Biochimica et Biophysica Acta 1844 (2014) 1145–1152

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Differential role of molten globule and protein folding in distinguishing unique features of botulinum neurotoxin

Raj Kumar, Roshan V. Kukreja, Shuowei Cai, Bal R. Singh *

Department of Chemistry and Biochemistry, University of Massachusetts, Dartmouth, MA 02747, USA

ARTICLE INFO

Article history:
Received 23 October 2013
Received in revised form 15 February 2014
Accepted 17 February 2014
Available online 22 February 2014

Keywords:
Fluorescence
Enzyme catalysis
Protein folding
Protein denaturation
Protein conformation

ABSTRACT

Botulinum neurotoxins (BoNTs) are proteins of great interest not only because of their extreme toxicity but also paradoxically for their therapeutic applications. All the known serotypes (A-G) have varying degrees of longevity and potency inside the neuronal cell. Differential chemical modifications such as phosphorylation and ubiquitination have been suggested as possible mechanisms for their longevity, but the molecular basis of the longevity remains unclear. Since the endopeptidase domain (light chain; LC) of toxin apparently survives inside the neuronal cells for months, it is important to examine the structural features of this domain to understand its resistance to intracellular degradation. Published crystal structures (both botulinum neurotoxins and endopeptidase domain) have not provided adequate explanation for the intracellular longevity of the domain. Structural features obtained from spectroscopic analysis of LCA and LCB were similar, and a PRIME (PReImminent Molten Globule Enzyme) conformation appears to be responsible for their optimal enzymatic activity at 37 °C. LCE, on the other hand, was although optimally active at 37 °C, but its active conformation differed from the PRIME conformation of LCA and LCB. This study establishes and confirms our earlier finding that an optimally active conformation of these proteins in the form of PRIME exists for the most poisonous poison, botulinum neurotoxin. There are substantial variations in the structural and functional characteristics of these active molten globule related structures among the three BoNT endopeptidases examined. These differential conformations of LCs are important in understanding the fundamental structural features of proteins, and their possible connection to intracellular longevity could provide significant clues for devising new countermeasures and effective therapeutics.

1. Introduction

Botulinum neurotoxins (BoNTs), produced by Gram-positive bacteria, *Clostridium botulinum*, are the most toxic substances known. BoNTs are responsible for human and animal botulism. The lethal effect of BoNT is mediated by intracellular cleavage of SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment proteins receptor) [1] protein in presynaptic nerve endings, which are essential for neuroexocytosis [2]. Cleavage of SNARE proteins results in the blockage of acetylcholine release at nerve–muscle junctions resulting in flaccid muscle paralysis, and eventually respiratory collapse. Paradoxically, while BoNT is a very toxic potential warfare agent, it also has a wide range of medical applications related to neuromuscular disorders, including blepharospasm, hyperhidrosis, and cervical dystonia [3–5]. BoNT-based products are also being used for cosmetic purposes, such as removal of wrinkles [6].

Eight distinct serotypes (A-H) of BoNT are produced by *Clostridium botulinum* as single chain 150 kDa molecules, which are changed to dichain molecules by proteolytic cleavage at the Lys–Ala peptide bond, either by proteolytic enzymes in tissue or in bacterial cytosol. This cleavage creates a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) connected via a disulfide bond and non-covalent interactions [7,8]. Heavy chain can be hydrolyzed into two 50 kDa domains, C-terminus binding domain (H_c) and N-terminus translocation domain (H_N). BoNT is composed of three domains: binding domain, translocation domain, and catalytic or endopeptidase domain. These three domains, respectively, allow BoNT to bind to cell surface receptors, pass through the endosomal membrane, and cleave the protein(s) involved in the synaptic vesicle docking. [1]. BoNT/A, BoNT/B, and BoNT/E neurotoxins are known to cause human botulism, and cleave the SNAP-25 (BoNT/A and E; 9) and VAMP (BoNT/B) [10] component of SNARE. While all the serotypes have similar domain organization and function, and show only immunological distinction [10], duration of their intracellular action (longevity) and potency vary substantially. BoNT/A, BoNT/B, and BoNT/E intoxication is persistent for about 180 days, 90 days, and 30 days, respectively [9,11,12]. Persistence of the BoNT/A intoxication

Abbreviations: BoNT, botulinum neurotoxin; PRIME, Pre-imminent molten globule enzyme; CD, circular dichroism; MG, molten globule; SNARE, soluble NSF attachment protein receptor, NSF, *N*-ethylmaleimide-sensitive factor; VAMP, vehicle associated membrane protein

* Corresponding author at: Botulinum Research Centre, Institute of Advanced Sciences, 78-540, Faunce Corner Road, North Dartmouth, MA 02747, USA. Tel.: +1 508 992 2042; fax: +866 709 1689.
E-mail address: bsingh@inads.org (B.R. Singh).

http://dx.doi.org/10.1016/j.bbapap.2014.02.012
1570-9639/

is because of the stability of its catalytic domain, the light chain, inside neuronal cells [13,14]. The basis of differential stability of light chains of different BoNT serotypes is critical to develop countermeasures against botulism, and more effective therapeutic products. Possible reasons for the long term persistence of BoNT/A light chains could be phosphorylation and ubiquitination [15,16], which may depend on the structural and conformational state of the light chains.

Crystal structures of BoNT LCs suggest a high level of similarity in secondary and tertiary structures, as well as identical active site motif (HEXXH) and other active site participating residues (Y, R and E), which have little to offer as an explanation for the differential intracellular behavior of these molecules. Since light chains are the catalytic moieties of BoNTs, which upon entering the cell are responsible for the toxicity in neuronal cells, it is important to examine the structural features of the light chains of different BoNT serotypes. Comparison of LC structure of BoNT/A, BoNT/B, and BoNT/E may provide us clues to understand the differential behavior of these molecules.

In this work, we have examined the structural features of BoNT/A, B and E light chains to look for the elements of the PRIME structure, which has already been established for the BoNT/A light chain [17]. We have addressed the question as to whether the PRIME structure is a common structural feature involved in the function of these molecules. Our observations suggest that the BoNT/B light chain exhibits the PRIME structure which is responsible for its optimal activity at 37 °C, similar to BoNT/A LC. However, existence of PRIME conformation is not clearly visible for BoNT/E LC, despite its optimal activity at 37 °C.

2. Materials and methods

2.1. Expression and purification of BoNT/A, BoNT/B and BoNT/E light chains

All the light chains were purified on Ni^{2+} column according to the method described earlier [18]. BoNT/A and BoNT/B light chains were purified in phosphate buffer (10 mM sodium phosphate pH 8.0, containing 300 mM NaCl) but due to the solubility problem BoNT/E was purified in Tris-buffer (50 mM Tris pH 8.0, containing 500 mM NaCl). Prior to conducting experiments, all light chains were dialyzed in 10 mM phosphate buffer, pH 7.3, containing 150 mM NaCl and 1 mM DTT.

2.2. CD spectroscopy

CD spectra were measured at 25 °C on Jasco J715 CD spectrophotometer (Jasco Inc., Easton NJ) equipped with a Peltier type temperature controller (Model PTC-348 W). For secondary and tertiary structure measurements by CD spectral analysis in far-UV and near-UV regions, respectively, 0.2–0.3 mg/ml and 0.5 mg/ml concentrations of LC were used. For secondary structure analysis, spectra were recorded between 250 nm and 190 nm, and for tertiary structure, spectra were recorded between 310 nm and 250 nm. Spectral recording was carried out at a speed of 20 nm/min with a response time of 8 s, using cuvettes with different pathlength for far-UV (1 mm) and near-UV (1 cm). A total of three scans were recorded and averaged to increase the signal to noise ratio. The final spectra were obtained after correcting the buffer contribution. Secondary structure content was determined by using the Jasco secondary structure estimation program, which is based on the algorithm of Yang et al. [19].

For thermal denaturation experiments, protein was heated to a given temperature, and the CD spectrum was recorded after incubating the sample at that temperature for 5 min.

For thermal unfolding of the secondary structure, CD signal at 222 nm was monitored with a heating rate of 1 °C/min between 25 °C and 90 °C. High concentration of protein required for the near-UV CD signal resulted in protein aggregation at high temperatures. Therefore, it was not possible to monitor tertiary structure changes with CD.

2.3. Urea denaturation of BoNT endopeptidase

Recombinant full length LCA, LCB and LCE were dialyzed against the phosphate buffer (10 mM sodium phosphate, 150 mM NaCl, 1 mM dithiotritol, pH 7.3), and diluted to a fixed concentration of LC (0.2 mg/ml) by a series of varying urea concentrations (at least 0.5 M apart) from a 10 M stock urea solution yielding a final urea concentration in the sample between 0 and 8 M. All the mixtures were equilibrated for at least 2 h at 25 °C before recording CD spectra. CD spectrum was recorded between 250 nm and 190 nm.

Circular dichroism (CD) spectra were recorded in the far-UV region at 25 °C under similar conditions as described above, and the data was plotted for signal at 222 nm for each LC–urea concentration.

2.4. Fluorescence spectroscopy

Fluorescence measurements of BoNT LCs were carried out using the ISS K2 fluorimeter. Protein solutions (0.1 mg/ml) were excited at 280 and 295 nm, and emission spectra were recorded between 310 and 400 nm. Experiments were carried out in 10 mm fluorescence cuvette. Excitation and emission slits width were fixed at 4 nm.

Thermal denaturation of tertiary structure was monitored by fluorescence. For measuring thermal denaturation by fluorescence we used the ratio of F_{351}/F_{316} and F_{351}/F_{306} for LCB and LCE, respectively. F is fluorescence intensity, and numbers represent wavelengths of emission. Samples were heated from 25 to 90 at a rate of 1 °C/min. Excitation wavelength for thermal denaturation experiment was 280 nm.

2.5. Second derivative UV spectroscopy

Absorption spectra of LC dissolved in buffer (10 mM sodium phosphate pH 7.3, containing 150 mM NaCl and 1 mM DTT) were recorded between 225 and 330 nm on a Simadzu UV spectrophotometer, attached with a water bath to control temperature. The spectra were derivatized to the second order having 0.2 nm resolution and delta lambda. The ratio of a (an arithmetic sum of the negative $d^2A/d^2\lambda$ at 284 nm and positive $d^2A/d^2\lambda$ 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.

2.6. ANS binding

ANS (1,8-anilinonapthalenesulfonate) is a dye whose fluorescence is strongly quenched by water, and undergoes a dramatic increase in fluorescence intensity when it binds to hydrophobic regions of a protein molecule. ANS binding was performed for BoNT/B and BoNT/E LCs. ANS was titrated into 1 ml of 1 μM of protein solution in 10 mM sodium phosphate pH 7.3, containing 150 mM NaCl and 1 mM DTT, in a 1 cm path length cuvette. BoNT/B and BoNT/E LC each was titrated to 70 μM and 80 μM ANS, respectively, to reach the fluorescence saturation. Excitation wavelength was 370 nm, and emission spectra were recorded from 410 nm to 510 nm. Excitation and emission slit widths were fixed at 4 and 8 nm, respectively. Fluorescence intensities were measured at different temperatures, after the solution was incubated for 5 min at each temperature.

2.7. Endopeptidase assay

BoNT/B LC (LCB) was assayed for endopeptidase activity by using Vamptide (OBZ/DNP; List Biological, Campbell, CA) as its substrate. This peptide is intramolecularly quenched by FRET (fluorescence resonance energy transfer). The FRET assay was carried out at a given temperature with excitation at 321 nm and emission 418 nm. Excitation and emission slit widths were fixed at 4 nm. Before adding substrate, LCB was incubated at the designated temperature for 30 min. For the endopeptidase activity of LCB, the concentrations of the Vamptide and