Botulinum neurotoxins (serotypes A–G), the most toxic substances known to humankind, cause flaccid muscle paralysis by blocking acetylcholine release at nerve–muscle junctions through a very specific and exclusive endopeptidase activity against SNAP-25 proteins of presynaptic exocytosis machinery. We have examined polypeptide folding of the endopeptidase moiety of botulinum neurotoxin/A (the light chain) under conditions of its optimal enzymatic activity and have found that one of its stable conformational states is a molten-globule, which retains over 60% of its optimal enzyme activity. More importantly, we have discovered that the light chain acquires a novel pre-imminent molten-globule enzyme conformation at the physiologically relevant temperature, 37 °C. The pre-imminent molten-globule enzyme form also exhibited the maximum endopeptidase activity against its intracellular substrate, SNAP-25 (synaptosomal associated protein of 25 kDa). These findings will not only open new avenues to design effective diagnostics and antidotes against botulism but also provide new information on enzymatically active molten-globule or molten-globule like structures.

Botulinum neurotoxins (BoNTs) are the most potent natural toxins known to humankind with a mouse 50% lethal dose range of 0.1–1 ng/kg of body weight (1). Apart from being the sole causative agent of one of the oldest and most frightening food-poisoning diseases, botulism (2), BoNTs pose a major biological warfare threat (3). Interestingly, BoNTs are known to humankind with a mouse 50% lethal dose range of 0.1–1 ng/kg of body weight (1). Apart from being the sole causative agent of one of the oldest and most frightening food-poisoning diseases, botulism (2), BoNTs pose a major biological warfare threat (3). Interestingly, BoNTs are increasing used as therapeutic agents to treat several neurological disorders such as strabismus, blepharospasm, and torticollis (4), as well as for cosmetic purposes to remove facial wrinkles and frown lines (5). The efficacy of BoNT in the treatment of pain syndromes, including migraine and myofascial pain, has been well demonstrated (6). Thus, BoNT remains a topic of relevant human health concern due to its expanding use in clinical medicine and scientific research, as well as to the continual threat of its use as a bioweapon.

The family of BoNTs comprises seven antigenically distinct serotypes (A–G) that are produced by various toxigenic strains of Clostridium botulinum (7). BoNTs are produced as single inactive polypeptide chains of 150 kDa, which subsequently undergo endogenous or exogenous proteolytic cleavage to yield the fully active di-chain molecule comprising of a 100-kDa heavy chain and a 50-kDa light chain linked via both non-covalent interactions and a disulfide bond(s), the integrity of which is essential for the neurotoxicity. The heavy chain plays a key role in cell binding, internalization, and translocation of BoNT into nerve cells, whereas the light chain exhibits its intracellular toxic activity (1). The light chain acts as a zinc-dependent endopeptase and selectively cleaves one of the three synaptic vesicle fusion proteins that are crucial components of the neuroexocytosis apparatus. This results in chemodervation due to the blockage of acetylcholine release at the myoneural junction, subsequently leading to flaccid muscle paralysis (7).

Like other zinc endopeptidases, BoNTs possess a conserved zinc binding motif (HEXXH) in the central region of the light chain subunit polypeptide (8, 9). However, BoNT endopeptidases display several unique characteristics, most prominent being the exclusive peptide bond cleavage selectivity for very specific and nearly exclusive substrates. BoNT serotypes A and E cleave SNAP-25 at the C terminus, serotypes B, D, F, and G each cleave vesicle-associated membrane protein at different sites, and serotype C cleaves syntaxin and SNAP-25, each at different sites (10–12). Additionally, BoNT endopeptidases prefer the whole protein as substrates over the short peptides encompassing the specific cleavage site, further enhancing substrate specificity. An understanding of the molecular basis for the unusual substrate specificity and cleavage site selectivity is critical to the development of antidotes against botulism, maximally sensitive diagnostics, and also to the expansion of the therapeutic uses of botulinum neurotoxins.

Three-dimensional structures of BoNTs published, including those with co-crystals of BoNTs or their light chains with substrates or inhibitors, have focused on understanding the active site structure to explain the unique endopeptidase activity and to develop inhibitors (13–16). However, none of the studies, so far, has provided a satisfactory explanation to the unique substrate requirements of BoNTs. The x-ray crystal structure data from BoNT/A and BoNT/B, whereas similar in overall folding, differ in key aspects of the depth and occlusion of the active sites (17, 18), a key element expected to explain specificity and disulfide reduction-based activation of the endopeptidase activity of BoNTs (17, 19, 20). Moreover, there are substantial differences between structural elements of BoNTs in crystals and solutions (21, 22). The endopeptidase activity of BoNTs is observed only after reduction of the disulfide bond between light and heavy chain. Interestingly, the BoNT crystal structure does not seem to undergo any noticeable change upon disulfide bond reduction, although spectroscopic analysis of BoNT in solution indicates dramatic changes in the polypeptide folding (22). Crystal structures, therefore, may not be adequate to either explain the molecular basis of the action or design effective inhibitors of BoNT.

As mentioned above, reduction of disulfide bond between light and heavy chains is essential for the endopeptidase activity of BoNT holotoxin. Based on structural analyses of the non-reduced and the reduced form of BoNT/A, it was recently discovered that BoNT/A adopts a molten-globule conformation for the optimum enzymatic activity (22). This suggests that the molten-globule structure plays a critical role in the toxic action of the BoNT. However, because the light chain is the...
catalytic moiety of BoNTs that enters the nerve cell and is solely responsible for intracellular toxicity, it is important to examine the conformational state and enzymatic activity of the light chain.

In this study, we have reported for the first time a prominently bio-logically active 61% molten-globule structure of an enzyme, the catalytic domain of BoNT/A. More importantly, we have discovered a novel pre-immerminent molten-globule enzyme (PRIME) state of BoNT/A endopeptidase that is optimally active. Under PRIME conformational state, the endopeptidase retained its secondary structure intact while acquiring fluidic tertiary structure, which is likely to accommodate specific interaction with its substrate, SNAP-25, to exhibit optimum and selective enzymatic activity.

**MATERIALS AND METHODS**

*Expression and Purification of Recombinant BoNT/A Light Chain—Escherichia coli* strain HB101 was transformed with the T5 RNA polymerase expression plasmid pBN3 (23), and the expression of BoNT/A light chain (LC) was induced with isopropyl-β-thiogalactopyranoside for 12 h at 30 °C. The recombinant LC had 6 histidine residues tagged at the C-terminal and was purified on a Ni²⁺-column according to the method described previously (23).

**CD Spectroscopy**—CD spectra were recorded at 20 °C on a Jasco J715 spectropolarimeter equipped with a Peltier type temperature control system (model PTC-348W) as described previously (22). The concentrations of LC samples in the range of 0.15–0.3 mg/ml and 0.8–1.2 mg/ml were used for far-UV and near-UV CD measurements, respectively.

Temperature-induced unfolding of the LC polypeptide was followed by monitoring the change with temperature in the CD signal at 222 and 280 nm. LC samples dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl, were heated with ellipticity recorded at 222 and 280 nm at every 0.1 °C between 20 and 90 °C. Temperature was raised at a rate of 2 °C/min. In a parallel experiment, entire far-UV and near-UV CD spectra were recorded at every 5 °C temperature interval and at 37 °C during the temperature ramping between 20 and 90 °C.

**UV Second Derivative Spectroscopy**—Absorption spectra of LC dissolved in 10 mM NaPB, pH 6.0, containing 150 mM NaCl were recorded between 240 and 320 nm on a Jasco UV/VIS spectrophotometer equipped with a temperature control system (Model PSC-498T) as a function of temperature. The spectra were derivatized to the second order. The ratio of $a$ (an arithmetic sum of the negative $d^2A/dλ^2$ at 284 nm and positive $d^2A/dλ^2$ at 289.5 nm) and $b$ (an arithmetic sum of the negative at 291 nm and the positive at 294 nm) was measured at different temperatures. The degree of tyrosine exposure was calculated according to the method of Ragone et al. (24) as described previously (25).

**Binding of 1-Anilinonaphthalene 8-Sulfonate (ANS)**—Interaction of the fluorescent dye ANS with BoNT/A light chain was analyzed by measuring the fluorescence intensity of the protein-bound dye. Fluorescence spectra were recorded on an ISS K2 fluorometer (Champaign, IL) according to previously established procedures (22). To determine the optimized binding ratio, ANS was titrated into 2 ml of 1.1 mM LC solution dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl in a 1-cm path-length cuvette. The emission spectra of ANS were recorded between 400 and 540 nm, and the excitation wavelength was fixed at 370 nm. The excitation and emission slit widths were fixed at 4 and 8 nm, respectively. To examine the temperature-induced structural change in LC, the optimized binding ratio was used, and the ANS emission spectra were recorded at different temperatures (25–54 °C).

**Quenching of Tryptophan Fluorescence**—The quenching of tryptophan fluorescence with a neutral quencher, acrylamide, was assessed to estimate the extent of exposure of the buried tryptophan residues in LC. Fluorescence spectra were recorded on an ISS K2 fluorometer. The slit widths on both the excitation and emission monochromators were set at 8 nm. An excitation wavelength of 295 nm was used to selectively excite tryptophan residues. To minimize inner filter effect, the absorbance of LC at 295 nm was always kept below 0.1. Increasing concentrations (0.0–0.1 M) of a 8 M stock solution of acrylamide were added to LC that was dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl, and fluorescence emission spectra were recorded after each addition. This procedure was carried out at three different temperatures of 20, 37, and 50 °C.

To examine the temperature-induced structural changes in LC, the topography of Trp residues was probed by quenching of fluorescence of these residues as a function of temperature. 0.08 M acrylamide solution was added to LC dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl, and the fluorescence emission spectra were recorded at different temperatures (25–65 °C). In a parallel experiment, fluorescence emission spectra of LC dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl without the addition of any acrylamide were recorded at increasing temperatures (25–65 °C).

**Endopeptidase Assay—BoNT/A LC was assayed for endopeptidase activity using SNAP-25 as its substrate. Recombinant His₆-tagged SNAP-25 was expressed and purified as described previously (23). The temperature effect on the endopeptidase activity of LC was examined by incubating 6.4 μM SNAP-25 with 100 nM LC at the designated temperature for 30 min in an assay buffer (10 mM sodium phosphate buffer, 150 mM, pH 6.0). The cleavage reaction was terminated by the addition of SDS-PAGE sample buffer, and the samples were then separated on 4–20% SDS-PAGE. The electrophoresis was run using a Mini Protean II system from Bio-Rad at a constant voltage of 200 V at room temperature (25 °C). The bands on the gel were visualized by Coomassie Blue staining. The amount of uncleaved SNAP-25 was scanned on a GEL LOGIC 100 Imager system and analyzed and quantified using the Kodak Image analysis software (Eastman Kodak Co.). The percentage of cleavage was calculated by comparing the density of the uncleaved SNAP-25 band with that of the control SNAP-25.

**RESULTS AND DISCUSSION**

**Temperature-activated Endopeptidase Activity of BoNT/A Light Chain**—To investigate the functional stability of BoNT/A light chain against temperature, we determined the endopeptidase activity of light chain on its intracellular substrate SNAP-25, as a function of temperature. It was observed that the endopeptidase activity of the light chain reached a maximum at 37 °C (Fig. 1A), as strikingly portrayed by the 71.91 ± 6.07% cleavage of SNAP-25 in Fig. 1B. As the temperature was further increased, it was observed that the light chain retained 61% of the optimum enzymatic activity at 50 °C and continued to display residual activity even at 60 °C.

**BoNT/A Light Chain, an Enzymatically Active Molten-Globule**—The effect of temperature on light chain stability was investigated using CD spectroscopy. We analyzed the thermal unfolding of the light chain at both secondary and tertiary structural levels by monitoring CD signals as a function of temperature at 222 and 280 nm, respectively. Results obtained from the far-UV and near-UV CD spectra indicate that the native (N) light chain undergoes a two-state unfolding (U) transition (N to U), characterized by a steep transition at the secondary and a relatively expanded (non-cooperative) transition at the tertiary structural levels (Fig. 2A). The $T_M$ of light chain for secondary structure unfolding...
was about 53 °C, and the $T_M$ for the tertiary structure unfolding was about 50 °C, suggesting the existence of a state for light chain where the tertiary structure was already collapsed, whereas the secondary structure remained mostly intact.

The loss of rigid tertiary structure while still maintaining a high degree of secondary structure is a distinctive feature of the molten-globule state in proteins arising due to the lack of tight packing of side chains of aromatic amino acid residues (26). To confirm this state, we extracted the CD spectra at 20, 37, 50, and 90 °C. Although the ellipticity at 222 nm, characteristic of $\alpha$-helical conformation, at 37 °C is virtually the same as that at 20 °C, only about 32% of the ellipticity is lost at 50 °C (Fig. 2B), suggesting that a significant amount of secondary structure persists at 50 °C. The CD spectrum in the near-UV region, monitored by ellipticity at 280 nm, however, showed a marked difference. At 20 °C, a strong ellipticity indicative of fixed orientations of the aromatic residues in the protein was observed. At 37 °C, there were significant structural alterations in the polypeptide folding in comparison with those at 20 °C, whereas at 50 °C, the CD spectrum was almost completely collapsed (Fig. 2C). The CD spectra at 90 °C represent the spectra of completely denatured BoNT/A LC. The loss of near-UV CD signal at higher temperatures suggests an increased mobility of side chains of aromatic amino acid residue side chains (27). These observations strongly indicate that BoNT/A light chain exists in a molten-globule state at 50 °C, and to a certain degree at 37 °C, where it exists in a state that is less compact than the native state but substantially more compact than the molten-globule state at 50 °C.

The semi-flexible nature of the molten-globule state allows the internal hydrophobic groups to become exposed to water, which can be probed spectroscopically by monitoring binding of the hydrophobic dye ANS (28, 29). A molten-globule conformation allows ANS to access the hydrophobic segments of the protein, resulting in the enhancement of the fluorescence of the dye. To further confirm the existence of temperature-induced molten-globule state in BoNT/A light chain, we investigated ANS binding properties to BoNT/A light chain by monitoring the ANS fluorescence as a function of temperature at an optimum constant ANS to protein molar ratio (60:1, data not shown). The ANS binding reached a maximum at 50 °C (Fig. 3), a temperature at which virtually no native tertiary structure remained, although a considerable amount of secondary structure still remained intact. Thus, the ANS binding results also suggest that the light chain exists in a molten-globule conformation at 50 °C. It is notable that the ANS binding curve showed a bump at 37 °C, indicating a special conformational state for the optimum enzymatic activity observed at that temperature (Fig. 1B), and that it binds more strongly to this state than to the native structure at 20 °C. Examination of the enzyme activity of light chain indicated that about 61% of the optimum enzymatic activity was maintained in the molten-globule state at 50 °C.

**FIGURE 1.** Endopeptidase activity of BoNT/A LC as a function of temperature. A, SDS-PAGE analysis of endopeptidase activity of 100 nM LC at different temperatures using 6.4 μM SNAP-25 as substrate. 6.4 μM SNAP-25 was incubated with 100 nM BoNT/A LC at different temperatures for 30 min. B, the percentage of cleavage of SNAP-25 by BoNT/A LC as a function of temperature. The error bars represent the standard deviation from three independent experiments.

**FIGURE 2.** A, thermal unfolding of BoNT/A LC dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl. Samples were heated at the rate of 2 °C/min. The unfolding was monitored by continuously recording the CD signal at either 222 or 280 nm. B, far-UV CD spectra of BoNT/A LC dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl at 20 (●), 37 (■), 50 (▲), and 90 °C (——). C, near-UV CD spectra of BoNT/A LC dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl at 20 (●), 37 (■), 50, and 90 °C (——).
residues using UV second derivative spectroscopy (24) and fluorescence spectroscopy. The second derivative UV absorption spectrum of light chain dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl at 25 °C is shown in Fig. 4A. The spectral region of interest for the determination of exposed tyrosine residues is 280–300 nm. The second derivative spectrum of the light chain at 25 °C (dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl) showed only one negative band at 284 nm. Two positive peaks were observed at 289.5 and 294 nm with a positive trough at 291 nm. To probe the effect of temperature on the tertiary structural changes, second derivative absorption spectra were obtained at different temperatures (20–44 °C). The a/b ratio was calculated at each of these temperatures to determine the change in the exposure of Tyr residues in the protein with increasing temperatures (Fig. 4B). It was observed that with increasing temperature, there was no significant shifting in the peak position, although the relative intensities of the positive and negative peaks were altered significantly (spectra not shown). The a/b ratios of the light chain at 25 and 41 °C were 4.46 ± 0.13 and 2.39 ± 0.11, respectively (Fig. 4B), reflecting a decrease of 46.9% in the a/b ratio at 41 °C from that at 25 °C. A marked decrease in the a/b ratio was observed with a Tm of 37 °C, which indicated lower exposure of tyrosine residues (30). A 50% decrease in Tyr exposure observed at 37 °C (Tm) reflected exposure of 4 Tyr residues as compared with 7 Tyr residues exposed at 25 °C. Lower exposure of tyrosine residues could result from either refolding in the protein or a tighter construction of the protein structure. This observation suggests that dramatic changes occur in the polypeptide folding at 37 °C and implies that the light chain at this temperature exists in a conformational state that is different from the native state. It was difficult to accurately measure the peak ratios beyond 41 °C due to noise in the spectrum, which may result from aggregation of the protein observed upon further heating.

Complete denaturation of light chain with 6 M guanidine-HCl (30 min at 25 °C) resulted in a significant alteration on the relative peak intensities at 288 and 295.5 nm, resulting in an a/b ratio of 9.8 ± 0.08 (Fig. 4C). The degree of tyrosine exposure after comparing the second derivative spectra of light chain in native state with those of the denatured state revealed that about 59.1% of Tyr residues of light chain are exposed to the polar solvent environment, an observation consistent with the results obtained earlier with native BoNT/A light chain (31).

The effect of temperature on the Trp emission spectra of BoNT/A light chain was also investigated. The Trp emission maximum was observed at 318 nm at 20 °C, indicating that the Trp residues are in a relatively hydrophobic environment (data not shown). Upon heating, the emission maxima showed a gradual red shift reaching a maximum of

![FIGURE 4. A, absorption (— — —) and second derivative (——) spectra of LC dissolved in 10 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl at 25 °C. a denotes the arithmetic sum of d2A/dλ2 at 284 and 289.5 nm. b denotes the arithmetic sum of d2A/dλ2 at 291 and 294 nm. B, the effect of increasing temperature on the second derivative ratio (a/b) of BoNT/A LC dissolved in 10 mM NaPB, pH 6.0 containing 150 mM NaCl. C, absorption (— — —) and second derivative (——) spectra of LC dissolved in 6 M guanidine-HCl at 25 °C. a denotes the arithmetic sum of d2A/dλ2 at 284 and 289.5 nm. b denotes the arithmetic sum of d2A/dλ2 at 291 and 294 nm.](image)
329 nm at 60 °C. A longer wavelength shift of the fluorescence spectrum could be induced by the conformational changes in the protein, leading to an increase in the polarity of the microenvironment of the fluorophore (32).

Thermally induced conformational changes in BoNT/A light chain protein unfolding were also investigated by quenching of Trp fluorescence. The degree of exposure of Trp residues in solution as a function of temperature, monitored by fluorescence quenching technique, provides valuable information concerning the dynamics of protein folding (33). Acrylamide is an efficient quencher that preferentially quenches the more solvent-accessible Trp residues in multi-tryptophan proteins (34, 35). It is postulated that conformational fluctuations in the polypeptide facilitate the inward movement of acrylamide into the interiors of the protein matrix (36). The results obtained from acrylamide quenching of BoNT/A light chain fluorescence at different temperatures (Fig. 5, inset) indicate that there are more quenchable fractions in light chain from 37 to 50 °C in comparison with the protein in the native state. The relative inaccessibility of the Trp residues in LC in the native conformation further confirms the strong hydrophobic environment in which they are located (37).

Quenching of Trp fluorescence in light chain treated with a fixed concentration of acrylamide as a function of increasing temperature is shown in Fig. 5. Acrylamide maximally quenches the Trp fluorescence in the light chain at about 40 °C, indicating that the peptide segments containing Trp residues are more mobile and accessible at this temperature than at 20 °C. Upon further heating, beyond 45 °C, there was an increase in the fluorescence intensity, which could be attributed to the release of intrinsic quenching (38). The polypeptide chain in a native protein is folded into a compact structure that strongly limits freedom of molecular movement, thus restricting fluctuations. Increase in temperature will enhance the fluidity of a medium since the intramolecular interactions that stabilize the globular structure of the protein are distorted, and the protein becomes loosened, forming pores or pathways for a large molecule like acrylamide to collide with the excited states of internal Trp residues (35). Thus, the measurement of the extent of quenching of Trp fluorescence provides valuable information about the dynamics of the protein and has been powerful in sensing the exposure of Trp residues in the molten-globule state of a protein (38). Increased quenching of Trp fluorescence observed in BoNT/A light chain at 37 °C (Fig. 5) thus indicates a molten-globule-like structure.

Changes in BoNT/A light chain Trp fluorescence as a function of temperature suggested increased intramolecular mobility in the light chain tertiary structure at 37 °C relative to that in the native state. Since the Trp residues are more mobile, increased dynamics and even slight expansion of the protein structure facilitated the inward movement of the quencher. In addition, the hydrophobic environment of Trp residues in native structure changed to polar, due to exposure of Trp residues to aqueous solvent. These observations suggested the existence of another intermediate state at 37 °C, which preceded the molten-globule state and which was more expanded and dynamically flexible but distinctly different from the molten-globule state. The hydrophobic interior of BoNT/A light chain in this state was more densely packed than the molten-globule state, which accounted for reduced accessibility for ANS. Acrylamide, however, being a neutral molecule, can readily penetrate the globular protein matrix, resulting in quenching of the Trp residues (35). This new intermediate retained virtually all the secondary structure of the native state, acquired flexible tertiary structural folding, and showed increased binding to ANS as compared with the native state. This intermediate conformational state at 37 °C exhibited optimum enzymatic activity. We refer to this intermediate structure as the PRIME conformational state.

Interestingly, the optimum enzymatic activity of light chain and the PRIME conformational state were attained at the physiological temperature of 37 °C. The complex dynamic flexibility of the PRIME structure could be critically important to facilitate maximum specific binding of SNAP-25, which ultimately leads to the cleavage of the latter by the endopeptidase activity of the light chain. Thus, we suspect that the PRIME conformational state plays a very critical role in the biological function of BoNT/A, especially in its intracellular toxic action.

**PRIME Structure and BoNT/A Endopeptidase Selectivity**—The schematic model of the temperature-induced PRIME conformational and molten-globule states of BoNT/A light chain, as represented in Fig. 6, reflects how these states in BoNT/A light chain may facilitate binding and cleavage of SNAP-25. The intramolecular mobility of the PRIME state is significantly higher than that of the native state, which can be attributed to increased dynamics and expansion of the protein core, facilitating a maximum specific binding of SNAP-25, leading to its cleavage. The molten-globule conformation, on the other hand, has a considerably intact secondary structure, loose packing of side chains in the protein core, and partial unfolding of loops. This structure also binds to SNAP-25, although to a lesser degree than the PRIME state, thus showing only about 61% of the optimum enzymatic activity.

The latest three-dimensional structure published with a double mutant of BoNT/A light chain (lacking enzyme activity) and a truncated form of SNAP-25 (residues 141–204) crystallized together (16) provides a sound mechanism for BoNT/A endopeptidase specificity and selec-
Significance of PRIME and Molten-globule States in Proteins—For decades, there has been increasing conviction that the biological function of protein can be interpreted only in terms of their three-dimensional structure, and ordered native structure generally obtained from x-ray crystallography has been accepted as the standard structure. Stable three-dimensional structures are the ultimate goals for most of the modern protein engineering efforts to generate proteins for specific functions. Paradoxically, many de novo synthesized proteins exist as molten-globules (41), which are considered to be an obstacle to overcome in obtaining fully folded structures (41, 42). The molten-globule state has been widely recognized as a universal kinetic intermediate for in vitro protein folding and as an intermediate equilibrium state of protein molecules occurring between their unfolded and native states (43). This intermediate has been found for many proteins in mild denaturing conditions such as low or high pH, high temperatures, moderate concentrations of urea or guanidine hydrochloride, and so forth (44). Removing protein-bound ligands as well as chain truncation or amino acid replacements by genetic methods can also generate this equilibrium intermediate. In recent years, stable conformational states, including the pre-molten-globule and the molten-globule states, have also been experimentally observed between the unfolded and native states of several proteins (44).

Some proteins are known to undergo conformational changes, including to a molten-globule state, during activity such as transportation across membranes (45, 46) and possibly protein-receptor interactions (47). Of course, molten-globule conformation has also been shown to participate in recognition of proteins by chaperones (48). However, no molten-globule state has been observed for a fully active enzyme, except for BoNT/A, which adopts an enzymatically active molten-globule state under physiological conditions in which the native protein structure is destabilized by disruption of disulfide bonds (22). Since BoNT/A consists of enzymatic light chain linked through the disulfide bond to non-enzymatic heavy chain, analysis of the enzymatic domain by itself is important to the understanding of the role of the molten-globule in the enzymatic activity.

Our observation of a molten-globule structure with 61% enzymatic activity and a novel PRIME structure with optimum enzymatic activity is the first observation of enzymatically active molten-globule or molten-globule-like structure. The dynamic role of such a structure is more significant in view of the extreme specificity of the endopeptidase toward its substrate, SNAP-25, and its tremendous utility in designing specific antidotes against botulism threats.

The ability to obtain a stabilized and structurally defined molten-globule provides a useful model for studying the folding and unfolding pathways of proteins. Structural analysis of protein intermediates or molten-globules provides valuable information on biophysical and biological aspects of proteins. The presence of the PRIME conformational state, although being reported for the first time in BoNT/A endopeptidase, may play a major role in the biological functions of wide variety of proteins.

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REFERENCES
1. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) Physiol. Rev. 80, 717–755
2. Ergbuth, F. J. (2004) Movement Disorders 19, 52–56
3. Arnon, S. S., Schechter, R., Ingelsby, T. V., Henderson, D. A., Barlett, D. A., Ascher,
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M. S. Eitzen, E., Fine, A. D., Hauer, J., Layton, M., Lillibridge, S., Osthermol, M. T., O’Toole, T., Parker, G., Perl, T. M., Russell, P. K., Sverdlov, D. L., and Tonat, K. (2003) J. Am. Med. Assoc. 285, 1059–1070
4. Wheeler, A. H. (1997) Am. Fam. Physician 55, 541–545, 548
5. Klein, A. W. (2004) Dermatol. Surg. 30, 452–455
6. Cordivari, C., Misra, P. V., Catania, S., and Lees, A. J. (2004) Movement Disorders 19, 157–161
7. Singh, B. R. (2000) Nat. Struct. Biol. 7, 617–619
8. Hooper, N. H. (1998) FEBS Lett. 354, 1–6
9. Vallee, B. L., and Auld, D. S. (1990) J. Biol. Chem. 265, 637–664
10. Schiavo, G., Santucci, A., DasGupta, B. R., Mehta, P. P., Jones, J., Benfenati, F., Wilson, M. C., and Montecucco, C. (1993) FEBS Lett. 335, 99–103
11. Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., Das-Gupta, B. R., and Montecucco, C. (1992) Nature 359, 832–835
12. Schiavo, G., Shone, C. C., Benett, M.K., Scheller, R. H., and Montecucco, C. (1995) J. Biol. Chem. 270, 10566–10570
13. Eswaramoorthy, S., Kumaran, D., and Swaminathan, S. (2002) Biochemistry 41, 9795–9802
14. Agarwal, R., Eswaramoorthy, S., Kumaran, D., Binz, T., and Swaminathan, S. (2004) Biochemistry 43, 637–664
15. Segelke, B., Knupp, M., Kadkhodayan, S., Ballhorn, R., and Rupp, B. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 6888–6893
16. Breidenbach, M. A., and Brunger, A. T. (2004) Nature 432, 925–929
17. Lacy, D. B., Tepp, W., Cohen, A. C., Das-Gupta, B. R., and Stevens, R. C. (1998) Nat. Struct. Biol. 5, 898–902
18. Swaminathan, S., and Eswaramoorthy, S. (2000) Nat. Struct. Biol. 7, 693–699
19. Lacy, D. B., and Stevens, R. C. (1999) J. Mol. Biol. 291, 1091–1104
20. Singh, B. R. (2002) in Scientific and Therapeutic Aspects of Botulinum Toxin (Brin, M. F., Jankovic, J., and Hallet, M., eds) pp. 75–88, Lippincott Williams and Wilkins, Philadelphia
21. Cai, S., and Singh, B. R. (2001) Biochemistry 40, 4693–4702
22. Cai, S., and Singh, B. R. (2000) Biochemistry 50, 15327–15333
23. Li, L., and Singh, B. R. (1999) Protein Expression Purif. 17, 339–344
24. Bagone, R., Colonna, G., Balestrieri, C., Servillo, L., and Irace, G. (1984) Biochemistry 23, 1871–1875
25. Singh, B. R., and DasGupta, B. R. (1989) Mol. Cell. Biochem. 85, 67–73
26. Ogihashi, M., and Wada, A. (1983) FEBS Lett. 164, 21–24
27. Doshpande, S. S., and Damodaran, S. (1989) Biochim. Biophys. Acta 998, 179–188
28. Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F., and Gilmanashin, R. I. (1991) Biopolymers 31, 119–128
29. Pitsyn, O. B. (1995) Adv. Protein. Chem. 47, 83–229
30. Singh, B. R., Wassac, F. M., Strand, S., Jacobsen, R. J., and DasGupta, B. R. (1990) J. Protein Chem. 9, 705–713
31. Singh, B. R., and DasGupta, B. R. (1989) Biochem. Chem. 34, 259–267
32. France, R. M., and Grossman, S. H. (2000) Biochem. Biophys. Res. Comm. 269, 709–712
33. Eftink, M. R., and Ghiron, C. A. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3290–3294
34. Demchenko, A. P. (1986) Essays Biochem. 22, 120–157
35. Eftink, M. R., and Ghiron, C. A. (1976) Biochemistry 15, 672–680
36. Eftink, M. R., and Ghiron, C. A. (1977) Biochemistry 16, 5546–5551
37. Li, L., and Singh, B. R. (2000) Biochemistry 39, 6466–6474
38. Dubey, V. K., and Jaganadham, M. V. (2003) Biochemistry 42, 12287–12297
39. Washbourne, P., Pellizari, R., Baldini, G., Wilson, M. C., and Montecucco, C. (1997) FEBS Lett. 418, 1–5
40. Vaidyanathan, V. V., Yoshino, K., Jahnz, M., Dorries, C., Bade, S., Nauenburg, S., Niemann, H., and Binz, T. (1993) J. Neurochem. 74, 327–337 Betz, S. F., Raleigh, D. P., and DeGrado, W. F. Curr. 999 Curr. Opin. Struct. Biol. 3, 601–610
41. Vamvaka, K., Vogeli, B., Kast, P., Fervushin, K., and Halvæt, D. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 12860–12864
42. Bryson, J. W., Betz, S. F., Lu, H. S., Suich, D. J., Zhou, H. X., O’Neil, K. T., and DeGrado, W. F. (1995) Science 270, 935–941
43. Bychkova, V. E., and Pitsyn, O. B. (1993) Chem. Bio. and Mol. Biol. 4, 133–163
44. Arai, M., and Kuwajima, K. (2000) Adv. Protein Chem. 53, 209–282
45. van der Goot, F. G., Gonzalez-Manas, J. M., Lokej, J. H., and Pattus, F. (1991) Nature 354, 408–410
46. Bychkova, V. E., Pain, R. H., and Pitsyn, O. B. (1988) FEBS Lett. 236, 231–234
47. Nakagawa, S. H., and Tager, J. H. S. (1993) Biochemistry 32, 7237–7243
48. Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A., and Hartl, F. (1991) Nature 352, 36–42