Enhancement of the Endopeptidase Activity of Purified Botulinum Neurotoxins A and E by an Isolated Component of the Native Neurotoxin Associated Proteins

Shashi Kant Sharma‡ and B. R. Singh*

Department of Chemistry and Biochemistry, and Center for Marine Science and Technology, University of Massachusetts Dartmouth, Dartmouth, Massachusetts 02747

Received August 29, 2003; Revised Manuscript Received February 23, 2004

ABSTRACT: In botulism disease, neurotransmitter release is blocked by a group of structurally related neurotoxin proteins produced by *Clostridium botulinum*. Botulinum neurotoxins (BoNT, A−G) enter nerve terminals and irreversibly inhibit exocytosis via their endopeptidase activities against synaptic proteins SNAP-25, VAMP, and Syntaxin. Type A *C. botulinum* secretes the neurotoxin along with 5 other proteins called neurotoxin associated proteins (NAPs). Here, we report that hemagglutinin-33 (Hn-33), one of the NAP components, enhances the endopeptidase activity of not only BoNT/A but also that of BoNT/E, both under in vitro conditions and in rat synaptosomes. BoNT/A endopeptidase activity in vitro is about twice as high as that of BoNT/E under disulfide-reduced conditions. Addition of Hn-33 separately to nonreduced BoNT/A and BoNT/E (which otherwise have only residual endopeptidase activity) enhanced their in vitro endopeptidase activity by 21- and 25-fold, respectively. Cleavage of rat-brain synaptosome SNAP-25 by BoNTs was used to assay endopeptidase activity under nerve-cell conditions. Reduced BoNT/A and BoNT/E cleaved synaptosomal SNAP-25 by 20% and 15%, respectively. Addition of Hn-33 separately to nonreduced BoNT/A and BoNT/E enhanced their endopeptidase activities by 13-fold for the cleavage of SNAP-25 in synaptosomes, suggesting a possible functional role of Hn-33 in association with BoNTs. We believe that Hn-33 could be used as an activator in the formulation of the neurotoxin for therapeutic use.

Botulinum neurotoxins (seven serotypes, A−G) are a group of large proteins with mutually exclusive immunological properties but share pharmacological characteristics that cause flaccid muscle paralysis in the botulism disease (*1*). Botulinum neurotoxins (BoNTs)[1] are extremely toxic proteins (mouse LD_{50}, 10^8 mg^{-1} kg^{-1} for type A) of 150 kDa and consist of a 100 kDa heavy chain and a 50 kDa light chain linked through a disulfide bond (*2*). Type A BoNT is produced along with 6 neurotoxin associated proteins (NAPs) to form a complex. NAPs are known to protect BoNT from the acidity and proteases of the GI tract (*3*−*5*), and thus make BoNT one of the most dreaded food poisoning agents (*3*, *6*). In addition, because of the extreme toxicity and stability of BoNT in the presence of NAPs, BoNT complexes are considered as a group of the most dangerous biological warfare agents (*7*, *8*). Sadly, despite their critical role in food poisoning and biological weapons, little is understood as to how these nontoxic accessory proteins (i.e., NAPs) play a critical role in the toxicoinfection process of botulism.

* To whom correspondence should be addressed: Department of Chemistry and Biochemistry, University of Massachusetts Dartmouth, 285 Old Westport Road, Dartmouth, MA 02747. Phone: 508-999-8588. Fax: 508-999-8451. E-mail: bsingh@umassd.edu.

‡ Present address: Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy, HFS-300, College Park, MD 20740-3835.

[1] Abbreviations: BoNT, botulinum neurotoxin; NAP, neurotoxin associated protein; SNAP-25, synaptosomal associated protein of 25 kDa; DTT, dithiothreitol; GST, glutathione-*S*-transferase; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbant assay; Hn-33, hemagglutinin-33.

Recent discovery that BoNTs are Zn^{2+}-endopeptidases (*2*, *9*, *10*) 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 process. Cellubrevin, SNAP-25 (synaptosomal associated protein of 25 kDa), and syntaxin form the SNARE complex during docking of synaptic vesicles to the plasma membrane. Different BoNT types proteolytically cleave cellubrevin (BoNT/B, BoNT/C, BoNT/D, and BoNT/F), SNAP-25 (BoNT/A, BoNT/C, and BoNT/E), and syntaxin (BoNT/C) as part of their mode of action to block neurotransmitter release (*2*). Because of their specificity to inhibit neurotransmitter release at neuromuscular junctions, BoNT is increasingly being used to treat various neuromuscular disorders such as strabismus, torticollis, and blepherospasm (*11*). Interestingly, BoNT only in its complex form with NAPs (present in all BoNT serotypes) is used as a therapeutic agent, which is a more effective drug in this form than the pure BoNT (*12*). Again, the molecular basis of the superior therapeutic efficacy of BoNT complex is not known. Stabilization of BoNT/A by NAPs has been proposed as one possible explanation for the higher efficacy of the BoNT/A complex compared to the pure BoNT/A (*11*, *12*).

The endopeptidase activity of pure BoNT/A is expressed only after its interchain disulfide bond is reduced (*2*, *9*, *10*). We have previously reported (*13*) that the BoNT/A complex, in contrast to pure BoNT/A, is enzymatically active even under nonreducing conditions and the endopeptidase activity of BoNT/A complex is 17-fold higher than that of the pure

10.1021/bi0355544 CCC: $27.50
Published on Web 04/01/2004

BoNT/A. Under reducing conditions, the BoNT/A complex is significantly (about 15%) more active than the pure BoNT/A, and reduced purified BoNT/A has similar endopeptidase activity to the nonreduced BoNT/A in complex with NAPs (*13*). The higher endopeptidase activity of the BoNT/A complex is due to the presence of NAPs, suggesting a more than accessory role, such as protection against proteases in the GI tract, in the toxicoinfection process of botulism. The dramatically higher endopeptidase activity of the BoNT/A complex raises several questions: (1) Is the reduction of the disulfide bond of BoNT the only process that activates endopeptidase activity? (2) Is the higher therapeutic efficacy of the BoNT/A complex because of the enhanced endopeptidase activity? (3) Is the cumulative effect of all of the NAPs required for the BoNT/A complex to be enzymatically more active? (4) Can the NAPs-mediated activation of endopeptidase activity be observed with cross serotypes of BoNT?

In this paper, we present experimental evidence to suggest that the hemagglutinin-33 (Hn-33, also referred as HA-35 by some researchers) component of BoNT/A NAPs can activate BoNT/A equivalent to the cumulative effect of the BoNT/A complex. Further, Hn-33 is also able to activate the endopeptidase activity of BoNT/E.

MATERIALS AND METHODS

Purification of BoNT/A and BoNT/E. BoNT/A from *C. botulinum* (strain Hall) and BoNT/E from *C. botulinum* (strain Alaska) were purified as described previously (*5*, *14*). Purified neurotoxins were precipitated with 0.39 g/mL of ammonium sulfate and stored at 4 °C until used. The precipitate was centrifuged at 10000*g* for 10 min, and the pellet was dissolved in a desired assay buffer, followed by dialysis to remove any residual ammonium sulfate.

Production and Purification of Hn-33. Hn-33 was isolated according to the procedure described by Fu et al. (*14*) with the use of a DEAE-Sephadex A-50 and a Sephadex G-100 column chromatography.

Concentrations of neurotoxin and Hn-33 were determined according to the extinction coefficients, ϵ mg/mL (278 nm) of 1.63 and 1.74, respectively (*14*, *15*).

Expression and Purification of Glutathione-S-Transferase (GST)/SNAP-25. SNAP-25 was expressed and purified according to Cai et al. (*13*). Cells expressing GST/SNAP-25 fusion protein were collected by centrifugation and resuspended in 10 mM phosphate-buffered saline (PBS) at pH 7.4, containing 1 mM phenylmethylenesulfonyl fluoride (PMSF). Cells were lysed by sonication for 2 min, 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 GST/SNAP-25 fusion protein was eluted with 10 mM glutathione in 50 mM Tris-HCl buffer at pH 8.0. The fusion protein was precipitated with ammonium sulfate, redissolved in the 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 at pH 7.6), and dialyzed against the same buffer, before being used for experiments.

Isolation of Rat-Brain Synaptosomes and Cleavage of SNAP-25. Frozen rat brains were purchased from RJO Biologicals, Inc. (Kansas City, MO) and were stored at −80 °C. Synaptosomes were prepared as described by Li and Singh (*16*). The synaptosomes were first washed with Hepes buffer at pH 7.4, containing 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, and were then resuspended in 2 mL of the same buffer. All of the procedures were carried out at 4 °C. Synaptosomes (50 μL) in Hepes buffer were incubated with 200 nM (final concentration) nonreduced or reduced [treatment with 20 mM dithiothreitol (DTT) at 37 °C for 30 min] BoNT/A or BoNT/E with a 1:1 molar ratio (BoNT/A:Hn-33, BoNT/E:Hn-33) and a 1:2 molar ratio (BoNT/A:Hn-33) at 37 °C for 4 h. The samples were then separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) gel and were immunoblotted using anti-SNAP-25 antibody raised against the 12 amino acid C-terminal residues in a rabbit (Stressgen Biotechnologies Corp., Victoria, Canada).

In vitro Cleavage of SNAP-25: Western Blot. The endopeptidase activity of BoNT was assayed according to a Western blot method established previously (*13*). GST/SNAP-25 fusion protein (5 μM) was incubated with 200 nM of BoNT/A or BoNT/E in the presence or absence of 200 nM of Hn-33 at 37 °C for 15 min in an assay buffer (50 mM Tris, 10 mM sodium phosphate, 300 mM NaCl, 2 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 0.1% NaN_3) at pH 7.6, under reducing and nonreducing conditions. For reducing conditions, the BoNT/A and BoNT/E were prepared by pretreatment with 20 mM DTT for 30 min at 37 °C. To investigate the effect of Hn-33, BoNT/A and BoNT/E were preincubated with Hn-33. Hn-33, nonreduced BoNT/A or BoNT/E, and reduced BoNT/A or BoNT/E were each separately dissolved in 0.05 M citrate buffer at pH 5.5 and were filtered through a 0.45 μm filter paper. Nonreduced BoNT/A or BoNT/E were mixed with Hn-33 (molar ratio 1:1) in a reaction volume of 3 mL. Similarly, reduced BoNT/A or BoNT/E were mixed with Hn-33 (molar ratio 1:1) in a reaction volume of 3 mL. The reaction mixture was incubated up to 15 min at room temperature (25 °C) because, at a 30-min incubation time, a complete cleavage of SNAP-25 was observed (data not shown). Samples were then separated on a 12% SDS−PAGE gel 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.) as described previously (*13*).

The amount of uncleaved SNAP-25 was estimated by scanning the Western blot band using an Image Analyzer (ITTI, St. Petersberg, FL) and a Multiscan-R, and the percent cleavage was calculated by comparing the density of the uncleaved band to that of the control SNAP-25.

Enzyme-Linked Immunosorbant Assay (ELISA). The cleavage of SNAP-25 was also determined by ELISA with the aim to determine the minimum concentration of BoNT/A and BoNT/E required to cleave the SNAP-25. Flat-bottom microtitter plates were coated (100 μL/well) with 10 μg/mL of SNAP-25 fusion protein dissolved in a 0.01 M phosphate buffer at pH 7.2. Plates were incubated at 4 °C overnight. The plates were then washed 5 times with 0.01 M phosphate buffer at pH 7.2. Serially diluted 200 nM of BoNT/A and BoNT/E, with or without 200 nM of Hn-33, were added into the plates to a total volume of 100 μL. After incubation at 37 °C for 30 min, plates were washed with 0.01 M phosphate

buffer at pH 7.2 containing 0.05% Tween-20 and were subsequently blocked by 3% bovine serum albumin dissolved in a 0.01 M phosphate buffer at pH 7.2. Rabbit anti-SNAP-25 antibody (Stressgen Biotechnologies Corp.), 3 ng/mL, (100 μL/well) was used as the primary antibody to bind with the SNAP-25 remaining in the plates followed by incubation at 37 °C for 30 min. The peroxidase-labeled anti-rabbit antibody, 1:10 000, was added as a secondary antibody onto the plates, and the plates were incubated at 37 °C for 30 min. A substrate solution containing 0.04% OPD (*o*-phenylenediamine dihydrochloride) and 0.012% hydrogen peroxide in a citrate phosphate buffer at pH 5.0 was added into the wells, and the plates were incubated for 15 min at room temperature (25 °C). The reaction was subsequently quenched with 50 μL of 2 M sulfuric acid, and the color was monitored by measuring the absorbance at 490 nm. Unless otherwise stated, the plates were washed 5 times with 0.01 M phosphate buffer at pH 7.2, containing 0.05% Tween-20, between the steps. The percent cleavage was calculated by comparing the absorbance of the uncleaved to that of the control SNAP-25.

For kinetic experiments, the ELISA method was used, as described above with the following modifications: after preincubation of Hn-33 with BoNT/A or BoNT/E separately, the BoNT/A or BoNT/E was added to the plate for different time intervals (0−40 min). The temperature of the plate was maintained at 37 °C during the addition of the samples. After incubation at 37 °C for 40 min, plates were washed with 0.01 M phosphate buffer at pH 7.2, containing 0.05% Tween-20, and subsequently blocked by 3% bovine serum albumin dissolved in 0.01 M phosphate buffer at pH 7.2. Primary and secondary antibodies were used as described above to estimate uncleaved SNAP-25.

Isothermal Titration Calorimetric Analysis of Hn-33 Interaction with BoNT/A. The binding isotherm of Hn-33 to the BoNT/A was generated employing the CSC isothermal titration calorimeter (Calorimetric Sciences Corp., Provo, UT). The binding was measured at 25 °C in a 10 mM phosphate buffer at pH 7.4. BoNT/A (0.7 μM) in a total volume of 1.3 mL was placed in the reaction cell. After temperature equilibration, the 0.7 μM of BoNT/A was titrated with 30 μM Hn-33 ligand. The 20 μL injections of Hn-33 ligand was mixed with BoNT/A at 400-s intervals in a reaction cell. The observed heat change accompanying titration was measured after each injection. The total observed heat effects were corrected for the heat of dilution of the ligand by performing control titrations in the buffer used for dissolving the protein. The resulting titration curve was deconvoluted for the best-fit model using Titration BindWork in the ITC (CSC) to obtain the affinity constant and the number of binding sites.

RESULTS

Hn-33 Induced Endopeptidase Activity of BoNT/A and BoNT/E in Vitro. Because SNAP-25 is the substrate for both BoNT/A and BoNT/E (*9*, *17*, *18*), endopeptidase activities of BoNT/A and BoNT/E were analyzed for their ability to proteolytically cleave recombinant GST/SNAP-25 fusion protein. The activity was determined by estimating the uncleaved SNAP-25 by Western blot analysis with a polyclonal antibody raised against the C-terminal 12 amino acid

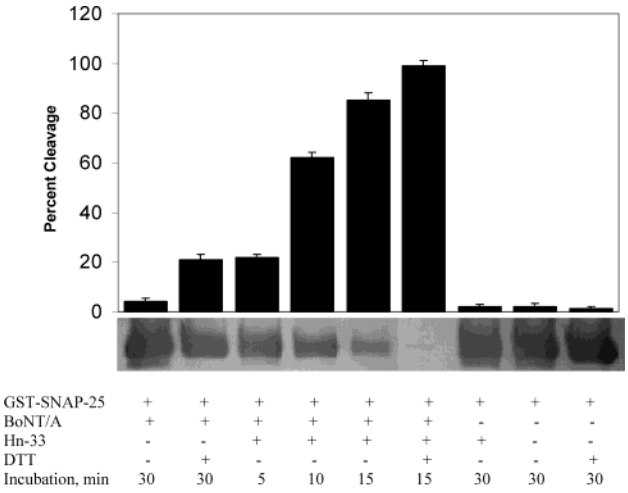

FIGURE 1: Comparative analysis of the endopeptidase activity of BoNT/A in the presence or absence of Hn-33. GST/SNAP-25 fusion protein (5 μM) was incubated with pure BoNT/A (200 nM) in the presence or absence of Hn-33 (1:1 molar ratio) for 30 min at 37 °C in an assay buffer. Samples were then separated by 12% SDS−PAGE and analyzed by Western blot using a polyclonal antibody raised against the C-terminal 12 amino acid residues of SNAP-25 (Stressgen Biotechnologies Corp.) as detailed in the Materials and Methods. The results were plotted by averaging the percentage of GST/SNAP-25 cleaved in three independent sets of experiments.

residue of SNAP-25. Under nonreducing conditions, 200 nM of pure BoNT/A exhibited negligible residual endopeptidase activity (4 ± 1% cleavage, $n = 3$), whereas BoNT/A, when reduced with 20 mM DTT, exhibited a 5.5-fold increase in its endopeptidase activity (22 ± 2% cleavage, $n = 3$). Preincubation of nonreduced BoNT/A with Hn-33 enhanced the endopeptidase activity by 21-fold (85 ± 2%, $n = 3$), while preincubation of reduced BoNT/A separately with Hn-33 enhanced its endopeptidase activity by 25-fold (99 ± 1%, $n = 3$) within 15 min of the reaction time at 37 °C (Figure 1, Table 1).

Similar to BoNT/A, under nonreducing conditions, 200 nM of pure BoNT/E exhibited minimal residual endopeptidase activity (2 ± 1% cleavage, $n = 3$), whereas, when reduced with 20 mM DTT, it exhibited a substantial (5-fold) increase in the endopeptidase activity (10 ± 1% cleavage, $n = 3$) against SNAP-25. (Figure 2, Table 2). Preincubation of nonreduced BoNT/E with Hn-33 enhanced the endopeptidase activity by 25-fold (50 ± 1% cleavage, $n = 3$), while preincubation of reduced BoNT/E with Hn-33 enhanced the endopeptidase activity by 43-fold (85 ± 2% cleavage, $n = 3$) within 15 min of the reaction time at 37 °C.

We performed a series of control experiments to exclude the possibility of SNAP-25 cleavage by Hn-33 itself. When Hn-33 itself was incubated with SNAP-25, no cleavage of the latter was observed. Two other proteins isolated from type E *C. botulinum*, a 120 kDa neurotoxin binding protein (*5*) and a 70 kDa protein (19), were used in place of Hn-33 to validate the assay of BoNT endopeptidase activity through the ELISA method used in this paper. No endopeptidase activity (cleavage of SNAP-25) was observed in these control experiments. Also, DTT by itself did not induce any cleavage of SNAP-25 (Figure 1).

To confirm the above cleavage results, the SNAP-25 cleavage by a range of BoNT/A or BoNT/E concentrations

Table 1: Comparison of SNAP-25 Cleavage by BoNT/A or BoNT/E with and without Hn-33 under Nonreduced and Reduced Conditions[a]

	percent cleavage							
	nonreduced BoNT		nonreduced BoNT + Hn-33		reduced BoNT		reduced BoNT + Hn-33	
neurotoxin	Western blot	ELISA	Western blot	ELISA	Western blot	ELISA	Western blot	ELISA
BoNT/A	4 ± 1	5 ± 2	85 ± 2	92 ± 2	22 ± 2	25 ± 1	99 ± 1	97 ± 4
BoNT/E	2 ± 1	4 ± 3	50 ± 2	52 ± 1	10 ± 1	15 ± 1	85 ± 1	89 ± 2

[a] The percent cleavage was monitored by two methods, Western blot and ELISA, and represents the average of three individual independent experiments (data derived from Figures 1−3).

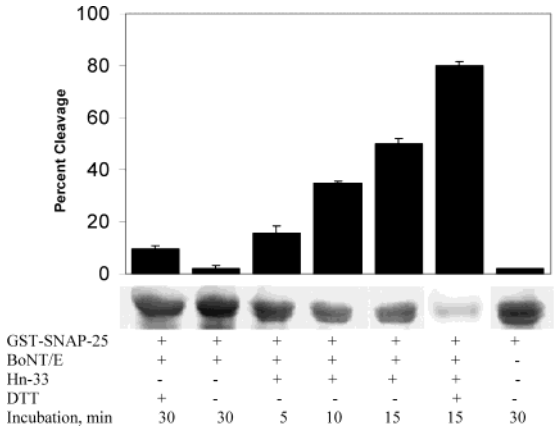

FIGURE 2: Comparative analysis of the endopeptidase activity of BoNT/E in the presence or absence of Hn-33. GST/SNAP-25 fusion protein (5 μM) was incubated either with pure BoNT/E (200 nM) in the presence or absence of Hn-33 (1:1 molar ratio) for 30 min at 37 °C in an assay buffer. Other experimental details were the same as those in Figure 1.

Table 2: Initial Reaction Rates of SNAP-25 Cleavage by Nonreduced and Reduced BoNT/A and BoNT/E with and without Hn-33[a]

	initial reaction rate (μM/min) of SNAP-25 cleaved			
neurotoxin	nonreduced BoNT	nonreduced BoNT + Hn-33	reduced BoNT	reduced BoNT + Hn-33
BoNT/A	3.9	37.0	12.3	47.8
BoNT/E	2.4	15.0	7.5	21.0

[a] The initial reaction rate (μM/min) was determined from the slope of the linear portion (4 min) of the SNAP-25 cleavage versus the time plots (Figure 5).

was determined by an ELISA method using SNAP-25 C-terminal polyclonal antibody, as described in the Materials and Methods section. Under a nonreducing condition, 200 nM of pure BoNT/A exhibited negligible residual endopeptidase activity (5 ± 2% cleavage, $n = 3$), whereas under reducing conditions it exhibited a 5-fold increase in its endopeptidase activity (25 ± 1% cleavage, $n = 3$). Preincubation of nonreduced BoNT/A with Hn-33 enhanced the endopeptidase activity by 18-fold (92 ± 2% cleavage, $n = 3$), while preincubation of reduced BoNT/A with Hn-33 enhanced its endopeptidase activity by 19-fold (97 ± 4% cleavage, $n = 3$) (parts A and B of Figure 3, Table 1).

In case of BoNT/E, under nonreducing condition, 200 nM of pure BoNT/E exhibited negligible residual endopeptidase activity (4 ± 3% cleavage, $n = 3$), whereas BoNT/E, when reduced with 20 mM DTT, exhibited a 4-fold increase in its endopeptidase activity (15 ± 2% cleavage, $n = 3$). Preincubation of nonreduced BoNT/E with Hn-33 enhanced the endopeptidase activity by 14-fold (52 ± 1% cleavage, $n = 3$), while preincubation of reduced BoNT/E with Hn-33 enhanced its endopeptidase activity by 25-fold (89 ± 2% cleavage, $n = 3$) (parts C and D of Figure 3, Table 1).

While the comparison is made at 200 nm BoNT concentrations, similar cleavage results were observed throughout the concentration range of BoNT used (Figure 3; Table 1). The ELISA results agree with the Western blot results.

Hn-33 Induced Endopeptidase Activity of BoNT/A and BoNT/E in Synaptosomes. To evaluate the physiological significance of BoNT endopeptidase activity enhanced by Hn-33, we compared the Hn-33-induced endopeptidase activity of BoNT/A and BoNT/E against SNAP-25 using rat-brain synaptosomes. Under nonreducing conditions, 200 nM of BoNT/A and BoNT/E each cleaved 6 ± 4% ($n = 3$) and 4 ± 2% ($n = 3$), respectively, of SNAP-25 at 37 °C after a 4-h reaction time. Reduced BoNT/A (200 nM) cleaved 20 ± 5% ($n = 3$) of SNAP-25 under reaction conditions identical to those listed above. Preincubation of nonreduced BoNT/A with Hn-33 at equimolar concentrations (BoNT/A:Hn-33, 1:1) enhanced the endopeptidase activity of BoNT/A over 13-fold (78 ± 5%, $n = 3$) (Figure 4). Incubation of BoNT/A with Hn-33 at a higher molar ratio (BoNT/A:Hn-33, 1:2) did not have any significant additional effect on the endopeptidase activity (79 ± 3% SNAP-25 cleavage, $n = 3$) compared to the effect of the equimolar concentration of Hn-33 on BoNT/A (Figure 4). Reduction of the disulfide bond of BoNT/E (200 nM) enhanced its endopeptidase activity substantially (4-fold) as indicated by 15 ± 7% cleavage of SNAP-25 ($n = 3$) at 37 °C after a 4-h reaction time. Preincubation of nonreduced BoNT/E with Hn-33 at equimolar concentrations (BoNT/E:Hn-33, 1:1) enhanced the endopeptidase activity of BoNT/E by over 13-fold (53 ± 8% cleavage, $n = 3$) (Figure 4).

In a preliminary effort to evaluate the influence of Hn-33 on the kinetics of BoNT endopeptidase activity, initial rates of the endopeptidase activity of BoNT/A and BoNT/E were determined by estimating the percent cleavage of SNAP-25 at different time intervals. The time course of SNAP-25 cleavage, obtained with the ELISA method, showed a plateau within 30 min of the reaction time (Figure 5). However, linearity of the curve was observed only for the first 4 min of the reaction time, especially for reactions where no Hn-33 was added. We, therefore, used the slope of the linear portion of the curve within the first 4 min of the reaction time to estimate the initial reaction rates catalyzed by BoNT endopeptidase. Under nonreducing conditions, BoNT/A and BoNT/E showed initial reaction rates of 3.9 and 2.4 μM/min, respectively. Under reducing conditions, initial reaction rates for the endopeptidase activity of BoNT/A and BoNT/E were 12.3 and 7.5 μM/min, respectively. Preincubation of nonreduced BoNT/A and BoNT/E separately with Hn-33 at an equimolar concentration (BoNT:Hn-33, 1:1) enhanced the rates of their endopeptidase activity by

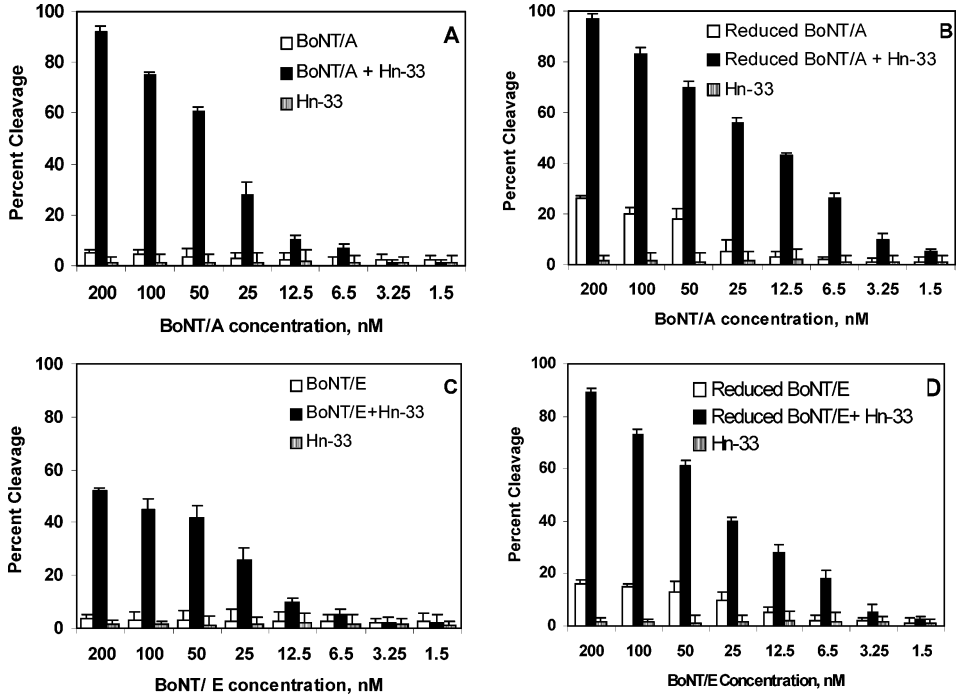

FIGURE 3: Cleavage of GST/SNAP-25 by nonreduced BoNT/A (A), reduced BoNT/A (B), nonreduced BoNT/E (C), and reduced BoNT/E (D) as analyzed by ELISA. Plates were coated with 10 μg/mL of GST/SNAP-25 and were incubated overnight at 4 °C. Nonreduced BoNT/A or BoNT/E was preincubated with or without Hn-33 (1:1 molar ratio) for 30 min at 37 °C before addition to the plates. Anti-SNAP-25 rabbit antibody (3 ng/mL) was added to the plates. Peroxidase-labeled anti-rabbit antibody was used as a secondary antibody as described in the Materials and Methods.

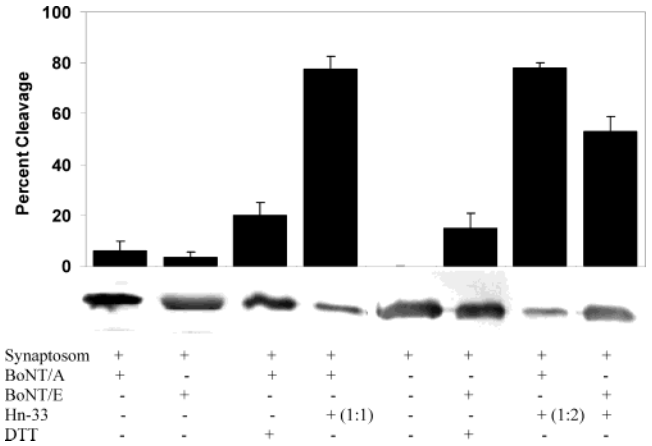

FIGURE 4: Comparative analysis of the endopeptidase activity of BoNT/A or BoNT/E in the presence and absence of Hn-33 on the level of SNAP-25 of rat-brain synaptosomes under nonreducing conditions. Synaptosomes (50 μL) in Hepes buffer were incubated with 200 nM (final concentration) nonreduced BoNT/A with 1:1 or 1:2 (BoNT/A−Hn-33) or BoNT/E with 1:1 (BoNT/E−Hn-33) at 37 °C for 4 h. The samples were then analyzed by 12% SDS−PAGE and were immunoblotted using anti-SNAP-25 antibody as described in the Materials and Methods. The results were plotted by averaging the percentage of SNAP-25 cleaved in three independent sets of experiments.

9.4- and 6.3-fold, respectively, with cleavage rates of 37 (BoNT/A) and 15 μM/min (BoNT/E). Preincubation of reduced BoNT/A or BoNT/E with Hn-33 at an equimolar concentration (BoNT:Hn-33, 1:1) enhanced the rates of their endopeptidase activity by 12.2- and 8.7-fold, respectively, with cleavage rates of 47.8 (BoNT/A) and 21 μM/min (BoNT/E) (Figure 5, Table 2).

Physical Interaction of Hn-33 with BoNT/A. Interaction of Hn-33 with BoNT/A was examined with isothermal calorimetry by measuring heat changes upon titration of the BoNT/A solution with Hn-33, both dissolved in a 10 mM phosphate buffer at pH 7.4. Figure 6 shows a typical titration curve. The concentration range chosen provided a very smooth curve of steady heat change with every injection until injection 15, after which it reached a saturation level (Figure 6A). The saturation level was reached at a molar ratio of 10:1 (Hn-33−BoNT/A). Considering that Hn-33 is known to exist as a dimer (*20*), this set of data suggests five Hn-33 dimeric molecules bound to one BoNT/A. Interestingly, the molar ratio of Hn-33 to BoNT/A in the BoNT/A complex was found to be 8:1 based on SDS−PAGE analysis (*21*). It is possible that in the absence of other NAPs Hn-33 is able to have more freedom to bind to BoNT/A thus leading to a ratio of 10:1. The number of binding sites estimated from the curve was 1.0, suggesting that the binding sites belong to a class of similar affinity.

The interaction between Hn-33 and BoNT/A is exothermic (Figure 6A), and ΔH estimated at -120 kJ mol^{-1} from the binding curve (Figure 6B) is within the typical range of strong protein−protein interactions (*22*, *23*). The binding constant was estimated to be 4.32×10^{-5} M^{-1}, which is within the range of interactions between specific proteins (*24*).

DISCUSSION

Although the discovery of endopeptidase activity of BoNT in 1992 (*10*) has opened up many avenues to the understanding of the biochemical mechanism of its toxic action, the endopeptidase activity itself has remained a subject of intense research to understand its unique characteristics (*2*, *25*, *26*). Among its unique features, it has been reported that reduction of the disulfide bond between the light and heavy chain is required for the endopeptidase activity (*9*, *10*). The active

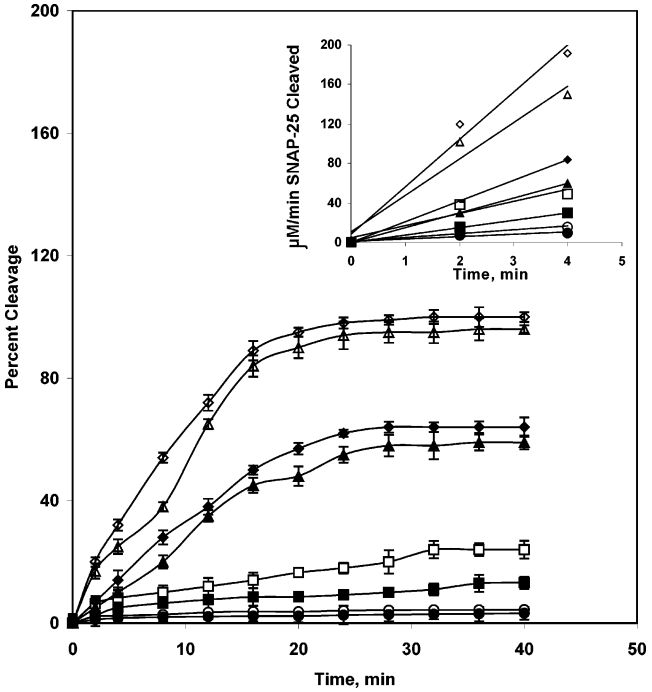

FIGURE 5: Time course of SNAP-25 cleavage by BoNT/A or BoNT/E in the presence or absence of Hn-33 analyzed by ELISA. Plates were coated with 10 μg/mL of GST/SNAP-25 and incubated overnight at 4 °C. Nonreduced BoNT/A or BoNT/E was preincubated with or without Hn-33 (1:1 molar ratio) for 30 min at 37 °C prior to addition to the plates. Nonreduced BoNT/A (○) Nonreduced BoNT/E (●), Reduced BoNT/A (□), Reduced BoNT/E (■), Nonreduced BoNT/A when preincubated with Hn-33 (△), Nonreduced BoNT/E when preincubated with Hn-33 (▲), Reduced BoNT/A when preincubated with Hn-33 (◇), Reduced BoNT/E when preincubated with Hn-33 (◆). For reducing conditions, BoNT/A and BoNT/E were prepared by pretreatment with 20 mM DTT for 30 min at 37 °C. Peroxidase-labeled anti-rabbit antibody was used as a secondary antibody as described in the Materials and Methods.

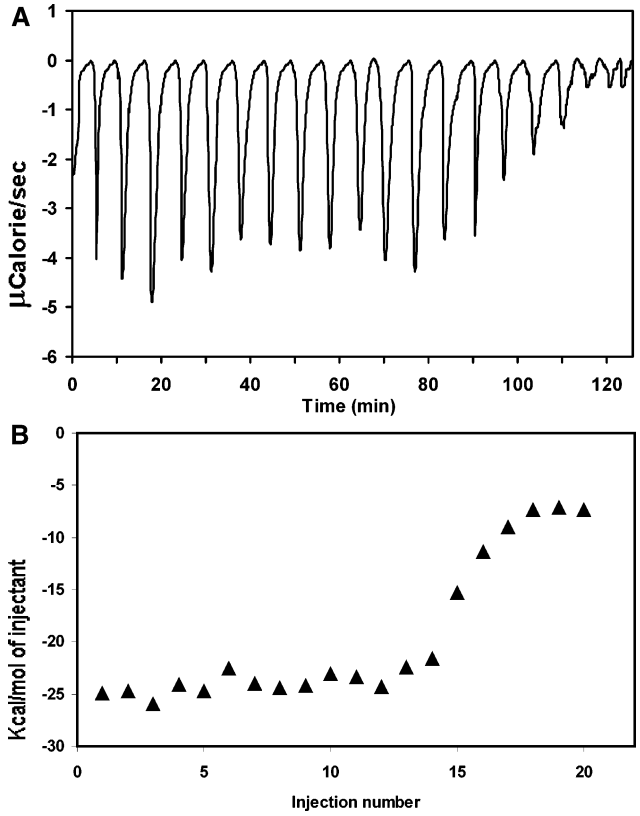

FIGURE 6: Typical isothermal calorimetric titration curve for BoNT/A with Hn-33. Sequential injections of 20 μL of Hn-33 ligand (concentration = 30 μM) was mixed with 1.3 mL of 0.7 μM BoNT/A at 400-s intervals in a reaction cell. The titration was performed at 25 °C in a 10 mM phosphate buffer at pH 7.4. (A) Total heat released is plotted in terms of microcalories per second versus time. The area underneath each injection peak is equal to the total heat released for that injection. (B) Integrated areas of the heat-release peaks in A plotted against the injection number. The solid line corresponds to the best-fit curve obtained by a nonlinear regression.

Zn^{2+} plays both a catalytic and structural role (*27*). In the presence of NAPs, there is no requirement of disulfide reduction for the endopeptidase activity (*13*) and the dimeric form of the BoNT has a lower enzymatic activity than the monomeric form (*28*). Furthermore, except for BoNT/C1, the seven serotypes of BoNT recognize only one specific protein substrate and selective cleavage site (*2*, *29*).

It is important to examine the structural basis of unique characteristics of BoNT endopeptidases. Understanding the molecular basis of the unique endopeptidase activity of BoNT is not only likely to provide clues to the mechanistics of the enzyme activity, but the information could lead to the development of antidotes to the most poisonous poison.

A significantly enhanced endopeptidase activity of BoNT/A in the presence of Hn-33 isolated from the BoNT/A complex was observed (Table 1) even under conditions where the disulfide bond between the light and heavy chains of BoNT was not reduced. Hn-33 does not reduce the interchain disulfide bond between light and heavy chains of BoNT/A (*20*). The BoNT/A complex itself is known to have a drastically enhanced endopeptidase activity against SNAP-25 in solution without reducing the interchain disulfide bond of BoNT/A (*13*). Interestingly, the enhanced endopeptidase activity of BoNT/A in the presence of Hn-33 is virtually the same as the whole BoNT/A complex, both quantitatively and qualitatively. In qualitative terms, the Hn-33-enhanced endopeptidase activity of BoNT/A did not require reduction of the disulfide bond (Figure 1, Table 1), just like the BoNT/A complex (*13*). It may be noted here that the disulfide bond of BoNT/A remains intact in the native complex form as well as in a complex with Hn-33 (*4*, *13*). Quantitatively, Hn-33 enhanced the endopeptidase activity of nonreduced BoNT/A by 21-fold for a 15-min reaction period, whereas BoNT/A complex is reported to exhibit endopeptidase activity 17-fold higher than nonreduced pure BoNT/A for a 10-min reaction period (*13*). Thus, Hn-33 seems to be able to imitate the presence of all of the NAPs in the BoNT/A complex with respect to the enhancement of the endopeptidase activity.

In the BoNT/A complex, there are five NAPs including Hn-33 (*13*); Hn-33 makes up the largest fraction (25%) of the NAPs (*20*) in the BoNT/A complex. Furthermore, Hn-33 strongly protects BoNT/A against proteases such as trypsin, α-chymotrypsin, subtilisin, etc. (*4*). These observations are consistent with the effect of Hn-33 on the BoNT/A endopeptidase activity and strongly suggest that Hn-33 directly interacts with BoNT/A in the BoNT/A complex.

Although the mechanism by which Hn-33 may enhance the BoNT/A endopeptidase activity is not discernible from the current data, a possible explanation derived from the known X-ray crystallographic structure of BoNT/A is as

follows: The active site of BoNT/A is known to be buried in a crevice about 24-Å deep, which is occluded by a 56 amino acid residue (residues 490−545) belt (*30*, *31*). It has been suggested that disulfide reduction opens up the belt and exposes the active site to the surface of the protein for its binding with the substrate peptide groups (*31*). We believe that the interaction of Hn-33 exposes the enzyme active site without any need to open up the belt. However, reduction of the disulfide bond and thus opening of the belt can further expose the active site to the substrate, as indicated by an approximately 17% additional increase in the endopeptidase activity of BoNT/A (Table 1). Exposure of the active site upon interaction with Hn-33 becomes more plausible in view of a recent report (*32*) suggesting the enzymatically active structure of BoNT/A in the form of a molten globule. A molten-globule-folded structure is significantly more flexible than a native-folded structure (*33*, *34*).

Interestingly, although Hn-33 was isolated from the BoNT/A complex (*14*), its effect appears common to all of the BoNT serotypes. This was demonstrated by a similar experiment of the endopeptidase activity of BoNT/E and BoNT/A by Hn-33 (Table 1). The Hn-33 effect on BoNT/E is especially noteworthy, because the latter does not have a comparative NAP in its complex form (*19*). Therefore, a similar effect of Hn-33 on the two serotypes of BoNT suggests a common mechanism involved in the accessibility of the active site of BoNTs to their respective substrates. Moreover, there must be common structure motifs of the surface of the BoNT/A and BoNT/E molecules. While the three-dimensional structure of BoNT/E is not yet solved, common structural motifs are well-known for BoNT/A and BoNT/B (*35*−*37*). Future work with the crystal structure of Hn-33 and the BoNT complex would be required to confirm the common structural motif on the BoNT surface for the interaction with Hn-33.

Molecular basis of Hn-33-enhanced BoNT endopeptidase activity can be discerned from an additional set of published data, related to the Hn-33 protection of BoNT/A from proteases (*4*). Hn-33 can completely protect BoNT/A from pepsin, trypsin, α-chymotrypsin, and subtilisin, suggesting Hn-33 either surrounds the protease cleavage sites on BoNT/A or introduces refolding in BoNT/A so that the cleavage sites become inaccessible. The dissociation constant of 0.4 μM derived from isothermal calorimetry experiments of BoNT/A and Hn-33 indicates a strong binding between the two proteins capable of introducing polypeptide refolding in BoNT/A. This is especially likely given a single binding site ($n = 1.0$) obtained from the isothermal titration curve (Figure 6). While it is possible that only one binding site is involved between BoNT/A and Hn-33, more than one binding sites could also lead to $n = 1$, as long as different binding sites have similar binding affinities. More than one binding site is also likely considering two dimers of Hn-33 bind to BoNT/A (*14*, *20*, *21*).

Each of the seven serotypes of BoNT has a group of associated NAPs, whose biological roles are not clearly understood. NAPs are known to protect the toxin from adversarial environmental conditions such as temperature, acidity, and proteases of the gastric juice (*3*, *38*, *39*). It is notable that Hn-33 represents the largest fraction of BoNT/A NAPs (*14*) and accounts for most of the hemagglutinin activity of the BoNT/A complex (*28*). Hn-33 by itself can protect BoNT/A from proteases (*4*). Thus, the influence of Hn-33 on the endopeptidase activity of BoNT/A and BoNT/E is consistent with the Hn-33 effect on other biological and physical features of BoNT/A and its complex with the NAPs. Hn-33 seems to imitate the role of all NAPs in the BoNT/A complex.

Comparison of SNAP-25 cleavage by BoNT/A and BoNT/E in the presence and absence of Hn-33 in synaptosomes representing nerve cell conditions (Figure 4) indicated the following: (a) SNAP-25 cleavage by both BoNT/A and BoNT/E is less in synaptosomes compared to the in vitro conditions (Table 1, Figure 4). (b) Hn-33 enhanced the endopeptidase activity of both BoNT/A and BoNT/E even in synaptosomes, albeit to a lesser degree (Table 1, Figure 4). These results suggest that the neurotoxin complex with Hn-33 enters the synaptosome and the lower cleavage of SNAP-25 (in comparison to in vitro conditions) is likely to result from the inaccessibility of SNAP-25 in the synaptosome. We have recently made an observation (Y. Zhou and B. R. Singh, unpublished data) that Hn-33 binds to synaptosomes through synaptotagmin, which will further support the possibility of Hn-33 entry into synaptosomes. These results are particularly significant to the possibility of the use of the BoNT complex with Hn-33 as a therapeutic agent. The entrance of Hn-33 and BoNT as a complex will enhance and stablize the endopeptidase activity inside the neuronal cell. Stable endopeptidase activity inside the neuronal is a critical phenomenon for the long lasting effects of botulinum either as a toxin or as a therapeutic agent (*40*, *41*).

In summary, our results show that (i) the Hn-33 component of BoNT/A NAPs is able to imitate the effect of all of the NAPs in enhancing the endopeptidase activity of BoNT/A; (ii) the Hn-33 of the BoNT/A complex is also able to enhance the endopeptidase activity of BoNT/E, suggesting a common structural motif of BoNT serotypes for Hn-33; and (iii) Hn-33 bound to BoNT/A and BoNT/E enhanced the endopeptidase activity in synaptosomes, suggesting a possible entry of Hn-33 and BoNT inside the synaptosome.

ACKNOWLEDGMENT

This work was in part supported by a U.S. Army Medical Research and Material Command under Contract No. DAMD17-02-C-001 and by National Institutes of Health through New England Center of Excellence for Biodefense (AI057159-01).

REFERENCES

1. Simpson, L. L. (1989) in *Botulinum Neurotoxin and Tetanus Toxins* (Simpson, L. L., Ed.) pp 153−178, Academic Press, San Diego, CA.
2. Li, L., and Singh, B. R. (1999) *J. Toxicol., Toxin Rev. 18*, 95−112.
3. Sakaguchi, G. (1983) *Pharmacol. Ther. 19*, 165−194.
4. Sharma, S. K., and Singh, B. R. (1998) *J. Nat. Toxins 7*, 239−253.
5. Singh, B. R., Foley, J., and Lafontaine, C. (1995) *J. Protein Chem. 14*, 7−18.
6. Middlebrook, J. L. (1989) in *Botulinum Neurotoxin and Tetanus Toxin* (Simpson, L. L., Ed.) pp 95−119, Academic Press, San Diego, CA.
7. Zilinskas, R. A. (1997) *J. Am. Med. Assoc. 278*, 418−424.
8. Singh, B. R., Li., B., and Read, D. (1995) *Toxicon 33*, 1541−1547.

9. Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., DeCamilli, P., Sudhof, T., Niemann, H., and Jahn, R. (1993) *Nature 365*, 160−163.
10. Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., de Laureto, P., DasGupta, B. R., and Montecucco, C. (1992) *Nature 359*, 832−835.
11. Johnson, E. A. (1999) *Annu. Rev. Microbiol. 53*, 551−75.
12. Schantz, E. J., and Johnson, E. A. (1992) *Microbiol. Rev. 56*, 80−99.
13. Cai, S., Sarker, H. K., and Singh, B. R. (1999) *Biochemistry 38*, 6903−6910.
14. Fu, F.-N., Sharma, S. K., and Singh, B. R. (1998) *J. Protein Chem. 17*, 53−60.
15. Dasgupta, B. R., and Sathyamoorthy, V. (1984) *Toxicon 22*, 415−424.
16. Li, L., and Singh, B. R. (1999) *J. Protein Chem. 18*, 89−95.
17. Schiavo, G., Rossetto, O., Catsicas, S., de Laureto, P., P., DasGupta, B. R., Benfenati, F., and Montecucco, C. (1993) *J. Biol. Chem. 268*, 23784−23787.
18. Schiavo, G., Santucci, A., Dasgupta, B. R., Mehta, P. P., Jontes, J., Benfenati, F., Wilson, M. C., and Montecucco, C. (1993) *FEBS Lett. 335*, 99−103.
19. Zhang, Z., and Singh, B. R. (1995) *Protein Sci. 4* (suppl 2), 110.
20. Sharma, S. K., Fu, F. N., and Singh, B. R. (1999) *J. Protein Chem. 18*, 29−38.
21. Inoue, K., Fujinaga, Y., Watanabe, T., Ohyama, T., Takeshi, K., Moriishi, K., Nakajima, H., Inoue, K., and Oguma, K. (1996) *Infect. Immun. 64*, 1589−1594.
22. Hibbits, K. A., Gill, D. S., and Willson, R. C. (1994) *Biochemistry 33*, 3584−3590.
23. Pierce, M. M., Raman, C. S., and Nall, B. T. (1999) *Methods 19*, 213−221.
24. Li, J., and Weis, R. M. (1996) in *Techniques in Protein Chemistry* (Marshak, D., Ed.) Vol. 7, pp 33−44, Academic Press.
25. Montecucco, C., and Schiavo, G. (1993) *Trends Biochem. Sci. 9*, 324−327.
26. Singh, B. R. (2002) in *Scientific and Therapeutic Aspects of Botulinum Toxin* (Brin, M. F., Jankovic, J., and Hallett, M., Eds.) pp 75−88, Lippincott and Wilkins, Philadelphia, PA.
27. Fu, F. N., Lomneth, R. B., Cai, S., and Singh, B. R. (1998) *Biochemistry 37*, 5267−5278.
28. Cai, S., and Singh, B. R. (2001) *Biochemistry 40*, 4693−4702.
29. Blasi, J., Chapman, E. R., Yamasaki, S., Binz, T., Niemann, H., and Jahn, R. (1993). *EMBO J. 12*, 4821−4828.
30. Lacy, D. B., Tepp, W., Cohen, A. C., DasGupta, B. R., and Stevens, R. C. (1998) *Nat. Struct. Biol. 10*, 898−902.
31. Lacy, D. B., and Stevens, R. C. (1999) *J. Mol. Biol. 291*, 1091−1104.
32. Cai, S., and Singh, B. R. (2001) *Biochemistry 40*, 15327−15333.
33. Christensen, H., and Pain, R. H. (1991) *Eur. Biophys. J. 19*, 221−229.
34. Nakagawa, S. H., and Tager, H. S. (1993) *Biochemistry 32*, 7237−7243.
35. Swaminathan, S., and Eswaramoorthy, S. (2000) *Nat. Struct. Biol. 7*, 693−699.
36. Hanson, M. A., and Stevens, R. C. (2000) *Nat. Struct. Biol. 7*, 687−690.
37. Singh, B. R. (2000) *Nat. Struct. Biol. 7*, 617−619.
38. Inoue, K., Fujinaga, Y., Honke, K., Yokota, K., Ikeda, T., Ohyama, T., Takeshi, K., Watanabe, T., Inoue, K., and Oguma, K. (1999) *Microbiology 145*, 2533−2542.
39. Fujinaga, Y., Inoue, K., Nomura, T., Sasaki, J., Marvaud, J. C., Popoff, M. R., Kozaki, S., and Oguma, K. (2000) *FEBS Lett. 467*, 179−183.
40. Keller, J. E., Neale, E. A., Oyler, G., and Adler, M. (1999) *FEBS Lett. 456*, 137−142.
41. Foran, P. G., Mohammed, N., Lisk, G. O., Nagwaney, S., Lawrence, G. W., Johnson, E., Smith, L., Aoki, K. R., and Dolly, J. O. (2003) *J. Biol. Chem. 278*, 1363−1371.

BI0355544