Crucial Role of the Disulfide Bridge between Botulinum Neurotoxin Light and Heavy Chains in Protease Translocation across Membranes*

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Clostridial botulinum neurotoxins (BoNTs) exert their neuroparalytic action by arresting synaptic exocytosis. Intoxication requires the disulfide-linked, di-chain protein to undergo conformational changes in response to pH and redox gradients across the endosomal membrane with consequent formation of a protein-conducting channel by the heavy chain (HC) that translocates the light chain (LC) protease into the cytosol. Here, we investigate the role of the disulfide bridge in the dynamics of protein translocation. We utilize a single channel/single molecule assay to characterize in real time the BoNT channel and chaperone activities in Neuro 2A cells under conditions that emulate those prevalent across endosomes. We show that the disulfide bridge must remain intact throughout LC translocation; premature reduction of the disulfide bridge after channel formation arrests translocation. The disulfide bridge must be on the trans compartment to achieve productive translocation of LC; disulfide disruption on the cis compartment or within the bilayer during translocation aborts it. We demonstrate that a peptide linkage between LC and HC in place of a disulfide bridge is insufficient for productive LC translocation. The disulfide linkage, therefore, dictates the outcome of translocation: productive passage of cargo or abortive channel occlusion by cargo. Based on these and previous findings we suggest a sequence of events for BoNT LC translocation to be HC insertion, coupled LC unfolding, and protein conduction through the HC channel in an N to C terminus orientation and ultimate release of the LC from the HC by reduction of the disulfide bridge concomitant with LC refolding in the cytosol.

Clostridium botulinum neurotoxins (BoNTs) inhibit synaptic exocytosis in peripheral cholinergic synapses, thereby causing flaccid paralysis (1). BoNTs are synthesized as a single polypeptide chain with a molecular mass of ~150 kDa. The BoNT polypeptide is then proteolytically cleaved by bacterial or host proteases into the activated di-chain form: an ~50-kDa light chain (LC) and an ~100-kDa heavy chain (HC). The LC and HC are cross-linked by a disulfide bond between the two chains. Structurally, BoNTs consist of three modules (1–4): The N-terminal LC is the catalytic domain, and the HC comprises the translocation domain (the N-terminal half) and the receptor-binding domain (the C-terminal half). The LCs of six of the seven isoforms of BoNT, designated A–G, have been crystallized and all share structural similarity to the Zn-containing metalloprotease thermolysin (2, 4–11). BoNT LCs are sequence-specific endopeptidases that cleave unique components of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, the synaptic vesicle fusion complex required for membrane fusion (12–14).

BoNTs enter cells by receptor-mediated endocytosis (1, 15). It has been widely recognized that, with the exception of BoNT/D, BoNT entry into neuronal cells requires surface receptors involving a specific ganglioside, namely GT1b (16–19), together with other protein components that determine the BoNT neurotropism. Recently, the identity of the neuronal protein receptors for BoNT/A (20, 21) and BoNT/B and BoNT/G (22) were uncovered as SV2 and syntaptotagmins I and II, respectively. The crystal structure of BoNT/B in complex with a peptide from the luminal domain of syntaptotagmin II has defined the surface determinants that account for the high specificity of binding of the toxin to neurons (23, 24).

A key step in the intoxication process is the translocation of endocytosed toxin across intracellular membranes to reach its cytosolic targets (1, 15). It was postulated that the acid pH of endocytic vesicles induces a conformational change that promotes insertion of the HC into acidic endosomal membranes, where the HC assembles into a protein-conducting channel with the LC as cargo translocated into the cytosol (1, 25, 26). Previously, we demonstrated that the HC of BoNT/A acts as both a channel and a transmembrane chaperone for the LC to ensure a translocation-competent conformation during its transit from the acidic endosome into the cytosol, thereby recovering the endopeptidase activity of BoNT LC (27).

Here, we probed the role of the disulfide bridge in LC translocation, focusing on the interactions between the HC channel/chaperone and its LC cargo under conditions that closely...
emulate those prevalent at the endosome. We utilized a previously developed assay in Neuro 2A cells to monitor interactions between the HC and the LC during translocation with single molecule sensitivity (28). This assay led to the identification of intermediate channel conductances that reflect permissive stages during LC translocation for both BoNT/A and BoNT/E (28). Further, we showed that productive translocation requires proteolytic cleavage of LC cargo from the HC channel (28). In this work we use the assay to examine the consequences of disulfide linkage disruption by chemical reductants accessible to different sides of the membrane. The disulfide linkage emerges as a crucial determinant required for chaperone function and LC translocation and release. These and previous findings indicate that BoNT translocation involves an acid pH-induced membrane insertion step coupled to LC unfolding and entry into the HC chaperone/channel, LC protein conduction through the HC channel in an N- to C-terminal orientation, and subsequent release of the LC cargo from chaperone by reduction of the disulfide bridge concomitant with LC refolding at the cytosol.

EXPERIMENTAL PROCEDURES

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich. Purified native BoNT/A holotoxin and...
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HC and BoNT/E holotoxin were from Metabiologics. The ING2 BoNT/A LC-specific monoclonal antibody was kindly provided by Dr. James Marks (University of California, San Francisco).

Cell Culture—Neuro 2A neuroblastoma cells were obtained from the American Type Culture Collection. Cells were passaged in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with penicillin 10 mM/streptomycin 10 μg/ml/ glutamine 2 mM (Invitrogen), and 5% newborn bovine serum (Invitrogen). Cells were plated onto Matrigel (BD Biosciences)-coated glass coverslips at a density of ~500 cells/cover slip and cultured at 37 °C, 5% CO₂ for 1–3 days prior to patch clamp recordings.

Patch Clamp Recordings—Patch pipettes were pulled from borosilicate glass (Hilgenberg, Lambrecht, Germany), fire-polished, and used at 3.5–7.0 MΩ resistance when immersed in recording solution. Excised patches in the inside-out configuration were used (29). After gigaohm (GΩ) seal formation, the patch was excised from the cell, removed, and reimmersed through the air-water interface to achieve an inside-out configuration. Current recordings were obtained under voltage clamp conditions by the application of consecutive voltage steps 800 ms in duration from +50 mV to −150 mV at a sampling frequency 20 kHz. Records were acquired and analyzed using the patch clamp amplifier system (List EPC-9; HEKA Electronik) fitted with an ITC-16 interface (Instrutech, Port Washington, NY) and the Pulse/PulseFit acquisition and analysis software (HEKA). Data were further analyzed using Clampfit v.9.2 software (Molecular Devices, Sunnyvale, CA), Microsoft Excel, and IGOR Pro (Wavemetrics, Portland, OR). All experiments were conducted at 22 ± 2 °C.

Solutions—To emulate endosomal conditions the trans compartment (bath) solution contained (in mM) NaCl 200, NaMOPS [3-(N-morpholino) propanesulfonic acid] 5, (pH 7.0 with HCl), tris-(2-carboxyethyl) phosphine (TCEP) 0.25, ZnCl₂ 1, and the cis compartment (pipette) solution contained (in mM) NaCl 200, NaMES [2-(N-morpholino) ethanesulfonic acid] 5, (pH 5.3 with HCl). The osmolarity of both solutions was determined to be 390 mosM. ZnCl₂ was used to block endogenous channel activity specific to Neuro 2A cells (30, 31). BoNT reconstitution and channel insertion were achieved by supplementing 5 μg/ml BoNT holotoxin or HC to the pipette solution, which was set to an endosomal pH of 5.3. BoNT/E experiments were performed with 40 μM trypsin in the trans compartment. GΩ seal formation was optimized as follows: The pipette tip was first dipped in the pipette solution in the absence of BoNT and then back-filled with solution containing BoNT.

Data Analysis—Analysis was performed on single bursts of each experimental record; a single burst is defined as a set of openings and closings lasting ≥50 ms bounded by quiescent periods of ≥50 ms before and after. BoNT channel activity occurs in bursts, and only single bursts were analyzed due to the random duration of quiescent periods between these bursts (32). The single channel conductance (γ) was calculated from Gaussian fits to current amplitude histograms. The total number of opening events analyzed was 184,923; the intermediate γ states were defined by a minimum of 500 events. For each experiment, the time course of channel γ change was calculated from γ of each record, where t = 0 s corresponds to onset of channel activity and average time course was constructed from the set of individual experiments for a single condition. Average occupancy time of occluded intermediate and unoccluded channel γ states was calculated from the total time the channel resides at a given γ value averaged over the set of individual experiments. The voltage dependence of channel opening was calculated from measurements of the fraction of time that the channel is open (P_o) as a function of voltage by integration of γ histograms where γ is 64 pS ≤ γ ≤ 68 pS. Statistical values represent means ± S.E. unless otherwise indicated. n and N denote number of experiments and number of opening events.

Fab Generation—The Pierce ImmunoPure Fab Preparation kit (44885) was used to generate Fabs from BoNT/A LC antibody ING2. In short, 3 mg/ml ING2 was incubated in the equiv-
alent volume of immobilized papain at 37 °C for 5 h. The digested ING2 was transferred to a Protein A column to separate the Fabs from the Fc and undigested IgG: the Fabs flow through the column whereas the Fc and undigested IgG are retained and elute later. BoNT/A LC Fabs were quality-checked using SDS-PAGE prior to the single molecule assays. Purified Fabs were concentrated to 1 mg/ml in patching bath solutions prior to incubation with BoNT/A holotoxin.

RESULTS AND DISCUSSION

Single Molecule Translocation Assay—Translocation of BoNT/A LC by the BoNT/A HC channel can be monitored in real time and at the single molecule level in excised membrane patches from Neuro 2A cells (28). Translocation requires pH 5.3 on the cis compartment, defined as the compartment containing BoNT/A, and pH 7.0 on the trans compartment, which is supplemented with the membrane-nonpermeable reductant TCEP, conditions that emulate those prevalent across endosomes. Translocation is then observed as a time-dependent increase in Na\(^+\)/H\(^+\) conductance through the HC channel, as illustrated by the experiment shown in Fig. 1A. The time course of change of the single channel conductance \(\gamma\) after insertion of BoNT/A holotoxin into the membrane displays multiple discrete transient intermediate conductances before achieving a \(\gamma\) of 67.1 ± 2.0 pS (Fig. 1C, red trace). The top panel of Fig. 1A shows that at the onset of translocation small, discrete events with a \(\gamma\) ~ 12 pS are clearly discerned, as indicated by the segment of the record designated with a black bar that is dis-
played at higher time resolution under the trace. Progressively, γ increases and, as shown in the middle panel of Fig. 1A, reaches a value of ~40 pS; note the insertion of two channels into the membrane, designated O1 and O2, which even during the short segment displayed undergo a continuous increase in conductance, ultimately reaching a stable value of 67 pS (bottom panel), a conductance at which they remain for the duration of the experiment (Fig. 1C, red trace). A γ of 67.1 ± 2.0 pS is also the characteristic conductance of isolated HC recorded under identical conditions; therefore it represents the conductance of the unoccluded HC in holotoxin experiments after translocation is complete. We interpret these different conductance events as reporters of discrete intermediate stages during the translocation of the LC across the membrane. During protease translocation, the protein-conducting channel progressively conducts more Na+ around the polypeptide chain before entering an exclusively ion-conductive state. This typical pattern of channel activity for holotoxin proceeds under conditions that mimic those across endosomes and lead to LC translocation and retrieval of protease activity after completion of translocation (27). Thus, we have used this assay to examine the role of the interchain disulfide linkage on the translocation process.

Premature Reduction of the Disulfide Bridge after Channel Formation Arrests LC Translocation—Previous work has demonstrated the important role of the disulfide bridge in the translocation process (27). We used the differential accessibility of the disulfide linkage between the HC and the LC to TCEP to identify requirements for translocation and showed that LC translocation requires both a pH gradient and a redox gradient, acidic and oxidizing on the cis compartment and neutral and reducing on the trans compartment. Significantly, addition of TCEP only to the cis compartment after acidification fails to evoke channel activity. This is indicative of disulfide shielding arising from the onset of LC translocation through the HC channel. Here, we pursue this strategy to determine how the disulfide bridge affects the progress of LC translocation. β-mercaptoethanol (βME) is a powerful tool for this task. First, βME does not modify HC channel activity, and preincubation of BoNT/A

**FIGURE 4. LC/A translocation arrest by an LC/A-specific Fab and relief from arrest by reduction of the LC-HC disulfide bridge.** Representative single-channel currents recorded at the indicated times and voltages; consecutive voltage pulses applied to the same patch. Black line designates the expanded region of the record displayed below the compressed record at a faster time scale, denoted by the green scale bar. A, BoNT/A holotoxin preincubated with a 5-fold molar excess of Fab on ice at pH 7 for 1 h prior to the experiment; channel activity begins 5 min after G1 seal formation. Multiple channel insertions occur with time; however, in contrast to unmodified holotoxin, the conductance never reaches the unoccluded HC γ = 66 pS. B, holotoxin preincubated with Fab; channel activity begins 10 min after G1 seal formation. Low conductance intermediate states persist until the addition of βME. Eighty seconds after onset of channel activity, 1 mM βME is added to the trans compartment. Within minutes γ increases to the larger intermediate conductance states and ultimately achieves the unoccluded HC channel γ = 66 pS. C, time course of channel γ change illustrated in Fig. 4B (black) and average time course of channel γ change for holotoxin preincubated with Fab (average N/data point for Fab preincubated with BoNT/A without addition of βME = 1,003 events and with addition of βME = 180 events; n = 5 for each condition, magenta). Addition of βME designated by the green arrow. Thin red line represents results for holotoxin without βME addition; γ values associated with raw data from panel B are indicated.
with reductants results in HC channel activity (data not shown). Additions to the cis compartment cannot be directly made after seal formation; therefore membrane-permeable reagents that equilibrate across both compartments are required. In contrast to TCEP (33), βME can traverse the lipid bilayer and reduce the disulfide bridge from either side of the membrane. If the disulfide bridge were translocated first across the membrane and were confined to the TCEP-containing trans compartment during the early steps of translocation, then addition of βME should have no effect on channel activity and growing conductance would end invariably in an unoccluded channel with γ ~ 67 pS. The conductance growth of holotoxin channels is interrupted by addition of βME immediately after its onset, as shown in Fig. 1B. The top panel shows a single BoNT/A channel undergoing a progressive increase in conductance, comparable with the entry events characteristically displayed by holotoxin (Fig. 1A). Addition of βME, however, precludes entry into the higher γ intermediates, as evidenced in the middle and bottom panels of Fig. 1B in which two channels that inserted into the membrane prior to βME addition remain in one of the low conductance states for the remainder of the experiment (Fig. 1C, black trace). The current transitions are faster and shorter-lived than those typical of unmodified, unoccluded holotoxin, giving the appearance of flickering between low and high conductance states, a characteristic feature of channel block (27, 34). Analysis of the results of five experiments of this type (Fig. 2) shows that LC translocation is arrested by reduction of the disulfide linkage after the initiation of translocation. Under these conditions, the holotoxin channel preferentially resides in one of the following conductance states (Fig. 2A): 10, 17, or 31 pS. The lowest two states detected correspond to the entry event identified for holotoxin if translocation is unperturbed (28); however, the 31-pS intermediate may correspond to a non-productive, dead-end state for the LC and HC. The increased P₀ of the low conductance states (γ ~ 10 and 30 pS, Fig. 2B) and the preponderant occupancy of these occluded intermediate states (Fig. 2C) further demonstrate that intermediate steps in the growing conductance of the holotoxin channel are stabilized, thereby precluding completion of LC translocation: the HC channel is occluded by the LC and translocation is arrested.

Premature Reduction of the Disulfide Bridge of Single-chain BoNT/E before Channel Formation Arrests LC Translocation—The finding that interruption of BoNT/A LC translocation results from premature reduction of the disulfide bridge leads to the hypothesis that the LC must be anchored to the HC during translocation. Is any linkage between the LC and HC sufficient to promote LC translocation or is the intact disulfide bridge specifically required? Whereas BoNT/A is cleaved to the mature di-chain within the Clostridium bacterium, BoNT/E is not cleaved before secretion. We previously demonstrated that, for the single-chain BoNT/E, completion of LC translocation proceeds only after proteolytic cleavage by trypsin and disulfide reduction in the trans compartment (28). Single-chain BoNT/E holotoxin is, therefore, an appropriate system to explore the linkage requirements for LC translocation. Accordingly, BoNT/E was incubated with 10 mM TCEP for 30 min at room temperature before the translocation assay. Prereduced BoNT/E displays channel activity as illustrated in Fig. 3A. Channel openings exhibit a γ similar to that of the early intermediates detected for unmodified BoNT/E (28); however, these low γ events do not undergo a transition to the higher γ intermediates or to unoccluded states (Fig. 3A). Despite the fact that trypsin is present in the trans compartment, the channel remains in low γ states for the lifetime of the experiment.

Analysis of four separate experiments under these conditions demonstrates that the channel activity of prereduced single-chain BoNT/E is different from that of the intact, disulfide cross-linked BoNT/E, never reaching the unoccluded channel state γ ~ 65 pS. BoNT/E with an intact disulfide bridge transi-
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FIGURE 6. Summary of BoNT/A channel characteristics and dependence upon LC translocation. A, P γ as a function of voltage for holotoxin bound to Fab with βME addition (black), unmodified holotoxin (red), and unoccluded HC (blue). V 1/2 for holotoxin bound to Fab + βME is −59.0 ± 4.5 mV, for unmodified holotoxin is −58.3 ± 1.7 mV, and for HC is −63.4 ± 2.4 mV (3 ≤ n ≤ 11/data point; average N/data point = 2,260 events). B, structure of BoNT/A holotoxin (2): LC, purple, translocation domain, orange, and receptor-binding domain, red, prior to insertion in the membrane (gray bar with magenta boundaries); then schematic representation of BoNT/A inserted during translocation of the LC through the HC channel (orange) with intact disulfide bridge (green) while located in the cis-compartment and the unoccluded HC channel in the membrane after LC dissociation. Occluded HC channels can be trapped in low conductance states under two experimental conditions: (i) LC translocation arrested by Fab and (ii) LC translocation arrested by premature reduction of the LC-HC disulfide bridge. Channels formed by holotoxin bound to an LC/A-specific Fab can be unoccluded by the subsequent βME-induced release of the LC-Fab complex into the acid pH compartment.

These results indicate that the disulfide bridge must be on the trans (cytosolic) compartment to achieve productive translocation of the LC; disulfide disruption on the cis compartment or within the bilayer during translocation aborts it. Disulfide disruption at different intermediate γ states resulted in arrested LC translocation; therefore, we infer that completion of LC translocation occurs as the disulfide bridge, C terminus of the LC, enters the cytosolic compartment. This analysis supports a model of N- to C-terminal orientation of cargo during translocation with the C terminus as the last portion to be translocated and exit the channel. We propose that an intact disulfide bridge is a necessary condition for translocation but not for channel insertion, as demonstrated by the facts that the isolated HC channel is unperturbed by chemical reductants (27) and that prematurely reduced single-chain BoNT/E exhibits low conductance channel activity. The tight coupling of translocation completion with disulfide reduction strongly argues in favor of the view that LC refolding precludes retrotranslocation. From this viewpoint, refolding in cytosol may be interpreted as a trap that prevents retrotranslocation and dictates the unidirectional nature of the translocation process. The disulfide linkage is, therefore, a crucial aspect of the BoNT toxicity and is required for chaperone function, acting as a principal determinant for cargo translocation and release.

An LC-specific Antibody Arrests LC Translocation—To selectively restrict the location of the disulfide linkage to the entry site into the HC and to probe its accessibility to βME, we exploited an LC-specific monoclonal antibody previously documented to block LC translocation (28). For this type of experiment Fab fragments are preincubated with BoNT/A for 1 h at pH 7 before the translocation assay. Under these conditions, low conductance channels are detected within a few minutes after patch excision (Fig. 4A, top panel). The initial conductances are comparable with those characteristic of the early events in unmodified LC translocation; however, the channels remain in the low conductance states throughout the experiment (Fig. 4A, bottom panel). We interpret these intermediates as early steps in translocation in which the HC has formed a channel that is partially occluded by the LC. Fab binding to the LC allows channel formation and early translocation, presumably stabilizing intermediate protein-protein interactions. However, it locks the channel and the LC in a translocating conformation that is irreversibly incomplete.

Reduction of the Disulfide Bridge Releases the Fab-induced HC Channel Block by βME—Reduction of the disulfide bridge between the LC and the HC may facilitate release of the Fab-LC complex, thereby unoccluding the HC channel. The disulfide on the Fab will be concurrently reduced; accordingly, only if the complex remains intact under reducing conditions will release of HC channel block ensue. This model was tested by preincubating the Fab with the BoNT/A, followed by supplementing βME to the trans compartment after the onset of the channel activity. Within minutes of βME addition the low conductance channel does enter the unoccluded channel state (Fig. 4B, bottom panel, and 4C, black) in sharp contrast to the Fab-induced block of channel activity (Fig. 4C, pink). The latency period for release of the HC channel from block by the LC estimated from these measurements is ~730 s (Fig. 4C, black) as compared with ~150 s for unabated LC translocation in unmodified holotoxin (Fig. 4C, red). Holotoxin channels under these conditions exhibit discrete transient intermediate conductances at γ = 13, 19, 50, and 57 pS before entering the unoccluded state at γ = 65 pS, as evidenced from analysis of four separate experiments (Fig. 5, A and B). These γ and P γ (Fig. 5C, pink) values approximate the low conductance intermediate states of holotoxin during productive LC translocation (Fig. 5C, black). However, the occupancy time in each conductance intermediate is longer as compared with unperturbed holotoxin (Fig. 5C) (28). The low γ intermediate states are stabilized by the Fab (Fig. 5A); however, the prolonged residency in the larger γ intermedi-
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