Role of the Disulfide Cleavage Induced Molten Globule State of Type A Botulinum Neurotoxin in Its Endopeptidase Activity†

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ABSTRACT: Botulinum neurotoxins are produced by anaerobic *Clostridium botulinum* in an inactive form. The endopeptidase activity of type A botulinum neurotoxin (BoNT/A) is triggered by reduction of its disulfide bond between its heavy chain and light chain. By using circular dichroism spectroscopy, we show that, upon reduction of BoNT/A and under physiological temperature (37 °C), the BoNT/A loses most of its native tertiary structure, while retaining most of its secondary structure. This type of structure is characterized as a molten globule type conformation, which was further confirmed for BoNT/A by the characteristic binding of 1-anilinonaphthalene-8-sulfonic acid. Under nonreducing conditions where the interchain disulfide bond is intact, the enzymatically inactive BoNT/A did not show a molten globule type of structure. A temperature profile of the structure and enzyme activity of BoNT/A revealed that, under reducing conditions, there was a strong correlation in the existence of the molten globule structure and optimum endopeptidase activity at about 37 °C.

Botulinum neurotoxins (BoNTs)[1] are highly potent toxins which inhibit neurotransmitter release from peripheral cholinergic synapses (*1*, *2*). They are secreted by *Clostridium botulinum* and are responsible for the neuroparalytic botulism syndrome characterized by flaccid muscle paralysis (*3*). The structurally related family of BoNTs consists of seven different serotypes (A−G) (*1*). BoNTs are synthesized as single polypeptide chains of approximately 150 kDa each, which are in a pretoxin form without much biological activity (*4*, *5*). The single chain pretoxin form of BoNTs is then cleaved endogenously or exogenously, resulting in a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) linked through a disulfide bond (*4*, *5*). The 150 kDa neurotoxin comprises of three functional domains related to its three-step mode of action (*6*, *7*): the C-terminal half of the HC responsible for receptor-mediated extracellular binding and internalization, the N-terminal half of the HC responsible for the membrane translocation to deliver the toxin into the cytosol of the neuronal cell, and the LC of the BoNTs responsible for the intracellular blockage of acetylcholine release through its endopeptidase activity on one of the three SNARE (soluble NSF attachment protein receptor) proteins, SNAP-25 (synaptosomal-associated protein of 25 kDa), syntaxin, and VAMP/synaptobrevin (*8*−*13*).

The dichain form of BoNTs behaves like a preenzyme (*5*). The enzyme active site of the LC is the zinc binding motif (HEXXH), which is the consensus sequence of zinc endopeptidases (*14*). Reduction of the disulfide bond between the HC and LC of BoNTs has been identified as the rate-limiting step during the toxification of BoNTs (*15*). However, the free thiol group itself does not play any role in the intracellular process (*16*), even though the reduction of the interchain disulfide bond is required for the endopeptidase activity of BoNTs (*8*−*13*, *17*).

In recent years, substantial progress has been made in defining the molecular basis of the action mode of BoNTs (*17*−*21*). The crystal structure of the preenzyme form of BoNT/A and BoNT/B (nonreduced form, leaving the disulfide bond intact) also has been resolved (*22*−*24*). On the basis of the X-ray crystallography of BoNT/A and BoNT/B under conditions where their respective disulfide bonds remain intact, the active site of BoNT/A is buried 20−24 Å deep in the protein and is partially shielded by the belt and main body of the N-terminal domain of the HC (*22*). In BoNT/B, the active site is buried about 16 Å, and the belt from the N-terminal domain of the HC does not shield the active site of the protein (*23*). This kind of structural organization may prevent BoNTs from accessing their protein substrates under nonreducing conditions. The structural information of BoNTs revealed from spectroscopic methods suggests that reduction of the disulfide bond results into a more flexible structure (*17*, *18*).

In this report, we present results of our investigation of the activation mechanism of BoNT/A by reduction of the interchain disulfide bond. We observed that, under physiological temperature conditions, the reduced BoNT/A exists in a molten globule state; the nonreduced BoNT/A, on the

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[1] Abbreviations: BoNT, botulinum neurotoxin; BoNT/A, botulinum neurotoxin type A; DTT, dithiothreitol; CD, circular dichroism; ANS, 1-anilinonaphthalene-8-sulfonic acid; SNAP-25, synaptosomal-associated protein of 25 kDa.

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other hand, does not have this state. Examination of their biological activity suggests that the molten globule state of BoNT/A under reducing conditions is the enzymatically active structure.

MATERIALS AND METHODS

Botulinum Neurotoxin Preparation. Botulinum neurotoxin type A was purified from the cultures of *C. botulinum* strain Hall according to the methods described previously (*25*). The purity of BoNT/A was checked with sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis. Pure BoNT/A showed only one single band at approximately the 145 kDa position on a 12% SDS−polyacrylamide gel under nonreducing condition. Pure toxin sample was then dialyzed against 50 mM Tris buffer, pH 7.2. Reduced toxin was prepared by treating it with 20 mM dithiothreitol (DTT) for 30 min at 37 °C. To remove the excess DTT, the sample was then dialyzed against 50 mM Tris buffer, pH 7.2, containing 1 mM DTT. The concentration of BoNT/A was determined by using the published extinction coefficient at 280 nm (*25*).

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were recorded with a Jasco J715 spectropolarimeter. The cuvettes used for far-UV and near-UV CD spectral recordings had path lengths of 0.1 and 1.0 cm, respectively. The scan speed was 20 nm min^{-1}, and the response time was fixed at 8 s. The concentrations of toxin samples in the range of 0.1−0.3 mg/mL and 0.5−1 mg/mL were used for far-UV and near-UV CD measurements, respectively. The CD spectra were recorded at 20 °C. In the near-UV CD region, a total of four scans were taken and averaged to increase the signal-to-noise ratio. In all CD experiments, the buffer blank was recorded separately and subtracted from the sample spectrum.

Thermal denaturation studies were performed by monitoring the CD signal at 222 and 280 nm, using 0.1 and 0.5 cm path-length cuvettes, respectively. The temperature was raised at a rate of 2 °C/min from 20 to 80 °C for the far-UV CD region and at a rate of 2 °C/min from 20 to 60 °C for near-UV CD region.

All CD experiments were repeated three times using different batches of BoNT/A.

ANS Binding. Interaction of the fluorescence dye 1-anilinonaphthalene-8-sulfonate (ANS) (Molecular Probes, Eugene, OR) with BoNT/A was analyzed by measuring the fluorescence emission of the protein-bound dye. Fluorescence was recorded on an ISS K2 fluorometer (Champaign, IL). A 10 mM ANS stock solution was prepared as follows. ANS was first dissolved into 100 μL of ethanol and was then diluted into 20 mM sodium phosphate buffer, pH 7.2. To determine the optimized binding ratio, ANS was titrated into 2 mL of 2 μM BoNT/A or reduced BoNT/A solution in a 1 cm path-length cuvette. Samples were gently stirred for 1 min at 25 °C using a stirring bar. To measure the emission spectra of ANS, the excitation wavelength was fixed at 370 nm, and the excitation and emission slits were fixed at 8 and 2 nm, respectively. Emission spectra were recorded between 400 and 540 nm. The final fluorescence intensity at any given ANS concentration was derived after correcting for the dilution effect and the blank signal of ANS fluorescence in the absence of protein. To examine the temperature-induced structural change in BoNT/A and reduced BoNT/A, the optimized binding ratio was used, and the ANS emission spectra were recorded at different temperatures (15−60 °C).

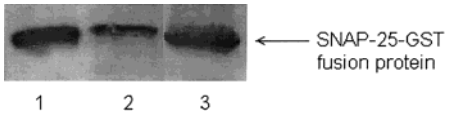

FIGURE 1: Comparison of proteolytic activities of BoNT/A under reducing and nonreducing conditions. Lanes: 1, SNAP-25−GST fusion protein control; 2, SNAP-25−GST fusion protein incubated with 100 nM BoNT/A at 37 °C for 1 h under reducing conditions; 3, SNAP-25−GST fusion protein incubated with 100 nM BoNT/A at 37 °C for 1 h under nonreducing conditions. The concentration of SNAP-25−GST fusion protein in all cases is 9 μM. The gel was analyzed by Western blot as described in Materials and Methods.

Endopeptidase Activity. The endopeptidase activity of BoNT/A was estimated using its intracellular target protein, synaptosomal-associated protein of 25 kDa (SNAP-25), as substrate. SNAP-25 used in our experiments was expressed as SNAP-25−glutathione *S*-transferase (GST−SNAP-25) fusion protein in *Escherichia coli* DH1αF′ strain (*17*). The GST−SNAP-25 fusion protein was purified according to the method described previously (*17*). To compare the endopeptidase activity of BoNT/A under reducing and nonreducing conditions, 9 μM GST−SNAP-25 fusion protein was incubated with 100 nM BoNT/A or reduced BoNT/A at 37 °C for 1 h. Samples were then separated by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The electrophoresis was run using a Hoefer SE 250 Mighty Small II Mini-Gel System (Amersham Pharmacia Biotech, Piscataway, NJ). The electrophoresis was run under a constant voltage of 200 V at room temperature (25 °C). The gel was then 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), followed by detection of the antibody−antigen complex using peroxidase-labeled secondary antibody and 4-chloro-1-naphthol as the substrate (*17*). 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 percentage of cleavage was calculated by comparing the density of the uncleaved band to that of control SNAP-25. To examine the temperature effect on the edopeptidase activty of BoNT/A, 5 μM GST−SNAP-25 fusion protein was incubated with 100 nM reduced BoNT/A at the designated temperature for 30 min. The cleavage was terminated by addition of SDS−PAGE sample buffer and analyzed using 12% SDS−PAGE and Western blot described as above.

RESULTS

Endopeptidase Activity of Reduced and Nonreduced BoNT/A. Figure 1 shows that only the reduced form of BoNT/A has endopeptidase activity, while the nonreduced form did not show any visible endopeptidase. This is consistent with the observations reported by other researchers in the literature (*13*).

Structural Difference between Reduced and Nonreduced BoNT/A. To investigate the mechanism of disulfide bond

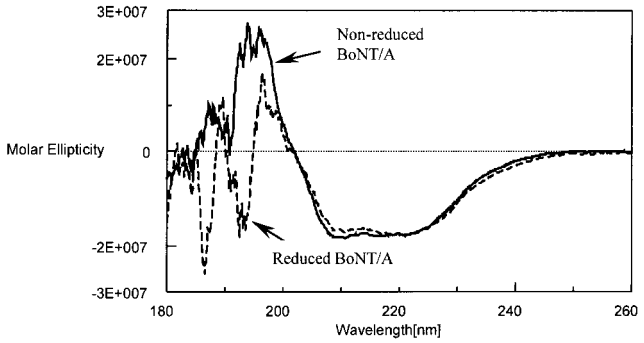

FIGURE 2: Far-UV CD spectra of nonreduced and reduced BoNT/A dissolved in 50 mM Tris buffer, pH 7.2. Spectral recordings were carried out at a speed of 20 nm/min with a response time of 8 s.

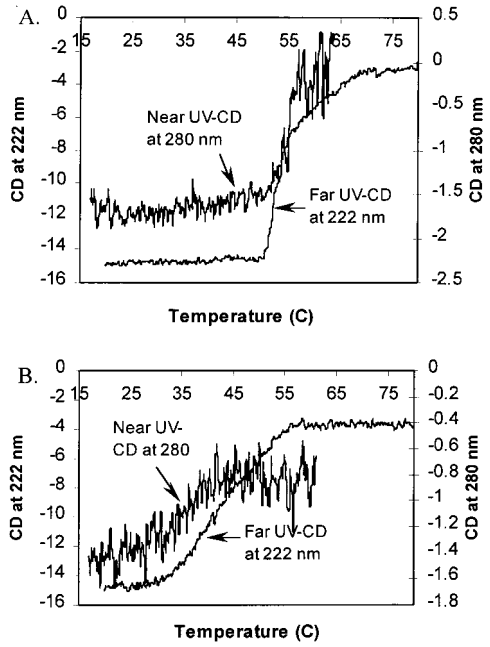

FIGURE 3: Thermal unfolding of BoNT/A in nonreduced (A) and reduced (B) forms. Samples were heated at a rate of 2 °C/min. The unfolding was monitored by continuously recording the CD signal at either 222 or 280 nm.

reduction, we examined structural differences between reduced and nonreduced BoNT/A. The secondary structure of reduced and nonreduced BoNT/A did not show any significant difference as revealed by far-UV circular dichroism (far-UV CD) spectroscopy (Figure 2). The changes seen in CD signals below 200 nm are likely to be artifactual due to strong absorbance by the buffer resulting in detector saturation. However, near-UV CD spectroscopy suggests a significant difference between reduced and nonreduced BoNT/A at the tertiary structural level (*17*).

To further understand the role of the disulfide bond, we analyzed the thermally induced unfolding process of BoNT/A, at both secondary and tertiary structural levels by monitoring CD signals at 222 and 280 nm as a function of temperature. Nonreduced BoNT/A shows a two-state unfolding transition (N to U) characterized by a steep transition at both secondary and tertiary structural levels, with a melting point (T_m) of 53 °C (Figure 3A). On the other hand, the reduced form of BoNT/A shows a dramatically different unfolding process (Figure 3B), exhibiting an expanded transition. The broad transition suggests a noncooperative unfolding, which would be consistent with a nonrigid structure. The T_m of reduced BoNT/A for the secondary structure was about 44 °C, indicating a relatively unstable structure of the reduced form of BoNT/A compared to its nonreduced form. The T_m of reduced BoNT/A for the tertiary structure was about 35 °C, which is about 9 °C lower than the T_m for the secondary structure. This observation suggested that there existed a state for reduced BoNT/A where the tertiary structure was already collapsed, while the secondary structure still remained mostly intact. To confirm this state, we extracted the CD spectra at 20 and 37 °C. For nonreduced BoNT/A, both far-UV and near-UV CD spectra did not indicate any significant change between 20 and 37 °C (Figure 4A,B). For reduced BoNT/A, while the secondary structure at 37 °C only shows a slight difference from that at 20 °C, the tertiary structure of BoNT/A is almost denatured at 37 °C, compared to that at 20 °C (Figure 4C,D). The presence of secondary structure and absence of tertiary structure are two of the characteristics of a molten globule type conformation. Therefore, it is strongly suggested that reduced BoNT/A exists in a molten globule state at 37 °C.

ANS has been used as a general probe for solvent-exposed hydrophobic clusters (*26*, *27*). Proteins with poorly packed interiors typically have a high affinity for ANS, the binding of which can be monitored spectroscopically (*27*). ANS also has been used to probe the molten globule structure of proteins (*28*). We investigated ANS binding properties of BoNT/A under reducing and nonreducing conditions. The binding of the apolar dye ANS to BoNT/A is associated with an enhanced fluorescence at 488 nm (Figure 5 inset). The ANS binding to reduced BoNT/A produced a higher fluorescence signal at 488 nm, in contrast to its binding with nonreduced BoNT/A, suggesting that ANS access to hydrophobic sites in reduced toxin is higher than that in the nonreduced toxin (Figure 5). The binding ratio of ANS to toxin under reducing and nonreducing conditions also showed a significant difference, while the ANS to protein molar ratio under nonreducing conditions saturated at about 50; it was about 110 under reducing conditions (Figure 5). This suggested that the reduced form of the toxin has more hydrophobic sites at the protein surface.

To investigate a possible temperature-induced molten globule state, we carried out experiments to monitor the ANS fluorescence emission as a function of temperature at a constant ANS to protein ratio. To compare the temperature-induced structural difference under reducing and nonreducing conditions, we kept the molar ratio of ANS to protein as 110 under both conditions (under this ratio, ANS binding to both reduced and nonreduced BoNT/A is saturated). The ANS fluorescence reaches a maximum at 35−37 °C for reduced BoNT/A (Figure 6), a temperature at which virtually no native tertiary structure is left, although a high amount of secondary structure still remains intact (Figure 4C,D). On the other hand, for nonreduced toxin, the ANS fluorescence reaches maximum at about 53 °C, a condition under which both secondary and tertiary structures are collapsed. Thus ANS binding experiments support the observation that the reduced toxin is in a molten globule type of conformation under physiological temperature (37 °C), whereas the nonreduced toxin is devoid of such conformation at the same temperature.

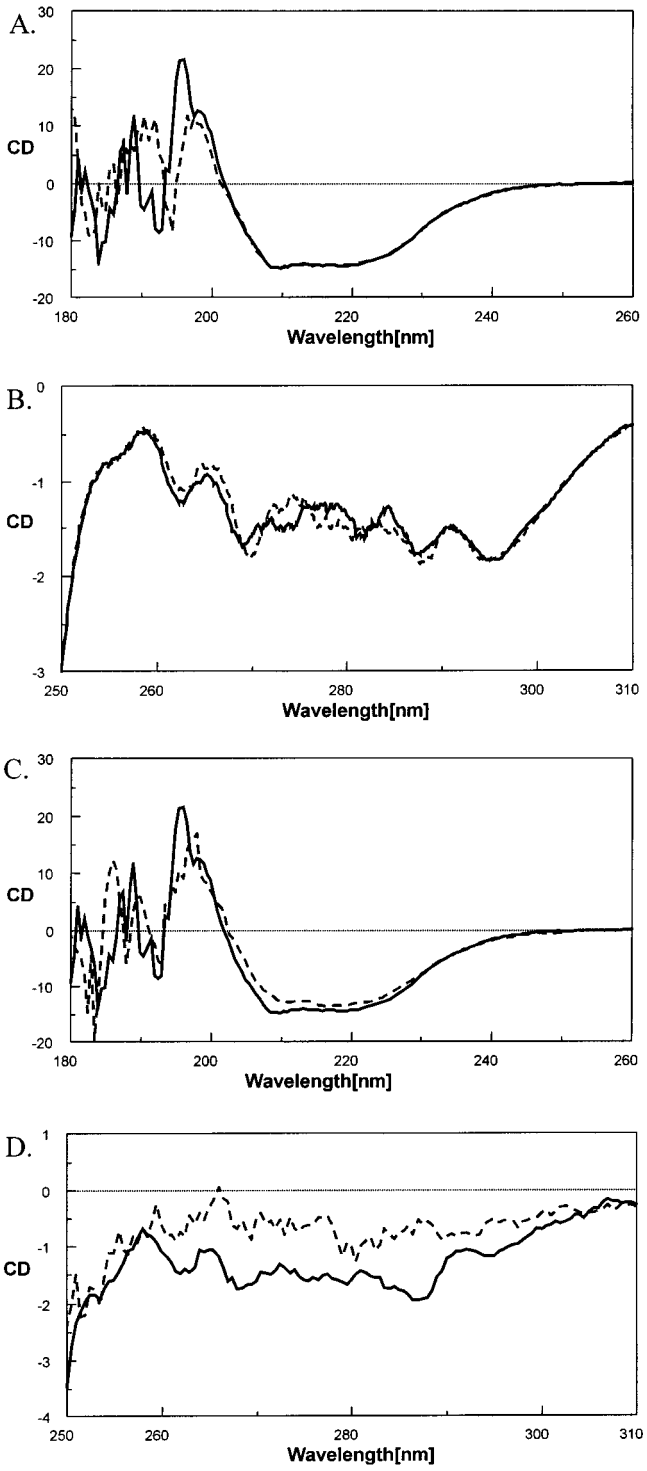

FIGURE 4: Far-UV CD and near-UV CD spectra of reduced and nonreduced BoNT/A at 20 and 37 °C. Panels: A, far-UV CD spectra of nonreduced BoNT/A; B, near-UV CD spectra of nonreduced BoNT/A; C, far-UV CD spectra of reduced BoNT/A; D, near-UV CD spectra of reduced BoNT/A. The solid line is the CD spectrum recorded at 20 °C, and the dashed line is the CD spectrum recorded at 37 °C.

Correlation between the Molten Globule State of Reduced BoNT/A and Its Endopeptidase Activity. To investigate the role of the molten globule type conformation of reduced BoNT/A in its biological activity, we determined the endopeptidase activity of reduced BoNT/A as a function of temperature. Under reducing conditions, the endopeptidase activity of BoNT/A reaches a maximum at temperatures between 35 and 37 °C, as revealed by 51 ± 4% cleavage of SNAP-25, the intracellular substrate of BoNT/A (Figure 7). When the temperature was increased to 42 °C, the endopeptidase activity of BoNT/A dramatically decreased, showing only 5 ± 2% cleavage of SNAP-25. The protein band showed a severe tailing, suggesting that the protein may begin to aggregate at this temperature (Figure 7). This observation suggests that the existence of a molten globule type conformation of reduced BoNT/A at physiological temperature is highly related to its endopeptidase activity and, therefore, plays a critical role in its intracellular toxification process.

DISCUSSION

BoNTs belong to a unique family of zinc endopeptidases. The only known intracellular substrates of BoNTs are SNARE proteins, which play a critical role in the exocytosis. Reduction of the disulfide bond between the L- and H-chains in BoNT/A is required for its endopeptidase activity (Figure 1).

Results of temperature-induced denaturation experiments suggest that reduced BoNT/A is under a two-stage (non-two-step) unfolding process monitored by near-UV CD at 280 nm and far-UV CD at 222 nm (Figure 3B). The first transition observed at 35 °C reflects the loss of a rigid tertiary structure. The second transition observed at 44 °C is related to the melting of the secondary structure. This observation suggests that a molten globule intermediate exists during thermal denaturation of reduced BoNT/A. On the other hand, nonreduced BoNT/A only shows a one-stage (two-step) transition, from a rigid native structure to a completely unfolded state (Figure 3A).

The structural difference between reduced and nonreduced BoNT/A has also been explored with ANS binding experiments. The higher fluorescence signal of ANS upon binding to reduced BoNT/A suggests that reduced BoNT/A has more hydrophobic clusters on its surface (Figure 5). This conclusion is consistent with our earlier study that suggested a more flexible structure for reduced BoNT/A (*17*). The differential ANS binding pattern observed as a function of temperature (Figure 6) indicates the existence of a molten globule state in the reduced BoNT/A at physiological temperature (37 °C), which is consistent with the results from CD experiments (Figure 3).

The biological significance of this molten globule structure in reduced BoNT/A is further confirmed by the corresponding variation in the endopeptidase activity with temperature. The endopeptidase activity of reduced BoNT/A reaches a maximum at the molten globule stage (Figure 7). This indicates that the molten globule type structure plays a very critical role in the biological function of BoNT/A, especially in its intracellular toxic action.

The molten globule type structure is characterized by the existence of a nativelike secondary structure and a nativelike folding pattern with a loosely packed nonpolar core without a rigid tertiary structure (*28*, *31*, *32*). The "semiflexible" structure permits the protein molecule to adapt to a wide variety of external conditions (*28*), while the nativelike folding pattern permits retention of the important features of its overall architecture despite these adaptations. It is

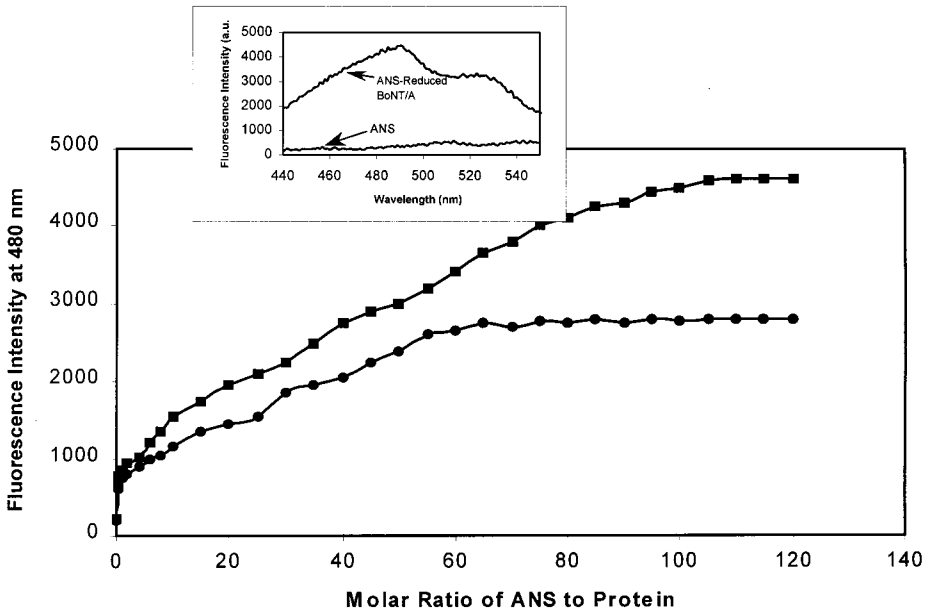

FIGURE 5: ANS binding to nonreduced BoNT/A (circles) and reduced BoNT/A (squares). The fluorescence intensity was measured at 488 nm with excitation at 370 nm. ANS fluorescence intensity vs molar ratio of ANS to protein is plotted. Insert: Fluorescence emission spectra of ANS in the absence and presence of reduced BoNT/A.

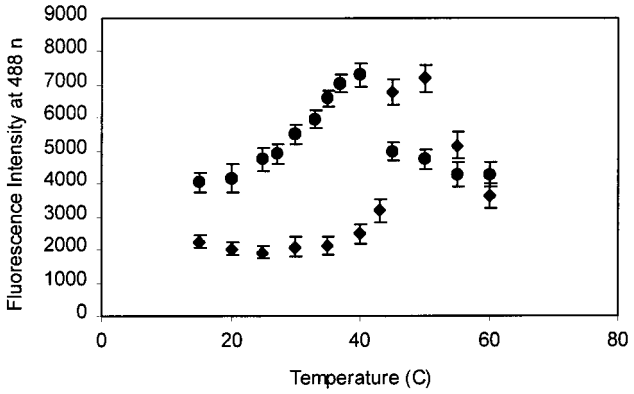

FIGURE 6: Fluorescence intensity of ANS bound to reduced (circles) and nonreduced (squares) BoNT/A as a function of temperature. The fluorescence intensity was recorded at 488 nm with excitation at 370 nm. The error bar represents the standard deviation from the experiments using three different batches of BoNT/A.

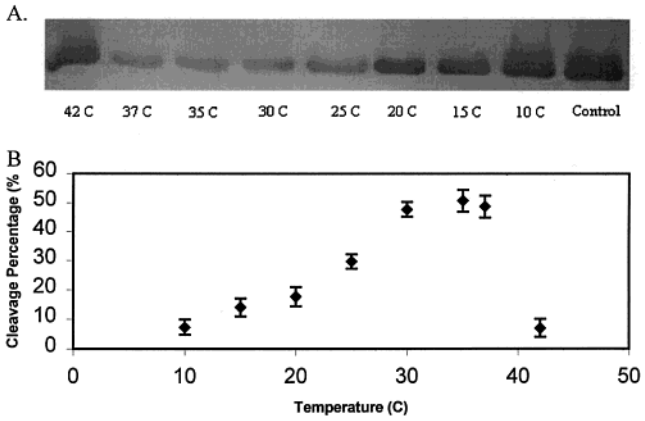

FIGURE 7: Endopeptidase activity of reduced BoNT/A as a function of temperature. Panel A: Western blot analysis of endopeptidase activity of reduced BoNT/A at different temperatures using SNAP-25−GST fusion protein as its substrate. Panel B: Cleavage percentage of SNAP-25−GST fusion protein by reduced BoNT/A as a function of temperature. The error bars represent the standard deviation of three independent experiments.

suggested that the semiflexible structure of the protein may play an important role in the protein−receptor interaction, since the semiflexible structure of both of these components may facilitate their mutual adjustments (*33*). For example, the functional form of insulin is in the molten globule state, which allows its effective binding to its receptor (*34*). Also, the conformational changes are known to accompany activation of matrix metalloproteinases, which are zinc-dependent endopeptidases (*35*, *36*). A similar situation in the BoNT may explain one of the most intriguing features of its enzymatic activity, viz., activation of its endopeptidase activity by the reduction of its disulfide bond. The molten globule conformation has been implicated in physiological processes such as membrane insertion and pore formation (*31*, *37*). The disulfide bond reduction induced activation of proteases may be another role of this type of protein conformation.

It is not experimentally known if both the L- and H-chains enter the neuronal cell. While the endopeptidase activity is exclusively the property of the L-chain, at least one report suggests the requirement of both L- and H-chains in *Aplysia* neurons for the blockage of neurotransmitter release (*38*). Even if the disulfide bond of BoNT is reduced, the two chains are still attached to each other through noncovalent interactions (*18*). Therefore, the disulfide bond reduction does not preclude the involvement of the two chains in the intracellular activity. The HC of BoNTs is known to bind to ganglioside and synaptotagmin on the presynaptic membrane for their internalization and translocation in the neuronal cells (*6*, *7*, *39*). During the translocation process, BoNT/A is believed to dissociate itself from its protein and ganglioside receptor complex (*2*, *5*). Once inside the cell, the BoNT is known to interact only with its endopeptidase substrates (*40*). Our observations of structural differences between the enzymatically active (reduced) and inactive (nonreduced) forms of BoNT/A are, therefore, relevant to both in vitro and in vivo conditions.

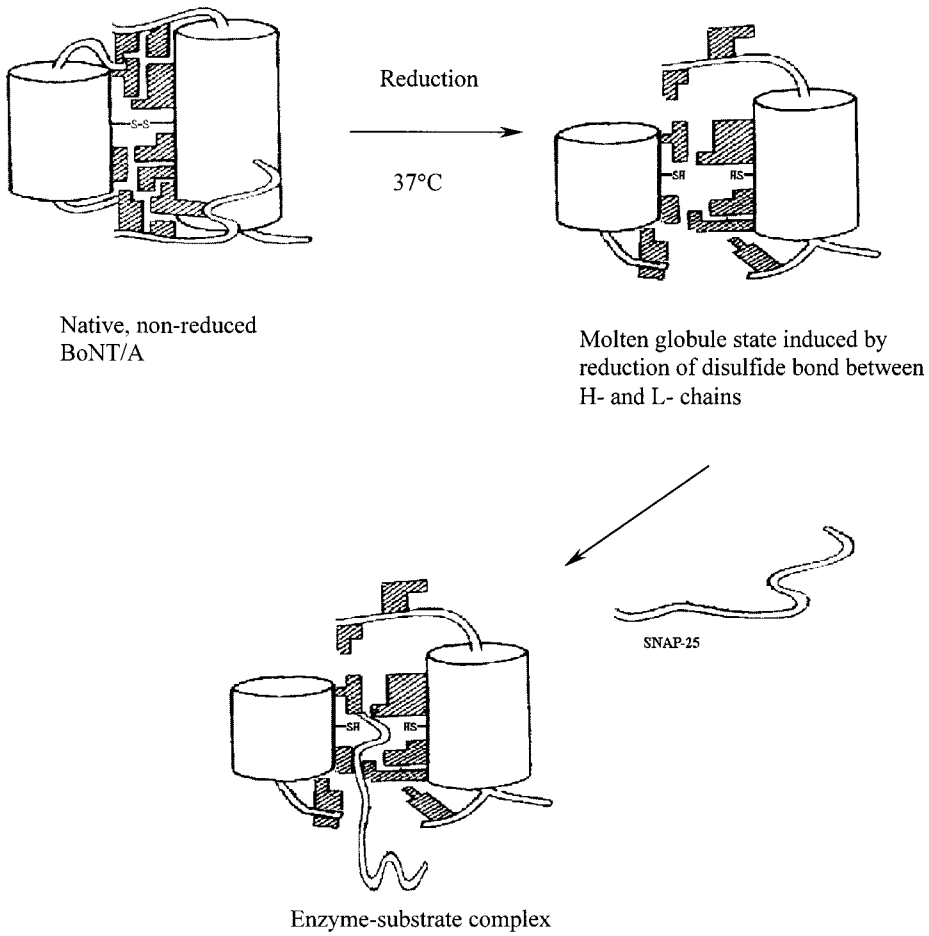

FIGURE 8: Schematic model of the reduction-induced molten globule state of BoNT/A facilitating binding of SNAP-25.

One of the unique features of BoNTs has been their exclusive substrates and cleavage sites (*7*). The molecular basis of this specificity is not clearly understood. The substrate specificity of different serotypes of BoNTs is believed to be due to a secondary recognition site beyond the cleavage site of SNARE proteins, which apparently interacts with the active site of BoNTs (*41*). A conserved region called the SNARE motif in SNARE proteins has been identified and has been proposed to serve as the recognition sequence for binding between SNARE proteins and BoNTs (*41*). However, the corresponding recognition sites in BoNTs have not been located. The cocrystal structure of synaptobrevin-II bound to the BoNT/B light chain has shown that the substrate is in a random coil conformation in the protease−product complex, and no major structural change of the BoNT/B L chain was apparent (*24*). SNAP-25 is also reported as an unstructured protein (*42*, *43*), and the BoNT/A structure is similar to that of BoNT/B (*23*). The molten globule conformation of BoNT/A observed at physiological temperature may facilitate the binding of unstructured SNAP-25, thus facilitating its cleavage by the endopeptidase activity of BoNT/A. This mechanism will reconcile apparent inaccessibility of the BoNT/A endopeptidase active site, as suggested by X-ray crystallographic structure (*22*). Figure 8 presents a model showing how the molten globule state of BoNT/A may facilitate binding and cleavage of unstructured SNAP-25.

So far, it has not been possible to crystallize proteins in the molten globule state. The crystal structure of BoNT/B under reducing conditions suggests no major structural change compared to its structure under nonreducing conditions (S. Swaminathan, personal communication). This observation is in apparent conflict with our current report, which shows a major tertiary structural difference between nonreduced and reduced BoNT/A. It is possible that this reflects a structural difference between BoNT/A and BoNT/B. Although the overall crystal structure of BoNT/A and BoNT/B under nonreducing conditions is very similar, the main difference is in the belt region from the translocation domain (*22*, *23*). In BoNT/A, the catalytic site is partially covered by the belt from the translocation domain; this enables the zinc ion to be shielded from the environment (*22*). In BoNT/B, the belt region does not shield the zinc ion, thus making it completely accessible to substrate molecules (*23*). Nevertheless, BoNT/B needs reduction of its disulfide bond between the heavy and light chains to activate its endopeptidase activity (*8*). Thus the disulfide bond reduction in BoNT/B may play a very critical role in binding of the unstructured region of synaptobrevin to BoNT/B. This hypothesis needs to be further investigated. Assuming a similar structural basis of endopeptidase activity in BoNT/A and BoNT/B, the cumulative information available, so far, suggests that the crystal structure of BoNT/A at low temperature (4 °C) is quite different from its enzymatically active structure at physiological temperature (37 °C) (*22*). The molten globule structure of BoNT/A observed at 37 °C exists only under reducing conditions, making it critical for BoNT/A endopeptidase activity.

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