Biochimica et Biophysica Acta 1854 (2015) 321–326

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Resolution of sub-nanosecond motions in botulinum neurotoxin endopeptidase: An evidence of internal flexibility☆

Raj Kumar [a], Shuowei Cai [b], Emmanuel Ojadi [b], Bal. R. Singh [a,*]

[a] Botulinum Research Center, Institute of Advanced Sciences, Dartmouth, MA 02747, USA
[b] Department of Chemistry and Biochemistry, University of Massachusetts, Dartmouth, MA 02747, USA

ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form 2 December 2014
Accepted 5 December 2014
Available online 9 January 2015

Keywords:
Fluorescence anisotropy decay
Molten globule
Protein flexibility
Botulinum toxin
Circular dichroism
Protein denaturation

ABSTRACT

Botulinum neurotoxins (BoNTs) are the most poisonous substances known to mankind, which act on the peripheral nervous system leading to flaccid paralysis. Although co-crystal structure of BoNT/A light chain (LC) reveals some unique features of the biological function of this molecule, structural characteristics in solution reveal its dynamic features, not available through the published crystal structures. In this study, we have examined internal flexibility of this molecule by measuring rotational correlation time as a function of viscosity, using frequency domain fluorescence anisotropy decay technique. Fluorescence anisotropy decay of BoNT/A LC resolved sub-nanosecond local motion (faster component), interpreted as internal flexibility of the molecule was affected significantly with viscosity. Both local and global movements were affected by viscosity, which indicates the accessibility of protein core and flexibility of overall structure. In conclusion, this work demonstrates the presence of flexibility in the internal peptide segments, which appears to play a significant role in BoNT/A LC biological function.

1. Introduction

Botulinum neurotoxins (BoNTs) are metalloproteinases which act on peripheral nerves and cleave one of the crucial components of neuroexocytosis, causing inhibition of acetylcholine release at nerve–muscle junctions. Toxin molecules have evolved with unique characteristics of high stability, specificity, and selectivity. These molecules are currently used to treat several neuromuscular disorders, such as strabismus, blepharospasm, hemifacial spasm and cervical dystonia, because of their biological activity at very low concentration exclusively at neuro-muscular junctions [1,2]. Because of extreme toxicity, BoNTs are also considered Class A biothreat agents. While all confirmed serotypes of BoNT (A–G) are highly toxic, and can be used both as biothreat and therapeutic agents, botulinum neurotoxin A (BoNT/A) is the most toxic, and has been most successfully used for the treatment of many disorders, and also for cosmetic purposes [3]. Structure–function relationship of BoNTs plays a critical role in developing further therapeutic applications and also in devising antidotes against botulism.

Each of the BoNTs has a unique trimodular structure consisting of a 50 kDa binding domain (H_{CC}), a 50 kDa translocation domain (H_{CN}), and a 50 kDa catalytic domain (LC). Since this protein has evolved with amazing specificity, and very unusual mechanism of substrate recognition involving active site and exosites, it is likely to possess substantial flexibility in its structure to accommodate a large surface interaction with the substrate. The solution structure of BoNT/A is flexible and different from the crystal structure [4–8]. Silvaggi et al. [9] have in fact observed limited flexibility of the active site, although those changes would not necessarily explain differential interaction of BoNT endopeptidases with their respective substrates. Published crystal structures explain some of these phenomena [10–13], including the role of two exosites, α and β. However, there is a lack of consistent structural information of the biologically active enzyme, which appears to exist as a molten globule [5,14]. For example, the crystal structure does not explain satisfactorily the role of SNARE motifs of SNAP-25, which was established earlier by Washbourne et al. [8].

Structural characteristics in solution reveal a major role of the dynamic structure in the functioning of a protein molecule which is important for the design of effective inhibitors. It has been already observed that inhibitors identified for in vitro experiments failed to work under in vivo conditions and vice versa [15], the endopeptidase active site shows substantial flexibility [9], suggesting that structural dynamics may play a major role in the interaction of BoNT endopeptidase with the inhibitors. An understanding of molecular dynamics of the BoNT/A endopeptidase itself may reveal a variety of ways to inhibit its action and rescue poisoned nerve cells.

Abbreviations: BoNT, botulinum neurotoxin; BoNT/A LC, botulinum neurotoxin light chain (LCA); LED, light emitting diode; DTT, dithiothreitol; CD, circular dichroism; RCT, rotational correlation time

☆ This work was in part supported by a grant from the NIH (1U01A1078070-01) and the US Army Medical Research (W81XWH-08-P-0705 and W81XWH-14-C-0070).

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

http://dx.doi.org/10.1016/j.bbapap.2014.12.004
1570-9639/

To unravel the dynamic molecular features, we have examined the internal flexibility of BoNT/A LC. Structural changes in BoNT/A LC with viscosity have been monitored by circular dichroism, fluorescence anisotropy, and correlated with its endopeptidase activity. Fluorescence and circular dichroism showed significant conformational change in BoNT/A LC with the viscosity of the solution. Frequency domain fluorescence anisotropy decay analysis provided impressive resolution of rapid and complex decays of fluorescence anisotropy that allows discrimination between static and dynamic state of micro-environment of BoNT/A endopeptidase in solution. Results showed strong internal flexibility in BoNT/A LC molecule, which may be important for its substrate specificity and other biological functions.

2. Material and methods

2.1. Material

BoNT/A LC was purified according to the procedure described previously [6] with some modifications. All the samples were dialyzed in 10 mM sodium phosphate buffer, containing 150 mM NaCl and 1 mM DTT, pH 7.3, and purity was checked by SDS-PAGE. Coomassie blue staining showed essentially a single band for all the batches of LC used in this study. His-tag SNAG (a fusion protein consisting of SNAP-25, GFP, and HIS tag, batch no. SNAG-His6169SR, MW ~55 kDa) [16] was used as substrate for activity measurement.

2.2. CD spectroscopy

CD spectra were recorded at 25 °C on a Jasco J715 spectropolarimeter equipped with a Peltier type temperature control system (model PTC-348W). The concentrations of LC samples in the range of 20–300 μg/ml were used for far-UV CD measurements. Temperature-induced unfolding of the LC polypeptide was followed by monitoring the CD signal at 222. LC samples dissolved in a buffer (10 mM sodium phosphate buffer, pH = 7.3, containing 150 mM NaCl) alone or with buffer containing different concentrations (10–50%) of glycerol, were heated at a rate of 1 °C/min, and the ellipticity was recorded at 222 nm.

2.3. Fluorescence measurement

Thermal denaturation of tertiary structure was monitored by recording intrinsic Trp fluorescence. Fluorescence recording was carried out by ISS K2 fluorimeter (Urbana-Champaign, IL). Excitation and emission slit width was 8 nm. To minimize inner filter effect, the absorbance of BoNT/A LC at 295 nm was kept below 0.1. For following the thermal denaturation by fluorescence we plotted the ratio of fluorescence intensity at 351 nm and 324 nm as a function of temperature. Samples were heated from 25 to 90 °C at a rate of 1 °C/min.

2.4. Analysis of fluorescence anisotropy decay measurements

Frequency-domain fluorimetry has been demonstrated earlier for the measurement of complex and/or rapid decay of protein fluorescence anisotropy. In this experiment we wanted to distinguish local mobility of intrinsic Trp probe of the protein from the global rotation of the protein. Time-resolved anisotropies are obtained from the frequency (ω)-dependent phase angle difference between the perpendicular (φ) and parallel (φ_{II}) components of the modulated emission ($\Delta_\omega = \varphi - \varphi_{II}$) and from the ratio of the amplitudes of the modulated component of the emission ($\Lambda_\omega = m_{II}/m_\perp$). These values were fit through a multiexponential anisotropy decay law

$$r(t) = \sum r_i g_j e^{-t/\varphi i} \tag{A}$$

where φ_i is the correlation time, g_i is the associated amplitude, and r_i is the anisotropy of each component. The expected values of Δ_ω ($\Delta_{c\omega}$) and Λ_ω ($\Lambda_{c\omega}$) are calculated from the sine and cosine transforms of the individual polarized decays.

$$Ni = \int I_i(t)\ \sin\ \omega t\ dt \tag{B}$$

$$Di = \int I_i(t)\ \cos\ \omega t\ dt \tag{C}$$

The frequency-dependent values of Δ_ω and Λ_ω are given by

$$\Delta_{c\omega} = \arctan\{(D\,N - N\,D)/(N\,N + D\,D)\} \tag{D}$$

$$\Lambda_{c\omega} = \left\{\left(N^2 + D^2\right)/\left(N^2 + D^2\right)\right\} \tag{E}$$

where *Ni* and *Di* are calculated at each frequency. The parameters describing the anisotropy decay are obtained by minimizing the squared deviations between measured and calculated values,

$$\chi^2 = \sum (\Delta_\omega - \Delta_{c\omega})^2/\sigma^2{}_{\Delta\omega} + \sum\left((\Lambda_\omega - \Lambda_{c\omega})^2/\sigma^2{}_{\Lambda\omega}\right). \tag{F}$$

In this expression $\sigma_{\Delta\omega}$ and $\sigma_{\Lambda\omega}$ are the estimated experimental uncertainties in the measured quantities (Δ_ω and Λ_ω). The goodness-of-fit is usually judged by the value of reduced χ^2,

$$\chi_R{}^2 = \chi^2/\upsilon = \chi^2/2N\text{–}p$$

where υ, the number of degree of freedom, is given by $\upsilon = 2\,N - p$. N is the number of modulation frequencies and p is the number of floating parameters. If the assumed model is appropriate for the sample, and if the errors are random and properly estimated by $\sigma_{\Delta\omega}$ and $\sigma_{\Lambda\omega}$, then χ^2_R is expected to be near unity.

Eq. (A) can be expressed in another form:

$$r(\tau) = \frac{\alpha r_0}{1 + (1/\varphi_\tau + 1/\varphi_P)\tau} + \frac{(1-\alpha)r_0}{1 + \tau/\varphi_P}. \tag{G}$$

When the internal motion (φ_τ) of the protein is more rapid than the overall protein rotation (φ_P), then $\varphi_T \ll \varphi_P$, and the equation can be simplified as Eq. (H).

$$r(\tau) = \frac{\alpha r_0}{1 + \tau/\varphi_\tau} + \frac{(1-\alpha)r_0}{1 + \tau/\varphi_P} \tag{H}$$

Relative importance of each motion can be determined by the amplitude α and by the ratio of τ/φ_T and τ/φ_P. If $\alpha = 0$, the residue is immobile relative to the macromolecule, and the anisotropy is determined by the value of φ_P. If the residue is completely free to rotate, independent of the protein molecular motion, then $\alpha = 1$, and the anisotropy would be determined by φ_T. Now suppose the α is nonzero but less than one, and the overall rotational time is much larger than the fluorescence life time $\varphi_P \gg \tau$, then the rotation of the entire protein does not result in any loss of anisotropy, and the values for $r(\tau)$ are determined by the time scale of the internal motion (φ_T) and by the amplitude of this motion (α). If an internal motion is more rapid than the shortest quenched lifetime, $\varphi_T \ll \tau$, the effect of internal motion is complete prior to emission, and the overall rotation of the protein determines the decay of the residual anisotropy, $r_0(1 - \alpha)$. The existence of internal motion would be judged from the value of the anisotropy at $\tau = 0$.

In general, one can relate the anisotropy to the angular displacement (θ), also known as semicone angle, of the fluorophore according to

$$\theta = \cos^{-1}\left\{1/2\left[\left(1 + 8(r_\alpha/r_0)^{1/2}\right)^{1/2} - 1\right]\right\}. \tag{I}$$

A value of 0° corresponds to no rotational freedom, and an angle of 54.7° corresponds to complete depolarization.