Enhancement of the Endopeptidase Activity of Botulinum Neurotoxin by Its Associated Proteins and Dithiothreitol†

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ABSTRACT: Botulinum neurotoxins type A (BoNT/A), the most toxic substance known to man, is produced by *Clostridium botulinum* type A as a complex with a group of neurotoxin-associated proteins (NAPs), possibly through a polycistronic expression of a clustered group of genes. The botulinum neurotoxin complex is the only known example of a protein complex where a group of proteins (NAPs) protect another protein (BoNT) against acidity and proteases of the GI tract. We now report that NAPs also potentiate the Zn^{2+} endopeptidase activity of BoNT/A in both in vitro and in vivo assays against its known intracellular target protein, 25 kDa synaptosomal associated protein (SNAP-25). While BoNT/A exhibited no protease activity prior to reduction with dithiothreitol (DTT), the BoNT/A complex exhibited a high protease activity even in its nonreduced form. Our results suggest that the bacterial production of NAPs along with BoNT is designed for the NAPs to play an accessory role in the neurotoxin function, in contrast to their previously known limited role in protecting the neurotoxin in the GI tract and in the external environment. Structural features of BoNT/A change considerably upon disulfide reduction, as revealed by near-UV circular dichroism spectroscopy. BoNT/A in the reduced form adopts a more flexible structure than in the unreduced form, as also indicated by large differences in ΔH values (155 vs 248 kJ mol^{-1}) of temperature-induced unfolding of BoNT/A.

Botulinum neurotoxins are extremely toxic proteins (mouse LD_{50} of 10^{-8} mg^{-1} kg^{-1}). However, their potential for the most dreaded food poisoning disease, botulism, is negligible in the absence of NAPs[1] (*1*−*3*). NAPs protect BoNT from the damaging acidic environment and the proteases of the GI tract (*3*, *4*). NAPs are also essential for the stability of the BoNT in bacterial cultures under external environmental conditions. Due to the extreme toxicity and stability of BoNT (as a result of complex formation with NAPs), *Clostridium botulinum* is also considered one of the most dangerous biological warfare agents (*5*, *6*). Although NAPs are nontoxic (*3*), whether these accessory proteins play any critical role in the toxico-infection process of botulism has not been clearly established.

There are seven known serotypes (A−G) of BoNT, each approximately 150 kDa consisting of a 50 kDa light chain and a 100 kDa heavy chain linked through a disulfide bond (*7*). The recent discovery that BoNTs are Zn^{2+} endopeptidases (*8*, *9*) has led to the identification of several target proteins, which are critical for the docking and fusion of synaptic vesicles to the plasma membrane in the neurotransmitter release pathway (*10*, *11*). The endopeptidase activity of BoNTs is unique in that it requires the reduction of the disulfide bond between the light and heavy chains (*12*), and each of the BoNTs cleaves selectively only an exlusive peptide bond in a highly specific substrate (*13*). For example, BoNT/A cleaves SNAP-25 exlusively at the ^{197}Gln−^{198}Arg peptide bond (*14*), whereas BoNT/B cleave VAMP exclusively at the ^{76}Gln−^{77}Phe peptide bond (*9*). BoNT/E, on the other hand, cleaves SNAP-25, but at the ^{180}Arg−^{181}Ile peptide bond (*14*). BoNTs are zinc endopeptidases like thermolysin, but do not catalyze proteolysis of any proteins other than their known target proteins involved in the fusion and docking of the synaptic vesicles during exocytosis (*13*). Furthermore, BoNTs seem to be the only class of metalloproteases where Zn^{2+} plays both a catalytic and structural role (*15*). The structural bases of such unique behavior of BoNTs are not yet understood. In this study, we have examined the structural changes which follow the disulfide reduction.

Because of its specificity in inhibiting neurotransmitter release at neuromuscular junctions, BoNT is used increasingly to treat various neuromuscular disorders such as strabismus, torticollis, and blepharospasm (*16*−*18*). Interestingly, BoNT only in its complex form with NAPs is used as a therapeutic agent and is a more effective drug than the

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[1] Abbreviations: BoNT, botulinum neurotoxin; NAP, neurotoxin-associated protein; SNAP-25, 25 kDa synaptosomal associated protein; DTT, dithiothreithol; GST, glutathione *S*-transferase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism.

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pure BoNT (*19*). However, the molecular basis explaining the better therapeutic efficacy of the BoNT complex is not known.

Production of NAPs by *C. botulinum* has been retained through evolutionary time at an enormous cellular energy cost. NAPs must be useful to the organism in its competitive survival. A close functional relationship between BoNT and NAPs is perhaps strongly indicated by clustering of their genes on the *C. botulinum* chromosome, and by the possibility of polycistronic gene expression and regulation (*20*, *21*). This notion is further supported by our recent preliminary experiments which have indicated that one or more of NAPs might act as molecular chaperones (*22*). Therefore, a clear understanding of the functional role of NAPs vis-à-vis the biological activity of BoNT will provide a unique example of an evolutionary bacterial design of the most toxic biological system known to man.

In this paper, we present experimental evidence that reveals, for the first time, that the enzymatic activity of BoNT/A complexed with NAPs is several-fold higher than that of the uncomplexed purified BoNT/A. Our results also suggest that the enhanced endopeptidase activity of the BoNT/A complex is likely due to direct interactions between the BoNT/A and NAPs and the BoNT/A in the complex conforms to a structurally active state similar to that of the reduced BoNT/A.

MATERIALS AND METHODS

BoNT/A and BoNT/A Complex Purifications. The BoNT/A complex and BoNT/A were purified from the cultures of *C. botulinum* type A strain Hall according to the method described previously (*23*). BoNT/A and the BoNT/A complex were extensively dialyzed against the 50 mM Tris-HCl (pH 7.6) buffer prior to being used in the experiments. BoNT/A and the BoNT/A complex were analyzed on a 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel under reducing and nonreducing conditions. The protein bands were visualized by Coomassie blue staining. The zinc content of the BoNT/A complex was determined by using Perkin-Elmer 5100 PC atomic absorbance spectrophotometer with an HGA-600 graphic furnace and an AS-60 autosampler (*15*). The molecular mass of the BoNT/A complex was determined by using a Sephadex G-200 (Sigma Chemical Co., St. Louis, MO) gel filtration column as described previously (*24*).

Expression and Purification of the GST−SNAP-25 Fusion Protein. SNAP-25 cDNA was amplified from the total mouse brain mRNA by RT-PCR using two primers (forward primer, 5′-CCTTCCCTCCCTACCCGGCGGC-3′; reverse primer, 5′-GAGAGAAGCATGAAGGAGCTC-3′), which were designed from the published sequence (*25*). The amplified cDNA was cloned into the pCR II TA-cloning vector (Invitrogen) and was completely sequenced to verify its sequence integrity. The SNAP-25 cDNA insert was then excised with restriction enzymes *Bam*HI and *Eco*RI, and the digested fragment was cloned into the *Bam*HI- and *Eco*RI-digested glutathione *S*-transferase fusion vector pGEX-2T (Phamacia Biotech, Piscataway, NJ). A recombinant plasmid containing the cDNA insert in the correct fusion reading frame was transformed into the *Escherichia coli* DH1aF′, and a transformed colony was used to induce the overexpression of the glutathione *S*-transferase−SNAP-25 (GST−SNAP-25) fusion protein by isopropyl β-thiogalactopyranoside as described previously (*26*).

To purify the GST−SNAP-25 fusion protein, cells expressing the fusion protein were collected by centrifugation and resuspended in 10 mM phosphate-buffered saline (PBS) (pH 7.4) containing 1 mM phenymethanesulfonyl fluoride (PMSF). Cells were lysed by 2 min sonication, treated with 1% Triton X-100, and centrifuged to remove cell debris. The supernatant was applied to glutathione−agarose beads (Sigma Chemical Co., St. Louis, MO) and washed with PBS buffer to remove other cellular proteins, and the GST−SNAP-25 fusion protein was eluted with 10 mM glutathione in 50 mM Tris-HCl buffer (pH 8.0). The fusion protein was precipitated with ammonium sulfate, redissolved in desired assay buffer [50 mM Tris-HCl, 10 mM sodium phosphate, 300 mM NaCl, 2 mM $MgCl_2$, 0.3 mM $CaCl_2$, 1 mM mercaptoethanol, and 0.1% NaN_3 (pH 7.6)], and dialyzed against the same buffer, before being used for experiments.

Cleavage of the GST−SNAP-25 Fusion Protein. The GST−SNAP-25 fusion protein was incubated with a given concentration of BoNT/A (pure and complex) at 37 °C for a given time in the assay buffer under reducing and nonreducing conditions. For reducing conditions, the BoNT/A (pure and complex) was prepared by pretreatment with 20 mM DTT for 30 min at 37 °C. Samples were then separated by 12% SDS−PAGE and were analyzed by Western blot using a polyclonal antibody raised against the 12 C-terminal amino acid residues of SNAP-25 (Stressgen Biotechnologies Corp., Victoria, Canada) as described previously (*27*), followed by detection of the antibody−antigen complex using peroxidase-labeled secondary antibody and 4-chloro-1-naphthol as the substrate. The amount of uncleaved SNAP-25 was scanned using an Image Analyzer (ITTI, St. Petersberg, FL) and quantified by a Multiscan-R program, and the percent cleavage was calculated by comparing the density of the uncleaved band to that of the control SNAP-25.

Cleavage of SNAP-25 in Bovine Brain Synaptosomes. Synaptosomes were isolated from bovine cerebral cortex according to the method described previously (*28*). The synaptosomes were then diluted in Hepes buffer medium (HBM), which contains 140 mM NaCl, 5 mM KCl, 20 mM Hepes, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, and 10 mM glucose (pH 7.4). Synaptosomes (0.15 mg/mL) in HBM buffer were incubated with 200 nM (final concentration) nonreduced BoNT/A or BoNT/A complex at 37 °C for 4 h. The samples were then separated by 12% SDS−PAGE and were immunoblotted as described above. Staining was preformed with ECL chemiluminescent reagent (NEN Life Science Products, Boston, MA).

Circular Dichroism (CD) Spectra. Reduced BoNT/A was prepared by treating pure BoNT/A with 20 mM DTT for 30 min at 37 °C. The sample was then extensively dialyzed against 50 mM Tris-HCl (pH 7.6) buffer, containing 1 mM DTT. CD spectra were recorded using a Jasco model 715 spectropolarimeter (Jasco, Inc., Easton, NJ). Near-UV CD spectra of reduced and nonreduced BoNT/A were recorded between 320 and 245 nm at a speed of 20 nm/min, with a response time of 8 s. A baseline corresponding to the buffer solution was subtracted. For thermal unfolding, nonreduced and reduced BoNT/A were heated from 20 to 80

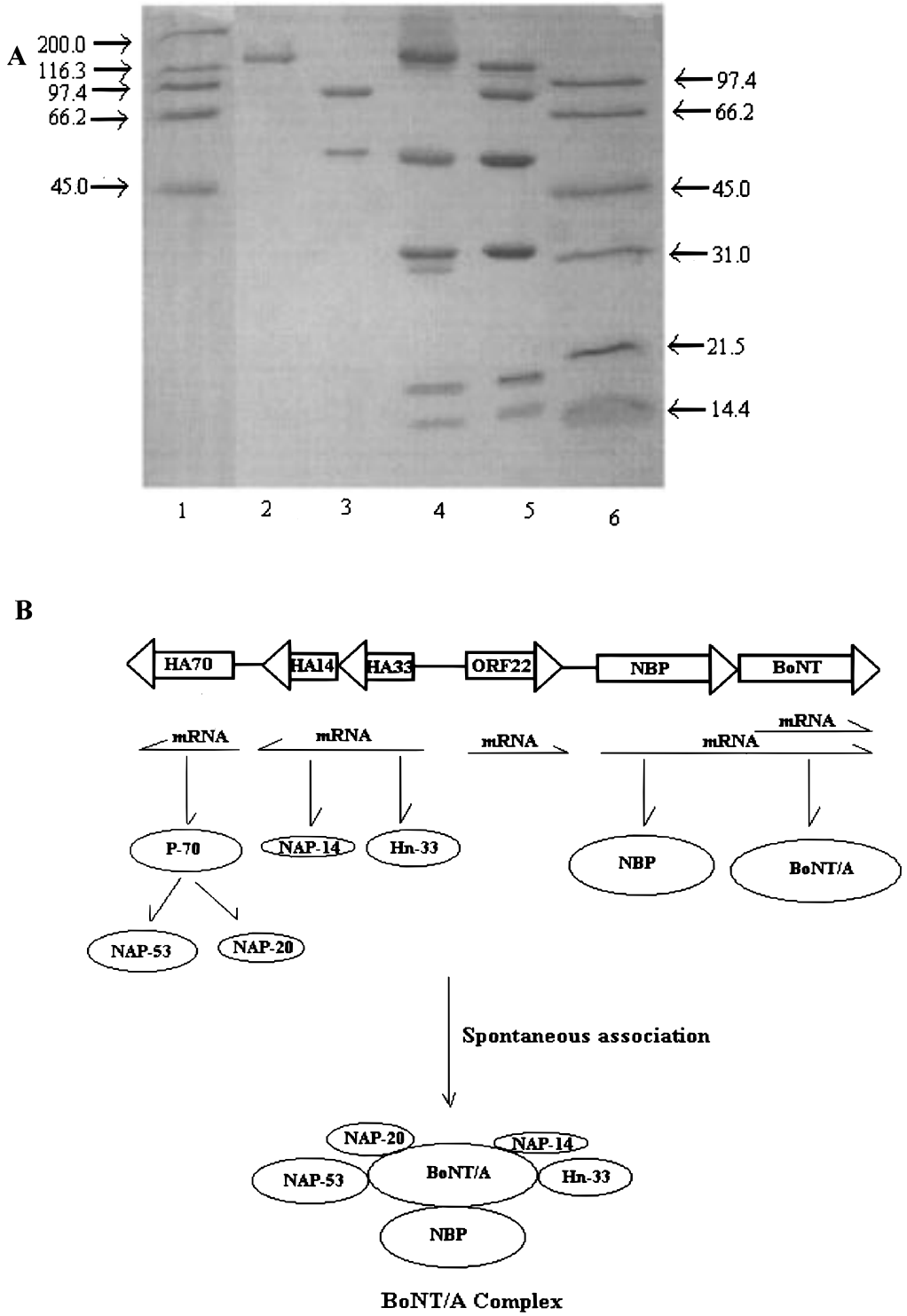

FIGURE 1: (A) SDS−PAGE analysis of BoNT/A and the BoNT/A complex under reducing and nonreducing conditions visualized by Coomassie blue staining: lane 1, high-molecular mass protein standards myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), and ovalbumin (45.0 kDa); lane 2, BoNT/A under nonreducing conditions; lane 3, BoNT/A under reducing conditions; lane 4, BoNT/A complex under nonreducing conditions; lane 5, BoNT/A complex under reducing conditions; and lane 6, low-molecular mass protein standards phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). (B) Schematic diagram showing the genomic organization of *C. botulinum* type A (*32*) and their expressed proteins in forming the BoNT/A complex. HA represents hemagglutinin, and the numbers following HA refer to the molecular masses of the protein expressed by these genes. ORF22 refers to the open reading frame of a gene that encodes a 22 kDa protein known to regulate BoNT gene expression (*33*).

°C at a heating rate of 2 °C/min. The unfolding was monitored by recording the CD signal at 222 nm, and the thermodynamic data were analyzed as described previously (*15*).

RESULTS AND DISCUSSION

BoNT/A and NAPs in the BoNT/A Complex. Freshly prepared BoNT/A complex exhibited seven Coomassie blue-

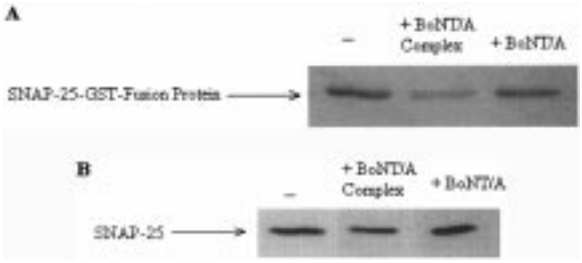

FIGURE 2: (A) Comparative analysis of proteolytic activities of pure BoNT/A and the BoNT/A complex under nonreducing conditions. The GST−SNAP-25 fusion protein (6 μM) was incubated with either pure BoNT/A (200 nM) or the BoNT/A complex (200 nM) for 2 h at 37 °C in assay buffer. Samples were then separated by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and were analyzed by Western blot using a polyclonal anibody raised against the 12 C-terminal amino acid residues of SNAP-25 (Stressgen Biotechnologies Corp.) as described in detail in Materials and Methods. The results that are shown are amounts of uncleaved SNAP-25 fusion protein remaining after the treatment. (B) Comparative analysis of the effect of pure BoNT/A and the BoNT/A complex on the level of SNAP-25 of bovine brain synaptosomes under nonreducing conditions as described in Materials and Methods.

stained protein bands when analyzed on a 12% SDS−PAGE gel under nonreducing conditions (Figure 1A). The protein bands correspond to 145 (BoNT/A), 120 [neurotoxin binding protein (NBP) (*29*)], 53 (NAP-53), 33 [hemagglutinin-33 (*23*)], 30 (NAP-30), 20 (NAP-20), and 14 kDa (NAP-14). Upon reduction, the band at 145 kDa disappeared and a band appeared at 90 kDa, corresponding to the heavy chain of BoNT/A. The light chain band of BoNT/A overlapped with the 53 kDa NAP band, and therefore, it was not seen separately. The band positions of light and heavy chains as well as that of the intact BoNT/A were verified by electrophoresing purified BoNT/A under nonreducing and reducing conditions (Figure 1A). The band observed at 30 kDa also disappeared, which indicated that it was formed as a result of a disulfide bond between either heterologous or homologous species of 20 and 14 kDa NAPs. The minor band observed at 120 kDa under nonreducing conditions shifted down to 110 kDa, and a major band appeared at 120 kDa. The band at 110 kDa is a partial degradation product of NBP (*30*). The NBP band observed at 120 kDa under reducing conditions appears to be closely overlapping with the BoNT/A band observed at 145 kDa under nonreducing conditions (Figure 1A, lanes 2 and 4), presumably due to the different molecular shape introduced by intramolecular disulfide bond(s). Other bands remained unaffected upon reduction with DTT. The assignments of bands observed on SDS−PAGE gels are based on previous biochemical and genetic analysis of the BoNT/A complex (*23*, *31*, *32*). A schematic model of BoNT/A genes and their expressed proteins is shown in Figure 1B.

Electrophoretic analysis of the BoNT/A complex under nonreducing and reducing conditions revealed that the disulfide bond between light and heavy chains of BoNT/A remained intact when it is complexed with the NAPs under nonreducing conditions. Since this disulfide intact form is the form of BoNT/A present in the bacterial culture, we will refer to it as the "native" BoNT/A form.

Zn^{2+} Endopeptidase Activity of BoNT/A and the BoNT/A Complex. The substrates for the zinc endopeptodase activity of different serotypes of BoNTs and tetanus neurotoxin (TeNT) are the constitutive components of the secretory machinery (*10*). The 25 kDa synaptosomal associated protein (SNAP-25) is the substrate for BoNT/A and BoNT/E (*7*, *10*). Endopeptidase activity of pure BoNT/A and the BoNT/A complex was analyzed by their ability to proteolytically cleave a recombinant glutathione *S*-transferase−SNAP-25 (GST−SNAP-25) fusion protein. The amount of uncleaved protein was determined by Western blot assay using a SNAP-25 anti-C terminal polyclonal antibody. Under nonreducing conditions, the pure BoNT/A exhibited virtually no proteolytic activity (average 4% cleavage, $n = 2$), whereas the BoNT/A complex was extremely active (Figure 2A) leading to an average cleavage of 66% GST−SNAP-25 ($n = 2$). The nonreduced BoNT/A complex also exhibited higher endopeptidase activity when bovine brain synaptosomes were used as the substrate (Figure 2B). While the nonreduced BoNT/A complex cleaved an average of 25% ($n = 2$) of the SNAP-25 in bovine brain synaptosomes after incubation for 4 h at 37 °C, nonreduced BoNT/A exhibited almost no endopeptidase activity. Thus, the enhanced endopeptidase activity of BoNT/A in the complex form was qualitatively similar under both in vivo (bound to natural membrane) and in vitro (solution) conditions. The lower activity of the BoNT/A complex against SNAP-25 in bovine brain synaptosomes, compared to that of the SNAP-25 in solution, is due to the lower accessibility of the BoNT/A complex to the substrate (SNAP-25) in synaptosomes. Although detergents such as 1-*O*-*n*-octyl β-D-glucopyranoside can overcome the problem of substrate accessibility without affecting the BoNT's endopeptidase activity when assayed under reducing conditions (*34*), 1-*O*-*n*-octyl β-D-glucopyranoside abolished the endopeptidase activity of the nonreduced BoNT/A complex (data not shown). This observation indicated that the detergent affected the BoNT/A complex structure.

Using in vitro assay system (GST−SNAP-25), we observed that under reducing conditions pure BoNT/A cleaved only about 18 ± 3% ($n = 3$) of the GST−SNAP-25 in 10 min whereas the BoNT/A complex was able to cleave 80 ± 4% ($n = 3$) of the GST−SNAP-25 in 10 min (Figure 3). Furthermore, a concentration of 50 nM of the BoNT/A complex cleaved 75 ± 5% ($n = 3$) of the GST−SNAP-25 after incubation for 2 h, whereas purified BoNT/A cleaved only 29 ± 3% ($n = 3$) of the GST−SNAP-25 (Figure 4). Even at 200 nM, purified BoNT/A cleaved only 69 ± 4% ($n = 3$) whereas the BoNT/A complex cleaved 92 ± 4% ($n = 3$) of the GST−SNAP-25 (Figure 4). Collectively, the above results suggest that (i) only the BoNT/A complex, and not the pure BoNT/A, is enzymatically active under nonreducing conditions and (ii) even under reducing conditions the BoNT/A complex is more than 4-fold more active than the pure BoNT/A.

To compare further the proteolytic activity of BoNT/A in pure and complex forms, we tested whether the BoNT/A complex also behaves as a Zn^{2+} protease by analyzing the effect of ethylenediaminetetraacetate (EDTA) on the enzyme activity of BoNT/A complex. As shown in Figure 5A, EDTA was equally effective in blocking the proteolytic activity of both pure and complexed BoNT/A, suggesting that the proteolytic activity of the BoNT/A complex is similar to the metalloprotease activity of the pure BoNT/A. To further confirm that the Zn^{2+} protease activity of the complex originates from the BoNT/A, we analyzed the Zn^{2+} content

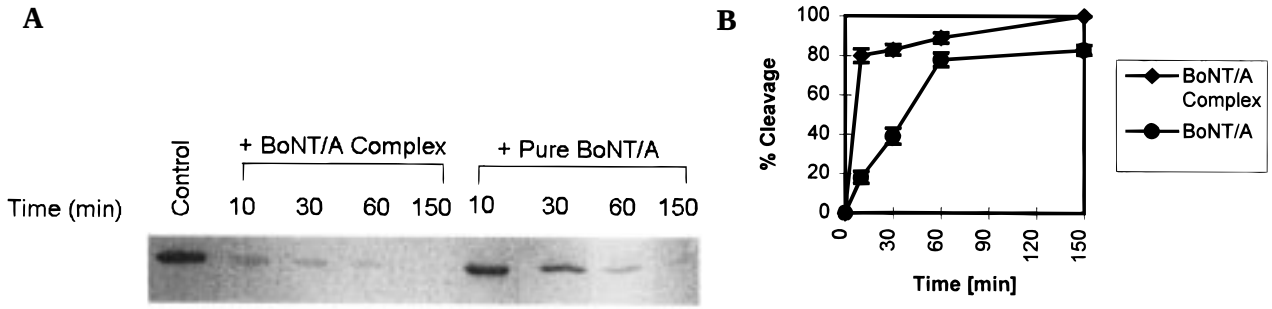

FIGURE 3: Time course of GST−SNAP-25 cleavage by pure BoNT/A and the BoNT/A complex under reducing conditions. (A) The GST−SNAP-25 fusion protein (4.9 μM) was incubated with either 200 nM pure BoNT/A or 200 nM BoNT/A complex for the indicated time periods, after which samples were separated by SDS−PAGE and separated proteins immunoblotted as described in the legend of Figure 1. (B) Percentage of GST−SNAP-25 cleavage as function of incubation time quantified by image analysis as described in Materials and Methods (average of three independent experiments; the bars represent standard deviations).

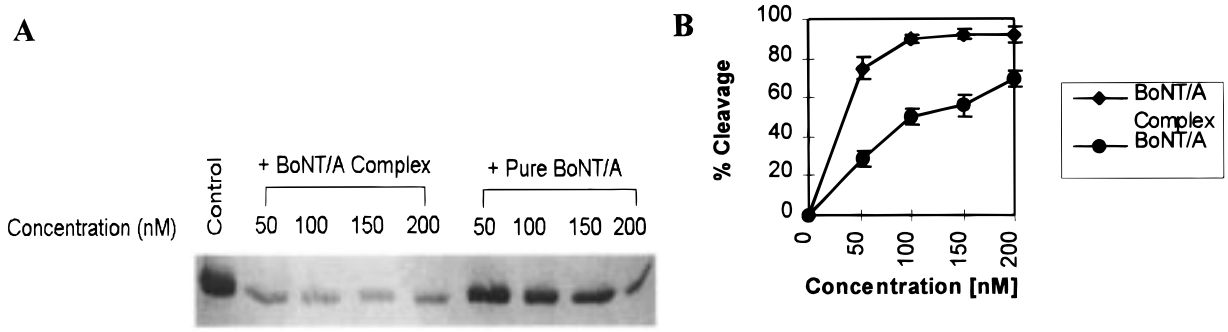

FIGURE 4: Concentration-dependent cleavage of GST−SNAP-25 (6.2 μM) by reduced BoNT/A and the BoNT/A complex after incubation for 2 h. Other experimental conditions were the same as described in the legend of Figure 1. (A) Immunoblot analysis of GST−SNAP-25 cleavage and (B) the percentage of GST−SNAP-25 cleaved (determined as described in Materials and Methods) as a function of BoNT/A concentration. The results depicted in panel B were plotted by averaging the percentage of GST−SNAP-25 cleaved in three independent sets of experiments whose results are depicted in panel A.

of the BoNT/A complex using atomic absorption spectroscopy. We found only one Zn^{2+} present per BoNT/A complex (Table 1), identical to the results with pure BoNT/A (*15*). The SDS−PAGE pattern of the cleaved GST−SNAP-25 fusion protein stained by Coomassie blue was identical for pure BoNT/A- and BoNT/A complex-catalyzed proteolysis (Figure 5B), indicating an identical cleavage site in GST−SNAP-25 for both BoNT reagents. Additionally, preincubation with a BoNT/A specific polyclonal antibody (rabbit anti-BoNT/A serum obtained from W. H. Lee, U.S. Department of Agriculture, Beltsville, MD) blocked the proteolytic activity of both pure BoNT/A and the BoNT/A complex (Figure 5C). Taken together, these results strongly support the possibility that the proteolytic activity of the BoNT/A complex is due to BoNT/A, and not due to any proteolytic activity of NAPs.

Alkaline pH reportedly leads to dissociation of the BoNT/A complex into BoNT/A and NAPs (*4*). Because we performed the endopeptidase assays at pH 7.6, we examined using a gel filtration column whether the purified complex retained its integrity at this slightly alkaline pH. The BoNT/A complex eluted as a single elution peak from a Sephadex G-200 gel filtration column, ahead of the molecular mass marker of 443 kDa, corresponding to the expected molecular mass (569 kDa) of the intact complex (Figure 6). This observation suggested that the BoNT/A complex remained intact under our experimental conditions, and the enhanced protease activity is likely to be the result of conformational alteration in the BoNT/A due to its interaction with NAPs. The possibility of NAP-mediated blockage of the disulfide bond between the light and heavy chains was ruled out as a cause of higher endopeptidase activity of BoNT/A in its complex form. This is because BoNT/A remained as a 145 kDa protein in the BoNT/A complex under denaturing SDS−PAGE analysis, and exhibited light and heavy chain separation only after DTT-mediated reduction of their disulfide bond (Figure 1A). Direct interaction between BoNT/A or BoNT/E and some components of NAPs leading to interaction-induced conformational changes in BoNT/A or BoNT/E have been demonstrated previously (*3*, *24*).

Structural Change Introduced by Disulfide Bond Reduction. BoNTs are now classified as an entirely new class of metalloproteases (*35*, *36*). One of their unique characteristics is that the reduction of the disulfide bond in the purified BoNT/A, linking the two subunit chains (50 kDa light chain and 100 kDa heavy chain) of the purified BoNT, is essential for its endopeptidase activity (*8*, *9*, *36*). However, the thiol group itself does not seem to participate in the catalytic site (*37*). Because the purified nonreduced BoNT is not enzymatically active when the light chain is linked through a disulfide bond in its dichain form, it is likely that polypeptide folding of the enzymatic subunit (light chain) is altered in its reduced and functionally active form. This possibility was probed by near-UV CD spectroscopy and thermal unfolding of BoNT/A in reduced and nonreduced forms.

The near-UV CD spectra of nonreduced and reduced BoNT/A (Figure 7) are remarkably different, particularly the bands corresponding to Phe (263 and 269 nm) and Trp residues (286 and 295 nm). For example, disulfide bond reduction resulted in a significant decrease (41%) in band

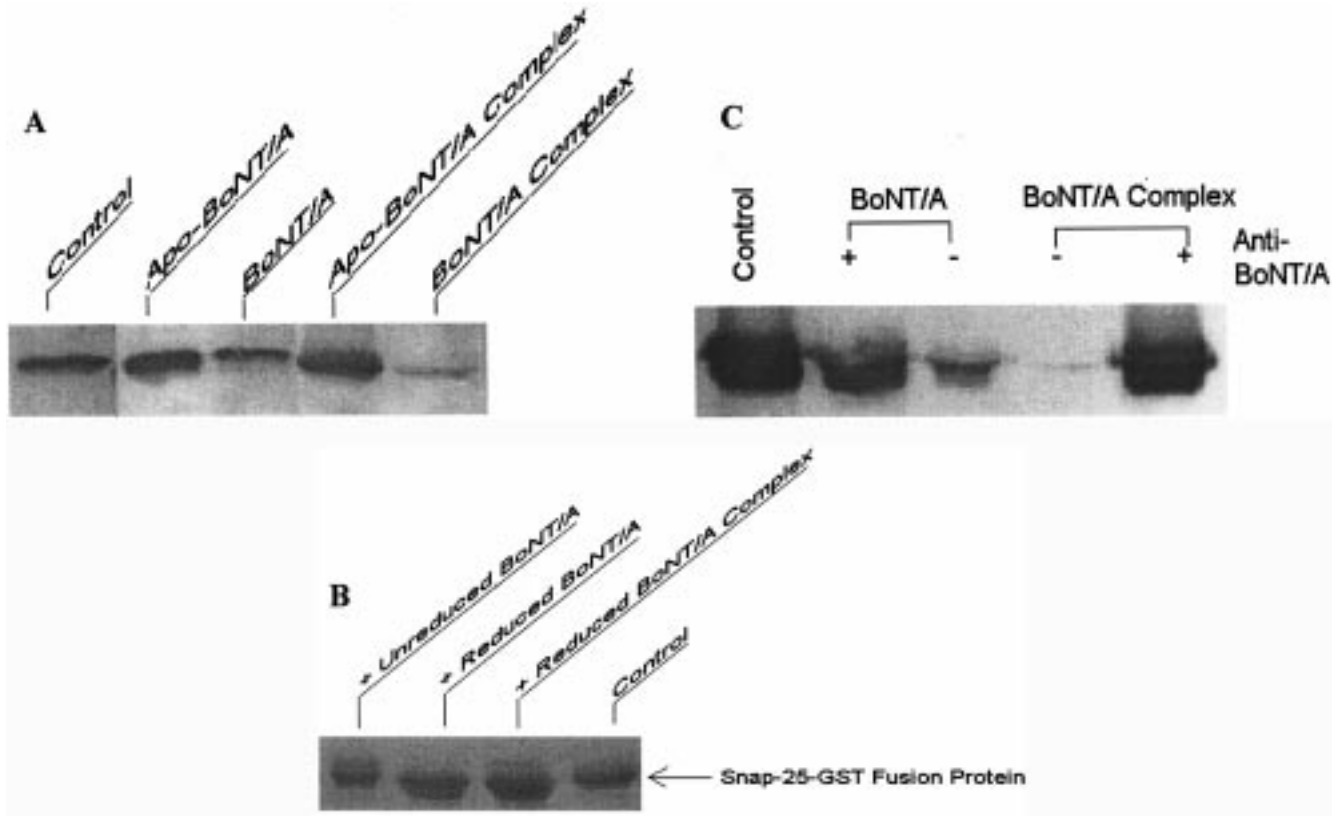

FIGURE 5: Effect of EDTA (A) and anti-BoNT/A serum (C) on the proteolytic activity of reduced BoNT/A and the BoNT/A complex. The cleavage of GST−SNAP-25 was analyzed by immunoblotting as described in Materials and Methods. (A) Pure BoNT/A and the BoNT/A complex were treated with 20 mM EDTA for 30 min at 37 °C and then extensively dialyzed in 50 mM Tris-HCl buffer (pH 7.6) at 4 °C overnight to prepare apo-BoNT/A and the apo-BoNT/A complex. The GST−SNAP-25 fusion protein (9 μM) was incubated with 200 nM apo-BoNT/A or apo-BoNT/A complex for 2 h at 37 °C. (B) SDS−PAGE analysis of GST−SNAP-25 cleavage by pure BoNT/A and the BoNT/A complex as revealed by Coomassie blue staining. The GST−SNAP-25 fusion protein (6 μM) was incubated with either pure BoNT/A (200 nM) or the BoNT/A complex (200 nM) for 2 h at 37 °C in 50 mM Tris-HCl buffer (pH 7.6). (C) The GST−SNAP-25 fusion protein (15 μM, control) was incubated for 2 h at 37 °C with either 200 nM reduced pure BoNT/A or 200 nM reduced BoNT/A complex with and without preincubation with anti-BoNT/A serum (0.2 mg/mL, 30 min, 37 °C).

Table 1: Zn^{2+} Content of the Holo- and Apo-BoNT/A Complex

BoNT/A complex	Zn^{2+} per BoNT/A complex
holo	1.04 ± 0.11
apo	0.26 ± 0.02

strength at 295 nm, which corresponds to the $'L_b$ transition of Trp residues (*15*, *38*). The band observed at 286 nm in nonreduced BoNT/A, corresponding to the $'L_a$ transition of Trp residues (*38*), was eliminated upon reduction of the disulfide bond. CD bands corresponding to Phe residues also registered a significant decrease at 263 nm (21%) and 269 nm (27%). The bands at 280−284 nm, corresponding to Tyr residues (*38*), remained virtually unchanged. A decrease in the near-UV CD signal generally results from increased flexibility of segments containing chromophores (*15*). The increased flexibility in BoNT/A polypeptide folding may help its binding with SNAP-25, and in the movement of the active site to the cleavage site of the substrate. A recent report of crystallographic structure of nonreduced BoNT/A predicts that its active site (HEXXH) is buried 20 Å deep within the protein matrix (*39*). Such a structure is difficult to reconcile with the large substrate molecule like the SNAP-25 for BoNT/A endopeptidase activity. The reduction-induced increase in the flexibility of polypeptide folding as observed in this study provides the rationale for the mechanism of BoNT/A endopeptidase activity against its exclusive target substrate, the SNAP-25.

The increased flexibility in the BoNT/A polypeptide folding with disulfide bond reduction was further examined by analyzing temperature-induced unfolding energetics (Figure 8). Nonreduced BoNT/A exhibited a typical unfolding transition with a melting temperature (T_m) of 53 °C. The sharp transition is indicative of a two-state (N $\rightleftharpoons$ U) unfolding. Reduced BoNT/A, on the other hand, exhibited a dramatically different transition curve with a smooth but slow unfolding transition. The broad transition suggests a noncooperative unfolding, which would be consistent with a nonrigid structure. The T_m for reduced BoNT/A was 43 °C, indicating a relatively unstable structure. Temperature-induced unfolding of nonreduced as well as reduced BoNT/A resulted in aggregate formation, which was observed for many large proteins (*40*). Therefore, it was not possible to estimate true thermodynamic parameters. Calculation of pseudothermodynamic parameters revealed ΔH, ΔS, and ΔG values of 248 kJ mol^{-1}, 761 J mol^{-1} K^{-1}, and 22 kJ mol^{-1} for nonreduced BoNT/A and 155 kJ mol^{-1}, 491 J mol^{-1} K^{-1}, and 9 kJ mol^{-1} for the reduced BoNT/A. A 37% decrease in the ΔH value suggests that considerable instability is introduced by the disulfide reduction. While disulfide bond reduction, in general, leads to protein instability (*41*, *42*), the extent of instability observed after reduction of the single

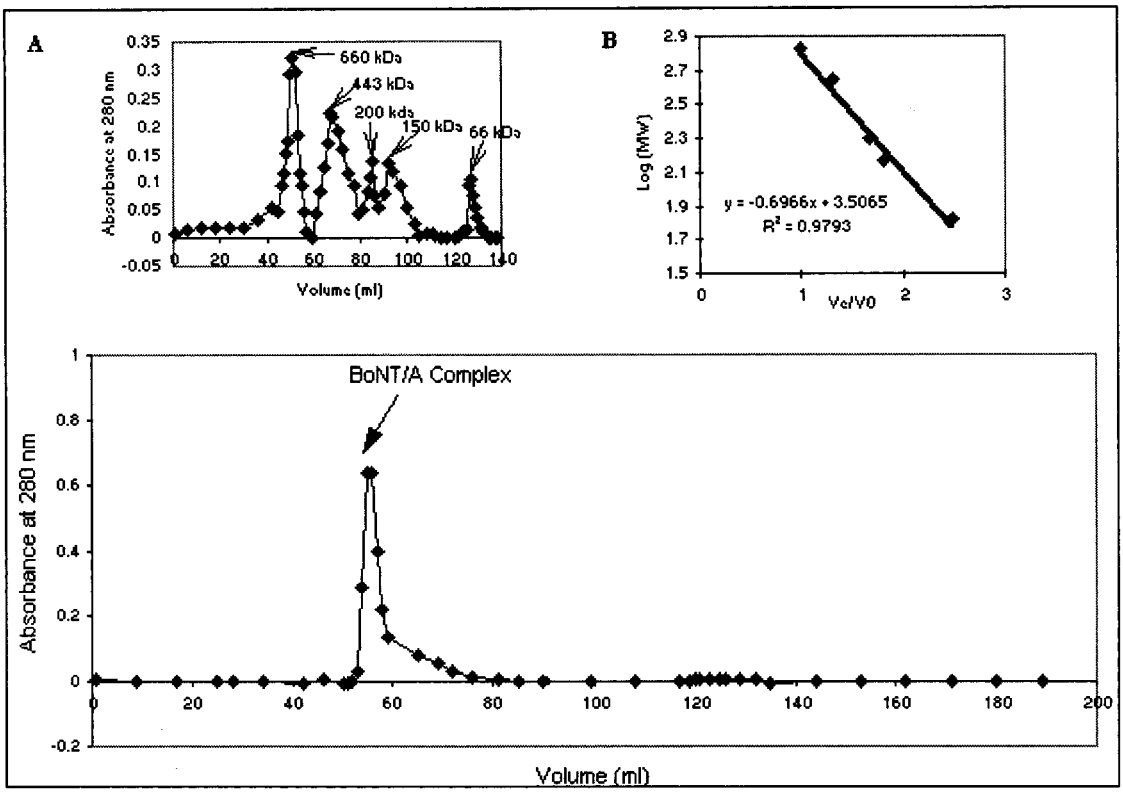

FIGURE 6: Elution profile of the BoNT/A complex determined with a Sephadex G-200 gel filtration column. (Inset A) The elution profile of the following molecular mass standards: 669 kDa thyroglobulin (bovine), 443 kDa apoferritin (house spleen), 200 kDa β-amylase (sweet potato), 150 kDa alcohol dehydrogenase (yeast), and 66 kDa albumin (bovine serum). (Inset B) The standard curve.

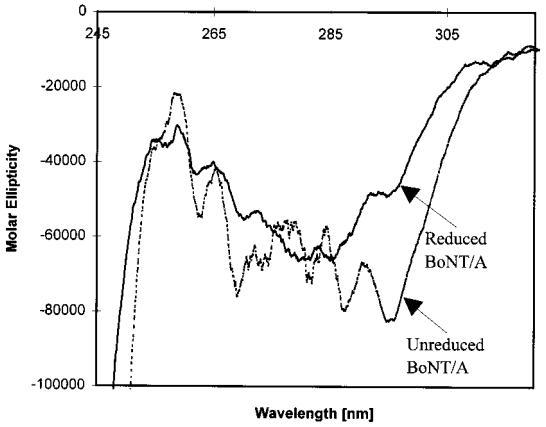

FIGURE 7: Near-UV CD spectra of pure BoNT/A in reduced and nonreduced forms. Spectral recordings were carried out at a speed of 20 nm/min and with a response time of 8 s.

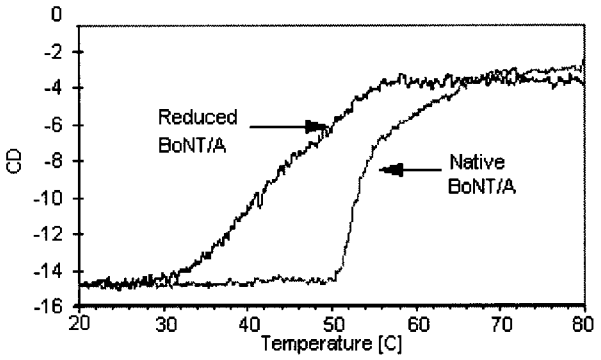

FIGURE 8: Thermal unfolding of pure BoNT/A in reduced and unreduced forms. Samples were heated from 20 to 80 °C at a heating rate of 2 °C/min. The unfolding was monitored by recording the CD signal at 222 nm.

disulfide bond in BoNT/A was quite significant. The extent of change in the protein folding notwithstanding, it must introduce specific changes in BoNT/A to enhance its enzymatic activity. The specific nature of these changes is also consistent with differential changes in the near-UV CD signal of BoNT/A. For example, while the Trp CD signal at 295 nm was reduced by 41%, no such decrease was observed in the Tyr CD signal at 280 nm.

In summary, the near-UV CD spectroscopy and temperature-dependent unfolding results of BoNT/A suggest that disulfide bond reduction introduces specific structural changes, which makes it possible for the active site of BoNT/A to interact with its substrate. Interestingly, the nonreduced BoNT/A complex cleaved 66% (Figure 2) whereas reduced pure BoNT/A cleaved 69% of the SNAP-25 after incubation for 2 h (Figure 4). Even though reduction of the BoNT/A complex with DTT further enhanced its activity, this increase (39%) was smaller than that for pure BoNT/A (17-fold). Thus, these results provide strong support for the idea that the enhanced endopeptidase activity of the BoNT/A complex, compared to the pure BoNT/A, is likely due to direct interactions between the BoNT/A and NAPs. As a result of this interaction, the BoNT/A in the complex conforms to a structurally active state similar to that of the reduced BoNT/A. It is possible that both of these conditions cause the exposure of the active site, which is found to be buried 20 Å within the crystal structure of the nonreduced purified BoNT/A (*39*).

Concluding Remarks. Unique characteristics of clostridial neurotoxins include induction of their endopeptidase activity by disulfide bond reduction. We provide direct evidence of

structural change which may be responsible for BoNT/A activity. To the best of our knowledge, this is the first direct experimental evidence that demonstrates that the active structure of BoNT in the reduced form is conformationally different from that of the nonreduced inactive form.

Our finding that BoNT/A activity in the complex form is dramatically higher than in the pure form reveals an additional role of NAPs in the toxicoinfection process of botulism. This finding is also very significant in formulating ideas about the molecular principles that underlie the better efficacy of BoNTs as a therapeutic agent. The fact that the difference in the endopeptidase activities of pure BoNT/A and BoNT/A complex remains qualitatively similar against both in vitro (GST−SNAP-25 fusion protein) and in vivo (synaptosomes) substrates makes the observation of higher enzymatic activity of the BoNT/A complex more significant both physiologically and therapeutically.

Clostridial neurotoxins have already proven to be highly effective tools for investigating the complex mechanism of neurotransmitter release (*27*, *43*). The availability of BoNT/A with at least 4-fold higher enzyme activity may prove to be a better tool in neurobiological research. Furthermore, one or more of the NAPs are likely to be important targets in developing therapeutic reagents for intervention and vaccine development.

Finally, demonstration of a direct role of NAPs in the activation of the endopeptidase activity of BoNT reveals a genetically designed biological system, which is a unique example of evolutionary design, structure, and multifunction (chaperonic folding, stabilization, protection, and activation of BoNT). Such a system also provides important clues for learning about the molecular design and folding of polypeptides and proteins.

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