F} adopts a structure essentially identical to that of wild-type BoNT/A, and is referred to here as BoNT/Ai (fig. S1 & Table S2). The free forms of BoNT/Ai and NTNHA-A are monomeric at pH 6.0 and 7.5 (fig. S2), and assemble into a monomeric M-PTC at pH 6.0 with K_d of ~30.8 nM and 1:1 stoichiometry as analyzed by analytic ultracentrifugation and isothermal titration calorimetry (fig. S2 & Table S1). By contrast, no assembly occurs at pH 7.5.

Free BoNT/Ai and NTNHA-A are inherently fragile, as shown by *in vitro* cleavage by digestive proteases such as trypsin and pepsin (Fig. 1A). Free wild-type BoNT/A is inactivated by trypsin or by incubation at pH 3 or less, based on *ex vivo* mice phrenic nerve hemidiaphragm (MPN) assays (Fig. 1B). In contrast, the structural integrity and activity of BoNT/A is protected in the *in vitro*-reconstituted M-PTC at low, but not at neutral or alkaline pH (Fig. 1A–B). Thus, the recombinant M-PTC faithfully mimics the behavior of native M-PTC.

To understand the molecular mechanism underlying BoNT/A protection and the pH-dependent assembly of the M-PTC, we determined a 2.7 Å resolution crystal structure of the M-PTC facilitated by a BoNT/A-specific nanobody, as well as the structure of a nanobody-free M-PTC at 3.9 Å resolution (Fig. 1C–D, figs. S3–4 and Table S2). Nanobodies are the smallest antigen-binding fragments (~12–15 kDa) of naturally occurring heavy-chain-only antibodies (VHH) present in camelids, and have been exploited as inhibitors of BoNTs (12). Here, the VHH binds on the surface of BoNT/Ai far from the NTNHA-A-interacting sites. It facilitates a more compact crystal packing and thus improves the diffraction quality of the crystals (fig. S5 and Table S3). The two independently solved crystal structures of the M-PTC are indistinguishable, demonstrating that the structure presented here represents the physiological conformation of the M-PTC, independent of VHH or crystal packing (fig. S3B).

BoNT/Ai and NTNHA-A form an interlocked compact complex, and bury an unusually large solvent-accessible area (~3,600 Å² per molecule) through multivalent interfaces (Fig. 1). BoNT/Ai is composed of a 50 kDa light chain (LC, a Zn²⁺-endoprotease) and a 100 kDa heavy chain (HC), which has two domains: the N-terminal domain (H_N) mediates LC translocation across the endosomal membrane, while the C-terminal domain (H_C) is the receptor-binding domain (1). Unexpectedly, the protective component NTNHA-A has a structure highly similar to BoNT/Ai despite a low sequence identity (~20%). NTNHA-A also displays three domains that, when compared pair-wise with LC, H_N, or H_C, yield root-mean-square deviations of 2.2 Å (314 Ca pairs), 2.3 Å (300), and 1.9 Å (319), respectively, and thus are termed nLC, nH_N, and nH_C (“n” indicates NTNHA) (Fig. 1C–D & fig. S6). At the center of the complex is the H_C fragment that is surrounded by all three domains of NTNHA-A (Table S4). Notably, H_C rotates about 140° around a linker connecting H_N and H_C, resulting in a distinct conformation in comparison to the free form of BoNT/A (Fig. 2) (13). Complementing this, H_N also forms many polar interactions with nH_N and nH_C (Fig. 3D–E). In contrast, LC does not bind NTNHA-A.

Despite their similarities, a structural comparison between NTNHA-A and BoNT/Ai revealed several features specific to NTNHA-A (fig. S6). NTNHA-A does not possess most of the characteristic structural features of BoNT/A that are crucial to its functions: (i) nLC does not have the catalytic zinc-binding “HExxH+E” signature motif that is conserved among all BoNT serotypes; (ii) NTNHA-A does not have the homologous long loop (Val⁴³¹–Leu⁴⁵³) that in BoNT/A connects LC and HC and is cleaved post-translationally to activate BoNT/A; (iii) a functionally essential disulfide bond (Cys⁴³⁰–Cys⁴⁵⁴ in BoNT/A) that is involved in translocation of LC and is conserved in all BoNTs is not present in NTNHA-A (14); (iv) nH_C maintains the core structure of H_C but has deletions in many surface areas that are not directly involved in BoNT/Ai binding; and (v) none of the ganglioside-binding residues of H_C, including the highly conserved ganglioside-binding motif (E+H+SxWY) found in many BoNT serotypes, is conserved in NTNHA-A (15, 16).

Intriguingly, NTNHA-A has a large insert in nLC (nGly¹¹⁶–nAla¹⁴⁸, termed the nLoop) that is not present in LC. Furthermore, the nLoop is conserved in NTNHA A1, B, C, D, and G, but is missing in A2, E, and F (fig. S7). Notably, many of the known spontaneous nicking sites in NTNHA are located in the nLoop (17), and consistent with this, we observed that recombinant NTNHA-A was nicked between nLys¹³³ and nLys¹³⁴ during long-term storage. The nicking sites in NTNHA are masked in the HA-bound PTC, and the M-PTC containing the nicked NTNHA can no longer assemble with HAs (17, 18). Moreover, NTNHA-A2, E, and F, which lack the nLoop, do not have accompanying HA proteins and only form the HA-negative M-PTC (19–21). The crystal structure reveals that the nLoop is fully exposed on the M-PTC surface and has no visible electron density, presumably due to its high flexibility (Fig. 1C). Collectively, these data suggest that the nLoop could participate in the interaction with HAs to assemble the larger PTCs.

The conformation of BoNT/Ai in the M-PTC brings the receptor-binding site located in the C-terminal subdomain of H_C close to the C-terminal boundary of the long rod-like H_N (Fig. 2). This conformation contrasts with crystal structures of the free BoNT/Ai (fig. S1), free BoNT/A, and free BoNT/B serotype, where the receptor-binding site in H_C points to the N-terminal boundary of H_N (13, 22–24). The H_C reorientation is mediated by a linker between H_N and H_C (Leu⁸⁴⁵–Thr⁸⁷⁶ in BoNT/A, referred to as the H_N–H_C linker), which adopts an essentially identical structure in free BoNT/A and BoNT/B but changes its conformation in the M-PTC (Fig. 2 & fig. S8). The conformational change is likely induced by NTNHA-A rather than pH because the same conformation is adopted by all structures of free BoNT/A or BoNT/B crystallized at pHs ranging from 5.0 to 7.0 (13, 22–24).

We speculated that the flexible H_N–H_C linker may play a role in coordinating H_C-mediated receptor binding and H_N-mediated translocation, given that the membrane-anchored receptors impose some geometric restrictions on the position of H_C with respect to the membrane surface (24, 25), and the long helical H_N needs to strategically orientate on the membrane to achieve efficient translocation of LC to the cytosol (26–28). To test this, we produced two mutants of BoNT/A in which the structure of the H_N–H_C linker was altered by point mutations. BoNT/A^{R861A/E868P/K871P}, containing the helix breaker proline, is expected to destabilize the helical linker, whereas BoNT/A^{L862K/L863E/T867K}, with extra ion pairs, will likely stabilize the helical structure. The two BoNT/A mutants showed wild-type-like characteristics with respect to protein folding, receptor binding capability, Zn²⁺-endoprotease activity, and NTNHA-A binding (fig. S9). However, the toxicity of both mutants was significantly decreased by ~6-fold in the MPN assay. Although the exact conformations of the two mutants are not known, it is clear that the observed decrease in toxicity is due to the disrupted coordination between H_N and H_C, leading to decreased efficiency of LC translocation. These data further suggest that any disturbance in the structural integrity of the H_N–H_C linker may affect the optimal positioning of H_C and H_N.

during BoNT intoxication. Thus the structural features of the H_N-H_C linker might represent a serotype-specific signature for the various BoNTs. For example, BoNT/E serotype has a more rapid LC translocation, which has been suggested to result from its intrinsically more flexible linker (fig. S8) (27, 29).

To validate the physiological relevance of the M-PTC structure, we performed systematic truncation studies. The isolated H_C fragment bound to NTNHA-A with high affinity at pH 6.0 ($K_d \sim 48.3$ nM) but not at pH 7.5, and thus largely replicated the binding behavior of full length BoNT/Ai. Consistent with this, the H_C-deleted BoNT/Ai (LH_N) no longer bound NTNHA-A (fig. S10). BoNT/Ai has a larger unfavorable binding entropy than H_C ($\Delta\Delta S \sim -16.8$ cal mol⁻¹ K⁻¹) (Table S1), which could be due to a loss of conformational entropy of BoNT/Ai upon NTNHA-A binding, and may be related to the reorientation of H_C. Truncating nH_C or the C-terminal subdomain of nH_C (nH_{CC}) in NTNHA-A abolished the protective function of NTNHA-A at pH 2, suggesting that nH_C is crucial for shielding the sensitive H_C of BoNT/A (Fig. 1B).

Additional support for the M-PTC structure is provided by structure-based mutagenesis studies. Interactions between H_C and NTNHA-A were gradually weakened as more intra-PTC charge-charge interactions were disrupted: the binding affinity at pH 6.0 decreased in the order of H_C-K1000A > H_C-K1000A/K1039A > H_C-K1000A/K1039A/K1121A (Fig. 3F & fig. S10). Even a single point mutation, H_C-K1000A, significantly decreased the binding affinity by ~7-fold ($K_d \sim 337.5$ nM). The H_C-centered protection by NTNHA-A is biologically relevant. While the integrity of H_C is crucial to enrich BoNT/A on the neuron surface at the early stage of intoxication, it is much more sensitive to proteolysis than LC and H_N in the free BoNT/A (30, 31).

To understand how a pH change can trigger the disassembly of the PTC, we focused on H_C because it largely replicates the binding features of full-length BoNT/Ai. We specifically concentrated on residues located in the complex interface that are titratable in an acidic environment (e.g., His, Glu, and Asp). Intriguingly, we found four acidic residues in H_C, each of which (shown in parentheses) is located in the vicinity of an intra-PTC salt bridge or a long-range electrostatic interaction: (Glu⁹⁸²)/Lys¹⁰⁰⁰-nAsp⁸⁰⁸, (Asp¹⁰³⁷)/Lys¹⁰³⁹-nGlu⁸¹⁰, (Asp¹¹¹⁸)/Lys¹¹²¹-nAsp⁴⁵⁵, and (Asp¹¹⁷¹)/Arg¹¹⁷⁵-nAsp¹¹¹⁰ (Fig. 4 & fig. S11). Our mutagenesis studies show that these charge-charge interactions are crucial for the assembly of the M-PTC (Fig. 3F). Analyzing the surface electrostatic potentials shows that BoNT/Ai is generally positively charged around these acidic residues, while the opposing NTNHA-A surface is largely negatively charged (fig. S11). Therefore, negatively charged BoNT/Ai residues in these areas pose an unfavorable force, which could potentially weaken the local electrostatic interactions in a pH-dependent manner.

To address the potential roles of these acidic residues in pH sensing, we mutated them individually to non-titratable residues. Single point mutants of the H_C fragment (E982A, D1037A, D1118A, or D1171A) all folded correctly (fig. S12) and bound NTNHA-A at pH 6.0 to a similar extent as the wild-type H_C. Remarkably, H_C-E982A and H_C-D1037A showed significant binding to NTNHA-A at pH 7.5 (Fig. 3F and fig. S10). Thermodynamic studies showed that H_C-E982A binds to NTNHA-A similarly to the wild-type H_C at pH 6.0, predominantly driven by enthalpy ($K_d \sim 39.4$ nM); it also binds to NTNHA-A at pH 7.5, driven by both enthalpy and entropy ($K_d \sim 279.5$ nM) (Table S1). Furthermore, a single mutation of E982A significantly increased binding between the full-length BoNT/Ai and NTNHA-A at pH 7.5, as measured in a pull-down assay (~20% binding by mutant vs. < 5% by the wild-type protein). These data suggest that Glu⁹⁸² and Asp¹⁰³⁷ are important pH-sensing residues that modulate the pH-dependent assembly of the M-PTC.

The pK_a of the ionizable groups in proteins may be substantially shifted from the intrinsic pK_a depending on the microenvironment surrounding these groups (32). Glu⁹⁸² and Asp¹⁰³⁷ of BoNT/A are predicted (33) to have clearly increased pK_a values upon binding to NTNHA-A, partly due to desolvation effect when they are buried in the context of the M-PTC. Thus, Glu⁹⁸² and Asp¹⁰³⁷ are likely protonated at pH 6.0 as their pK_a values increase during the BoNT/A–NTNHA-A interaction, leading to a stable M-PTC. In contrast, these residues are deprotonated in a neutral or alkaline environment, generating repulsive charge interactions with NTNHA-A to destabilize the M-PTC assembly. We replaced Glu⁹⁸² and Asp¹⁰³⁷ with the isosteric, non-titratable residues Gln and Asn, respectively, to mimic their protonated state. Both H_C-E982Q and H_C-D1037N showed significant binding with NTNHA-A at pH 7.5, further supporting the pH-sensing role of these two residues (Fig. 3F & fig. S10). At the same time, thermodynamic studies revealed a loss of enthalpy on binding of H_C-E982A to NTNHA-A at pH 7.5 compared to pH 6.0 ($\Delta\Delta H \sim 6.3 \text{ kcal mol}^{-1}$) (Table S1), suggesting that the protein-protein interaction network between H_C and NTNHA-A is partly impaired at pH 7.5. Thus, additional pH-sensing components might exist in H_C that become fully engaged in binding with NTNHA-A only at pH 6.0.

Collectively, these data suggest that the assembly of the M-PTC is dynamically regulated by key pH-sensing residues that switch protonation states in response to the environmental pH. These interaction sites would likely be the most malleable parts of the PTC, and could be specifically targeted for the development of small molecule inhibitors to break up the PTC in the acidic GI tract. A pharmacological approach such as this, which focuses on preventive countermeasures at the earliest stage of BoNT intoxication, would complement current efforts to design inhibitors that block the subsequent neuronal action of BoNT. Our results also suggest mechanisms for the development of an oral drug delivery system in which proteinaceous drugs could be conjugated to a BoNT fragment and protected from degradation with NTNHA.

Supplementary Material

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Acknowledgments

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Fig. 1.

The architecture of the M-PTC. **(A)** NTNHA-A protects BoNT/Ai against trypsin and pepsin digestion when they assemble into the M-PTC in an acidic environment. **(B)** NTNHA-A protects active BoNT/A against low pH-mediated inactivation. It also protects BoNT/A against trypsin inactivation at pH 6.0, but not at pH 8.0. **(C)** Cartoon presentation of the M-PTC. BoNT/Ai domains are blue (LC), orange (H_N), and green (H_C). NTNHA-A domains are yellow (nLC), cyan (nH_N), and red (nH_C). **(D)** Individual structures of BoNT/Ai and NTNHA-A in the M-PTC.

Fig. 2.

The H_C fragment of BoNT/A can rotate around a linker connecting H_N and H_C. **(A)** Superposition of the free and complex forms of BoNT/A based on Cα atoms in LC and H_N. H_C of free BoNT/A is violet; LC and H_N are omitted for clarity. Linkers in the free and complex forms of BoNT/A are gray and red, respectively. **(B)** Cartoon model showing two distinct conformations of BoNT/A that bring the receptor-binding site in H_C (red star) close to the opposite tip of the long helical H_N.

Fig. 3.

The M-PTC is stabilized by extensive intermolecular interactions. (A–C) H_C interacts with all three domains of NTNHA-A. The overall structure of the M-PTC is shown in (B) where NTNHA-A is in surface representation (gray) and BoNT/Ai is blue, orange, and green for LC, H_N, and H_C, respectively. Open-book views of the interfaces highlighted in the red or blue boxes are shown in (A) and (C), respectively. Residues in H_C that form hydrogen bonds or salt bridges with nLC, nH_N, or nH_C are yellow, cyan, and red, respectively. (D) Open-book view of the interface highlighted in the red box of panel E. H_N directly contacts nH_C (interacting residues shown in red) and nH_N (cyan), but not nLC. (F) Pull-down assays between full-length NTNHA-A (prey) and H_C variants (baits). Bar graph shows mean ± SD, n=3. Binding of samples 8–10 at pH 7.5 was not detectable.

Fig. 4.

A pH-sensing mechanism. A close-up view of pH-sensing residues Glu⁹⁸² and Asp¹⁰³⁷, which are buried in the M-PTC in the vicinity of intra-PTC charge-charge interactions. Key residues in the interface are shown as ball-and-stick models. Hydrogen bonds and charge-charge interactions are indicated by dotted lines. A simulated-annealing omit map of the M-PTC contoured at 1 σ was overlaid with the final refined model.