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Structural and functional analysis of botulinum neurotoxin subunits for pH-dependent membrane channel formation and translocation

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ABSTRACT

The structure–function relationship of Botulinum Neurotoxin (BoNT) proteins is greatly influenced by pH. While the low pH of endosome favors membrane interaction of the heavy chain (HC) for the formation of a membrane channel and translocation of the light chain (LC), the catalytic activity of the LC requires a neutral pH for cleavage of the soluble NSF attachment protein receptor (SNARE) complex in the cytosol. In this study, we monitored secondary structural characteristics of LC, HC and holotoxin at individual pHs 4.5 and 7.2 and at the transition pH 4.5 to 7.2 to identify the structural signatures underlying their function. The HC showed higher thermal stability at pH 4.5 with a melting temperature (T_m) of 60.4 °C. The structural analysis of HC in the presence of liposomes showed no difference in ellipticity with that of HC at pH 7.2 at 208 and 222 nm but a 25.2% decrease in ellipticity at 208 nm at acidic pH, indicating low pH-induced structural changes that might facilitate interaction with the membrane. Further, HC showed 18% release of K^+ ions from liposomes at pH 4.5 as against 6% at neutral pH, reinforcing its role in membrane channel formation. LC on the other hand, showed maximum ellipticity at pH 7.2, a condition that is relevant to its endopeptidase activity in the cytosol of the neurons. Also, the similarity in the structures at pH 7.2 and transition pH 4.5 to 7.2 suggested that the flexibility acquired by the protein at low pH was reversible upon exposure to neutral pH for cleavage of SNARE proteins.

1. Introduction

The 150 kDa BoNT causes flaccid muscle paralysis by inhibiting the acetylcholine neurotransmitter release at the neuromuscular junctions. The toxin molecule is made up of a 100 kDa HC subunit and a catalytic active LC domain of 50 kDa connected by a disulfide bond [1]. The mode of action of the toxin involves binding of the C-terminal portion of HC to the presynaptic nerve terminal through a dual protein and ganglioside receptor which then gets internalized by endocytosis [2]. Once inside the endosomes, the acidic pH of the lumen triggers structural changes in the N-terminal region of HC for the formation of an endosomal membrane channel and translocation of LC into the cytoplasm [3,4]. Subsequently, LC cleaves specific SNARE proteins involved in the fusion of synaptic vesicle with the neuronal membrane, thereby preventing the release of acetylcholine neurotransmitter from the vesicles [2,3,5,6]. In this mechanistic process, key questions that have been raised are 1) how does the low pH affect the membrane channel formation by HC and translocation of LC and 2) once exposed to acidic pH, does the light chain regain its structure to carry out its endopeptidase activity at neutral pH?

Low pH studies on HC pertaining to its role in the membrane channel formation have been conducted by various research groups using channel conductance assays [7,8]. Since BoNT is a water soluble protein, the interaction with the endosomal membrane requires it to acquire a hydrophobic character. Once internalized in the endosomes, acidic pH likely triggers conformation changes in the N-terminal HC portion, thus favoring hydrophobic interactions with the membrane [9,10]. The work by Cai *et al* (2006) on the effect of low pH on LC structure revealed a flexible molten globule conformation adopted by LC potentially for its endosomal translocation [11]. Further, Li *et al* (2000) demonstrated that after re-exposure to neutral pH, LC regains its structural features required for carrying out its endopeptidase activity in the cytosol [6]. In an earlier work from our group by Fu *et al* (2002), we had demonstrated the effect of low pH on BoNT/A HC and LC showed increased membrane interaction of these proteins with low pH exposure [12]. However, research in the current literature is limited to the investigation of only some structural aspects of the BoNT proteins, and secondary structural analysis of BoNT/A HC in its membrane bound state was not possible

Abbreviations: BoNT, botulinum neurotoxin; DrBoNT, deactivated recombinant botulinum neurotoxin; LD, lethal dose; LC, light chain; HC, heavy chain; CD, circular dichroism; SNARE, soluble NSF attachment protein receptor; T_m, melting temperature; PC, phosphatidylcholine; PS, phosphatidylserine; Chol, cholesterol

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due to liposome aggregation. Since there is a growing interest in using HC and LC for drug delivery, it is important to carry out detailed structural analysis of these proteins in its membrane bound state.

In this study, we carried out far-UV CD and thermal denaturation structural analysis of BoNT/ A LC, BoNT/A HC and BoNT/A toxin at acidic and neutral pH conditions, and have examined the reversibility of their structural characteristics. We further investigated membrane interaction and channel formation by HC using a liposomal model. The results indicate structural changes in HC and increased K^+ release from the liposome vesicles upon addition of HC at pH 4.5, indicating its involvement in membrane channel formation.

2. Experimental methods

2.1. Protein expression and purification

BoNT/A toxin was isolated from *Clostridium botulinum* type A (Hall strain) [13] and the BoNT/A HC and LC were isolated from the deactivated recombinant BoNT/A according to the method of Sathyamoorthy and DasGupta [14].

2.2. Secondary structural analysis of BoNT/A proteins

BoNT/A LC and holotoxin were dialyzed in respective pH buffers—10 mM sodium phosphate buffer containing 50 mM NaCl (pH 7.2) and 10 mM sodium phosphate buffer containing 50 mM NaCl (pH 4.5). For the transition pH, the proteins were dialyzed in each pH buffer and the concentration was estimated by BCA assay (Thermofisher Scientific, PA). BoNT/A HC was dialyzed in the same buffers but with a higher salt concentration of 200 mM NaCl since lower salt concentrations resulted in protein precipitation. CD data were collected using JASCO J-715 spectropolarimeter (Jasco Inc., MD) equipped with a Peltier temperature control. Far-UV CD spectra in the 190–250 nm wavelength region was recorded in a 1.0 mm pathlength cuvette for a total of 3 scans with a protein concentration of 0.1–0.3 mg/mL at 25 °C. The scan speed was 20 nm/min. and the response time was 8 s. The percentage of individual secondary structural elements was calculated using the method of Yang *et al* [15]. For the thermal denaturation analysis, the CD signal at 222 nm was monitored in the temperature range of 25–90 °C with a heating rate of 2 °C/min. The thermodynamic parameters were calculated from the melting curves using the Van't Hoff equation.

2.3. CD spectra of BoNT/A HC–liposome complex

Liposomes were prepared by extrusion procedure according the method of Mayer *et al* [16]. Briefly, 0.4 mg/mL of lipid containing phosphatidylcholine (PC) [17], phosphatidylserine (PS) and cholesterol (7:1:2 molar ratio) was purged with N_2 to remove the organic solvent chloroform, resulting in the creation of a thin film. The lipid film was then vacuum dried for 2 h to remove the traces of organic solvent and was rehydrated with 10 mM sodium phosphate buffer, containing 200 mM NaCl, pH 7.2 or 4.5. The liposome solution was then extruded through a mini extruder (Avanti polar lipids, Alabaster, AL) of 100 nm filter size for eleven times, leading to the formation of small and unilamellar liposomes. The liposomes in neutral and acidic pH buffers were incubated with 0.2 mg/mL of BoNT/A HC for 30 min at 37 °C and then subjected to CD analysis using the same conditions used for secondary structural analysis of pure proteins.

2.4. K^+ ion release assay for membrane channel formation by BoNT proteins

Liposome containing 12.5 mg/mL of asolectin and cholesterol lipids (Sigma Aldrich Inc., St. Louis, MO) in 2:1 mole ratio was prepared by thin film hydration approach [18]. A thin lipid film was formed by N_2 purging to remove the organic solvent. Residual traces of organic solvent were removed by vacuum drying for 2 h and rehydrated with K^+ rehydration buffer containing 100 mM potassium phosphate buffer, pH 7.2, containing 1.5 mM EDTA. Liposomes were incubated with 200 nM of HC and deactivated recombinant variant of BoNT toxin (DrBoNT) in pH 4.5 and pH 7.2 buffers for 1 min and the concentration of K^+ ions released was measured with the K^+ ion selective electrode (Orion Star, Thermo Scientific, Pittsburgh, PA). Gramicidin and trypsin served as positive and negative controls, respectively. For estimating the total release of potassium ions from liposomes, 0.5% of octylglucoside detergent (Sigma Aldrich Inc., St. Louis, MO) was used.

3. Results

3.1. Secondary structural analysis of BoNT proteins

3.1.1. BoNT/A LC

The far-UV CD spectra for LC at pHs 7.2 and 4.5 were similar with broad minima at 222 nm and 208 nm, which is typical for α-helical proteins. The molar ellipticities at 222 nm were -5×10^3 deg cm^2/dmol and -4×10^3 deg cm^2/dmol at pHs 7.2 and 4.5, respectively (Fig. 1a). The reversibility of pH from pH 4.5 to 7.2 also yielded CD pattern similar to that of pH 7.2 (Fig. 1a). It was observed that the acidic–neutral pH transition was not structurally as stable as the acidic pH transition and hence there was substantial protein precipitation observed. In such a case, the sample was centrifuged to remove aggregates before CD spectral recordings. From the CD spectra, the secondary structure estimations were carried out. At pH 7.2 and pH 4.5, the % of α-helix, β-sheet, β-turn and random coil were 21.2 ± 4.2%, 31.8 ± 7.1%, 22.5 ± 0.9% and 24.5 ± 1.9% and 22.4 ± 1.9%, 28.6 ± 0.2%, 23.9 ± 1.9%, and 25.1 ± 0.2%, respectively. The transition from acidic to neutral pH was marked by a decrease in β-sheet from 28.6 ± 0.2% to 6.6 ± 1.6% and increase in β-turn and random coil structures from 23.9 ± 1.9% and 25.1 ± 0.2% to 31.5 ± 3.3% and 38.2 ± 2.3%, respectively (Table 1).

The thermal denaturation pattern monitored at 222 nm showed a typical S-shaped denaturation pattern at pHs 7.2 and 4.5 to 7.2 with T_m at 48.0 ± 0.4 °C for pH 7.2 (Fig. 2a). However, at pH 4.5, no significant change in ellipticity with temperature was observed. In fact there was a slight increase in ellipticity from -3.5×10^3 deg cm^2/dmol to -4.0×10^3 deg cm^2/dmol (T_m = 44.4 °C) at acidic pH, which was then stabilized until the temperature reached 90 °C (Fig. 2a). From the thermal denaturation analysis, the thermodynamic parameters were calculated using the classical Van't Hoff equation. The thermodynamic parameters at pH 7.2 and pH 4.5 to 7.2 were expectedly in close correlation with each other with ΔH, ΔS and ΔG values of 277.7 ± 25.5 kJ/mol, 861.4 ± 79.5 J/mol K and 21.0 ± 1.8 kJ/mol at pH 7.2 and 294.7 ± 56.8 kJ/mol, 912.5 ± 176.5 J/mol K and 22.8 ± 4.2 kJ/mol at pH 4.5 to 7.2, respectively (Table 2).

3.1.2. BoNT/A HC

Far-UV CD spectra for HC at pHs 7.2 and 4.5 are shown in Fig. 1b. The molar ellipticity was similar at both acidic and neutral pH. From the secondary structural predictions, the % of α-helix, β-sheet, β-turn and random coil were calculated to be 27.9 ± 0.4%, 27.3 ± 0.9%, 17.2 ± 1.6% and 27.6 ± 0.1% and 29.5 ± 0.9%, 22.2 ± 2.8%,18.2 ± 2.2% and 30.8 ± 2.1% at pHs 4.5 and 7.2, respectively (Table 1).

Thermal melts recorded at 222 nm for pHs 7.2 and 4.5 are shown in Fig. 2b. A typical S-shaped denaturation pattern was observed at neutral and acidic pHs with T_m around 51.0 ± 0.4 °C at pH 7.2 and 60.4 ± 0.2 °C and at pH 4.5 indicating that the protein was thermally more stable under acidic pH condition. The thermodynamic parameters for HC unfolding also showed that the protein was conformationally more stable at acidic pH. The values of ΔH, ΔS and ΔG are listed in Table 2.

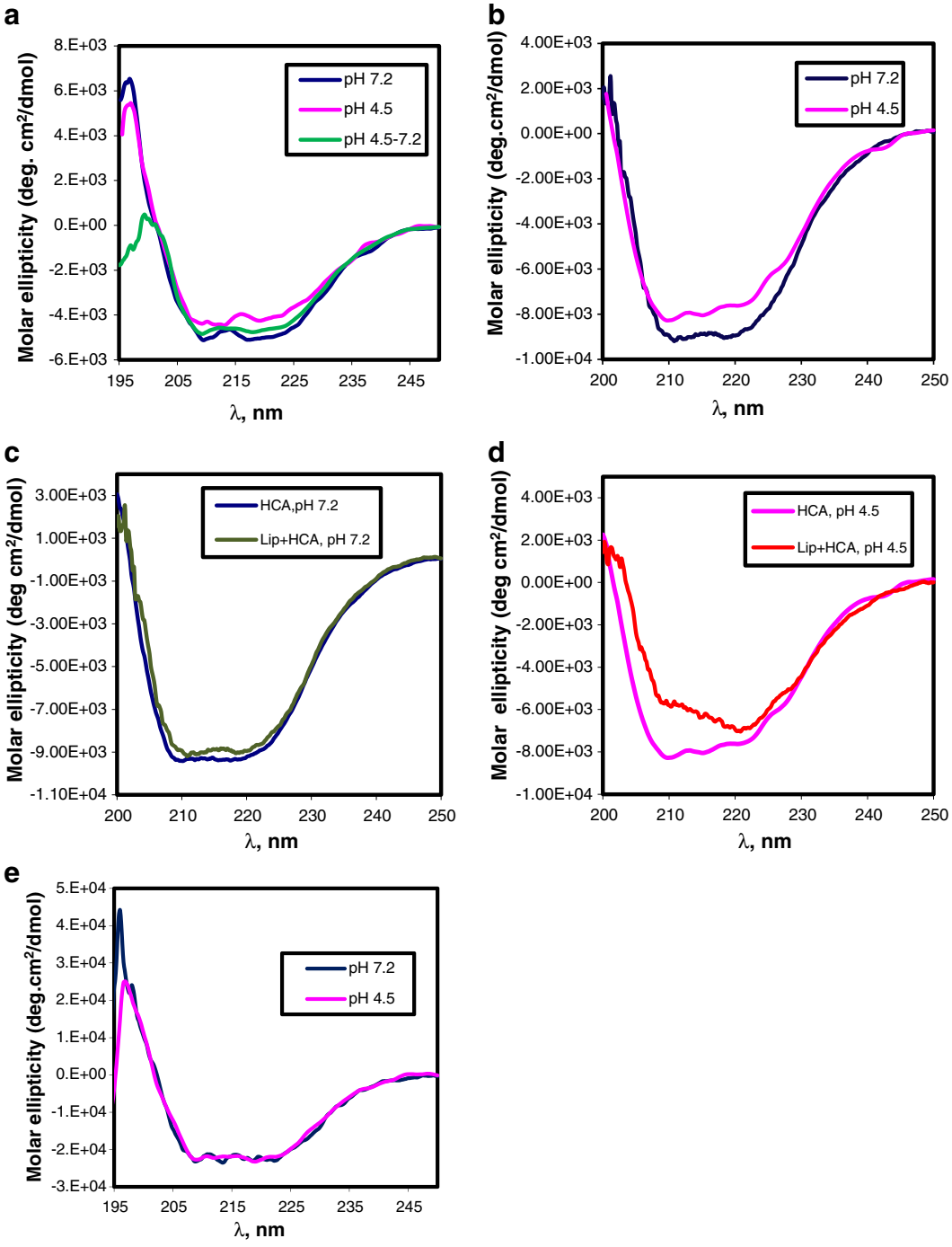

Fig. 1. Far-UV CD spectra of BoNT/A proteins—(a) purified BoNT/A LC, (b) purified BoNT/A HC, (c) HC–liposome complex, pH 7.2, (d) HC–liposome complex, pH 4.5 and (e) purified BoNT/A holotoxin. 0.2 mg/mL protein in 10 mM sodium phosphate and 50 mM NaCl pH 7.2 and 10 mM citrate-phosphate buffer and 50 mM NaCl, pH 4.5 was used for the spectral analysis in a 1.0 mm pathlength cuvette for a total of 3 scans at 25 °C. For liposome CD analysis, liposomes were synthesized using 0.4 mg/mL of lipid containing PC, PS and Chol (7:1:2 molar ratio) in 10 mM sodium phosphate buffer containing 200 mM NaCl. The liposomes were incubated with 0.2 mg/mL of BoNT/A HC for 30 min at 37 °C and then subjected to CD analysis. The scan speed was 20 nm/min and the response time was 8 s.

Table 1
% of secondary structural fractions from the far-UV CD spectra of three BoNT/A proteins—(a) BoNT/A LC, (b) BoNT/A HC and (c) BoNT/A holotoxin at different pH conditions estimated using the method of Yang *et al.*

pH	(a) BoNT/A LC				(b) BoNT/A HC				(c) BoNT/A holotoxin			
	α-helix (%)	β-sheet (%)	β-turn (%)	RC (%)	α-helix (%)	β-sheet (%)	β-turn (%)	RC (%)	α-helix (%)	β-sheet (%)	β-turn (%)	RC (%)
7.2	21.2 ± 4.2	31.8 ± 7.1	22.5 ± 0.9	24.5 ± 1.9	29.5 ± 0.9	22.2 ± 2.8	18.2 ± 2.2	30.8 ± 2.1	18.6 ± 0.7	42.0 ± 6.6	18.3 ± 3.1	21.2 ± 2.8
4.5	22.4 ± 1.9	28.6 ± 0.2	23.9 ± 1.9	25.1 ± 0.2	27.9 ± 0.9	27.3 ± 0.9	17.2 ± 1.6	27.6 ± 0.1	22.2 ± 3.8	27.4 ± 11.6	25.5 ± 4.6	24.8 ± 3.3
4.5–7.2	23.8 ± 0.5	6.6 ± 1.6	31.5 ± 3.3	38.2 ± 2.3	–	–	–	–	–	–	–	–

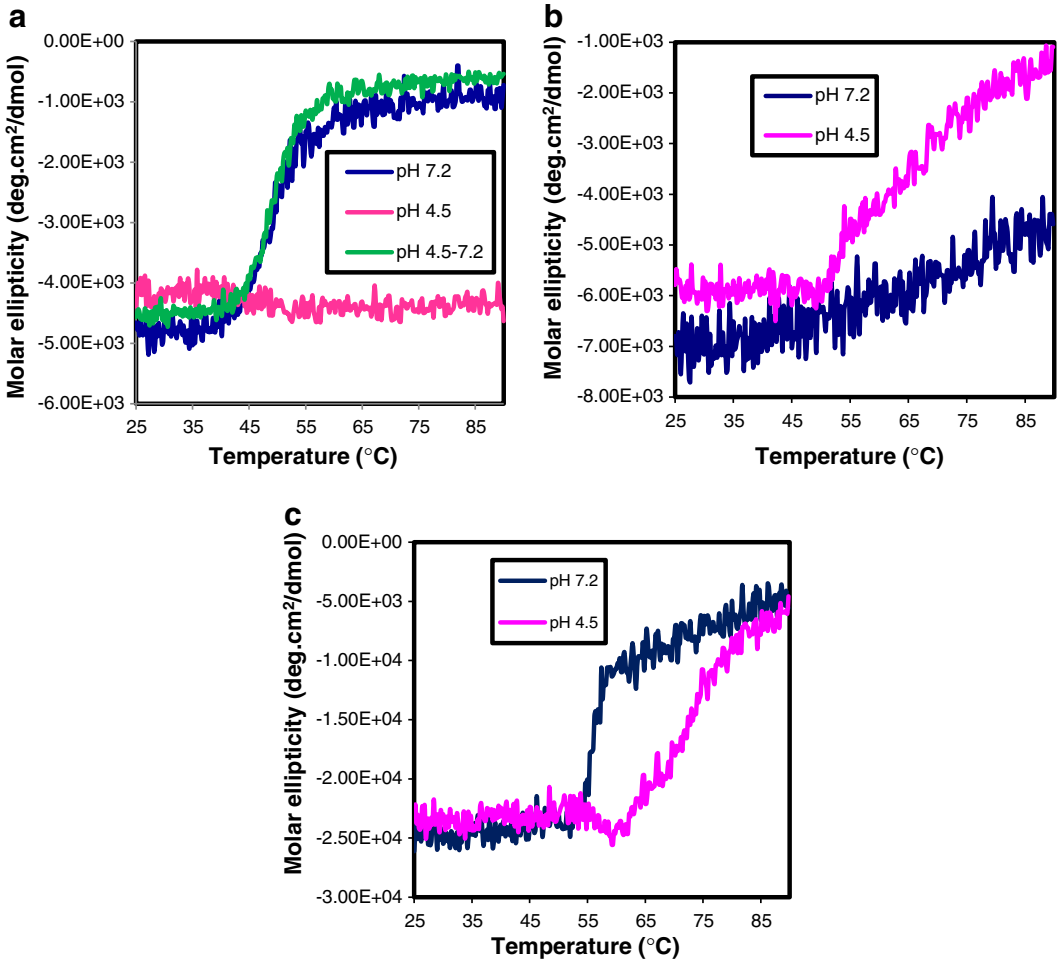

Fig. 2. Thermal unfolding pattern of BoNT/A proteins—(a) purified BoNT/A LC, (b) purified BoNT/A HC and (c) purified BoNT/A holotoxin measured at pHs 7.2, 4.5 and 4.5 to 7.2 by monitoring the ellipticity at 222 nm. The protein buffers used for spectral recordings were 10 mM sodium phosphate and 50 mM NaCl, pH 7.2 and 10 mM citrate-phosphate buffer and 50 mM NaCl, pH 4.5. The spectrum is an average of three spectral scans in the temperature range of 25 °C–90 °C. The rate of heating was 2 °C/min and the response time was 8 s.

3.1.3. BoNT/A HC–liposome complex

To determine the structural changes accompanying low pH induced membrane interaction of HC, the CD spectra of HC in the presence of liposomes were extracted at pHs 7.2 and 4.5 as shown in Fig. 1c & d. At pH 7.2, the secondary structures of HC and HC–liposome yielded a similar ellipticity value of -8.65×10^3 deg cm^2/dmol at both 222 nm. However, at pH 4.5, the CD spectra of HC in the presence of liposomes showed no significant changes in ellipticity at 222 nm but 25.2% decrease in ellipticity at 208 nm compared to that of HC. The blank liposomes not treated with the protein did not yield any CD signal at both neutral and acidic pHs (data not shown).

3.1.4. BoNT/A holotoxin

Fig. 1e depicts the far UV-CD spectra for purified BoNT/A at pHs 7.2 and 4.5. The CD signal intensity was similar at both pH 7.2 and 4.5 with the molar ellipticity at 222 nm at -2.0×10^4 deg cm^2/dmol., a value that was 4.0 and 2.3-fold higher than that of the constituent LC and HC subunits, respectively. The secondary structural analysis showed differences in the % of secondary structural elements with pH changes. The % of α-helix, β-sheet, β-turn and random coils were 18.6 ± 0.7%, 42.0 ± 6.6%, 18.3 ± 3.1% and 21.2 ± 2.8% at pH 7.2 and 22.2 ± 3.8%, 27.4 ± 11.6%, 25.5 ± 4.6% and 24.8 ± 3.3% at pH 4.5 (Table 1).

The thermal unfolding curves of BoNT/A showed a distinct denaturation pattern at neutral and acidic pH conditions as shown in Fig. 2c. At pH 7.2, a steep unfolding curve was observed with T_m of 55.6 ± 0.3 °C whereas at pH 4.5, there were two T_m observed at 57.0 ± 0.2 °C and 71.0 ± 3.0 °C. Quite uniquely, the first transition was marked by an increase in CD signal with temperature, which then decreased, giving rise to the second unfolding pattern.

Estimation of the thermodynamic parameters from the denaturation curve indicated that the protein is thermodynamically more stable at pH 4.5 than at pH 7.2. For the second transition, the enthalpy (ΔH) and entropy (ΔS) were 255.2 ± 7.8 kJ/mole and 737.9 ± 21.8 J/mole K at pH 4.5 and 589.3 ± 36.6 kJ/mole and 1782.8 ± 112.2 J/mole K at pH 7.2, respectively. The free energy change (ΔG) associated with thermal denaturation at pHs 4.5 and 7.2 was 35.3 ± 1.3 kJ/mole and 57.9 ± 3.2 kJ/mole, respectively (Table 2).

3.2. K^+ release assay for membrane channel formation by BoNT proteins

The results as shown in Fig. 3 revealed that the release of K^+ ions with BoNT/A HC was around 18.3% at pH 4.5 as against 6% at neutral pH. The positive control gramicidin showed the highest K^+ ion release with 49% and 45%, respectively at both the acidic and neutral pH. With trypsin as a negative control, the K^+ ion release was 3.8% at pH 7.2 and 6.0% at acidic pH. In the case of nicked form of the non-toxic variant of BoNT (DrBoNT), the % release of K^+ ions at acidic pH was 7%, which was less compared to HC (18.3%) at pH 4.5. The increased release of K^+ ions at pH 4.5 by BoNT/A HC suggests low pH induced membrane channel formation by the protein [12].

Table 2
Estimation of the values of the melting temperature (T_m) and the pseudo thermodynamic parameters-ΔH, ΔS and ΔG from the thermal denaturation profiles of (a) BoNT/A LC, (b) BoNT/A HC and (c) BoNT/A holotoxin using the Van't Hoff equation. T_m (°C) was measured as the mid-point of the S-shaped portion of the thermal denaturation curve.

pH	(a) BoNT/A LC				(b) BoNT/A HC				(c) BoNT/A holotoxin			
	ΔH (kJ/mol)	ΔS (J/mol K)	ΔG (kJ/mol)	T_m (°C)	ΔH (kJ/mol)	ΔS (J/mol K)	ΔG (kJ/mol)	T_m (°C)	ΔH (kJ/mol)	ΔS (J/mol K)	ΔG (kJ/mol)	T_m (°C)
7.2	277.7 ± 25.5	861.4 ± 79.5	21.0 ± 1.8	48.0 ± 0.4	169.4 ± 12.5	515.8 ± 39.9	15.7 ± 0.3	51.0 ± 0.4	589.3 ± 36.6	1782.8 ± 112.2	57.9 ± 3.2	55.6 ± 0.3
4.5	–	–	–	–	84.5 ± 0.2	248.2 ± 0.3	10.6 ± 0.06	60.4 ± 0.2	255.2 ± 7.8	737.9 ± 21.8	35.3 ± 1.3	71.0 ± 3.0 57 ± 0.2
4.5–7.2	294.7 ± 56.8	912.5 ± 176.5	22.8 ± 4.2	48.0 ± 0.4	–	–	–	–	–	–	–	–

4. Discussion

It has been established that low pH is a requisite for channel formation by BoNT/HC [19]. We looked for structural cues accompanying low pH exposure of BoNT holotoxin and its subunits by carrying out the secondary structural analysis. The CD spectroscopy results revealed structural changes in HC at low pH accompanied by an increase in the β-sheet structure to 27.3% at pH 4.5 from 22.2% at pH 7.2. Also, the higher T_m for thermal unfolding of HC at acidic pH (T_m of 51.0 ± 0.4 °C at pH 7.2 and 60.4 ± 0.2 °C at pH 4.5) indicates low pH-induced thermal stability attained by the protein, a condition that is relevant to its endosomal membrane association. However, among the three proteins studied, BoNT holotoxin showed the maximum molar ellipticity of -2.0×10^4 deg cm^2/dmol at both acidic and neutral pHs, a value that was 4.0 and 2.31 fold higher than that of the constituent LC and HC subunits under these pH conditions, respectively.

Several membrane–protein interaction studies have shown the association of BoNT/HC with planar lipid bilayers and liposomes at low pH for the formation of a transmembrane protein channel [20,21]. Similarly, we emulated the physiological conditions for membrane association of HC using liposomes as a membrane model and examined the resultant structural changes in protein–liposome complex using CD analysis. Comparison of the structures of HC at neutral pH, either in the absence or in the presence of liposomes revealed no structural changes. On the other hand, at low pH, HC in the presence of liposomes showed a distinct CD pattern (Fig. 1d) where the ellipticity decreased at 208 nm compared to the structure of HC alone at the same pH. The ratio of ellipticities at 208 nm and 222 nm for the secondary structures of HC–liposome complex and HC were 0.77 and 1.03, respectively which resulted in the decrease in helicity by 25.2% for HC–liposome complex in comparison with HC alone. This indicates that low pH induces conformational changes in the protein resulting in a species suitable for membrane interaction [7]. Another research led by Sun *et al* showed that in the presence of GT1b receptor-bound liposomes, BoNT/B showed more hydrophobicity and membrane association at low pH, marked by a decrease in α-helicity [22]. Though, it could be suspected that reduction in ellipticity at low pH might be related to light scattering and absorbance effects from liposomes and not due to actual conformational changes in the protein, examination of the voltage channel of the CD recording (which records enhanced absorbance or light scattering)

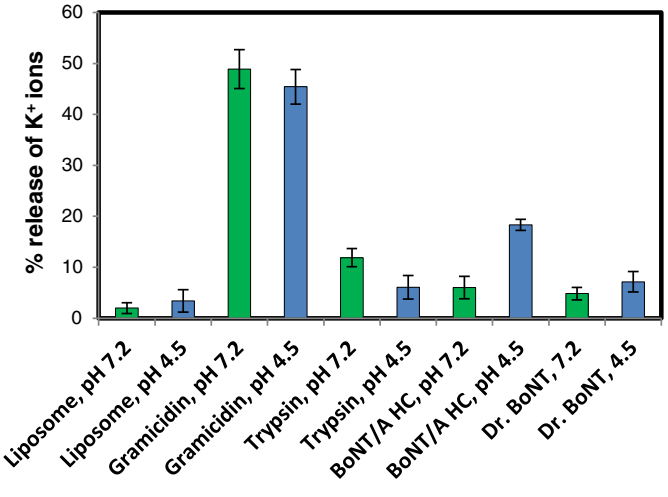

Fig. 3. K^+ ion release assay for membrane channel formation by BoNT proteins at pHs 7.2 and 4.5. Gramicidin positive control showed the maximum release of K^+ ions at both neutral and acidic pHs from asolectin liposomes after one min incubation. BoNT/A HC showed 18% release (3-fold higher) of K^+ ions at pH 4.5 and 6% release at neutral pH. The recombinant non-toxic variant of holotoxin (DrBoNT) showed 7% release of K^+ ions at pH 4.5 which was less compared to BoNT/A HC at the same pH. Trypsin negative control showed 7% release of K^+ ions at pH 4.5. The total lipid and protein concentrations used were 12.5 mg/mL and 0.02 mg/mL, respectively.

showed no saturation or any significant changes while recording the ellipticity of HC and liposome–HC mixture at pH 4.5, suggesting low pH-driven structural changes in the protein that converts it to a membrane interacting structure. Also, earlier attempts by Fu *et al* to study membrane association of HC at low pH using liposomes showed liposome aggregation, which also suggested membrane interaction of the protein at low pH. However, for the same reason it precluded the extraction of secondary structural information using CD analysis. The differences between our observations could be due to the difference in the physical properties of liposomes. The use of small, unilamellar (100 nm) vesicles in our study eliminated the spectral artifacts associated with differential light scattering and absorbance flattening by particulate suspensions and thus reflects the actual conformational changes in the protein upon association with the membrane at pH 4.5 [23]. Thus, the results of CD analysis of liposome–HC complex indicate that acidic pH triggers conformational changes in the protein, exposing hydrophobic species favorable for interaction with the endosomal membrane.

Given the low-pH triggered membrane interaction of HC, we then tested if the membrane association of HC is linked to its channel forming ability. Studies carried out in anthrax, diphtheria and tetanus toxins have shown low pH induced membrane channel formation by these proteins using channel conductance assays or cation release from lipid bilayer and vesicles, respectively [20,24,25]. In a similar experiment, we monitored the release of K^+ ions from liposomes upon treatment with BoNT proteins at acidic and neutral pH. BoNT/A HC showed a three-fold increase in the % of K^+ ion released at pH 4.5 (18% release at pH 4.5 vs. 6% release at pH 7.2) (Fig. 3). In an earlier work from our group by Fu *et al*, the membrane channel formation by BoNT/A HC at low pH was demonstrated by monitoring the fluorescent calcein molecule release from liposome vesicles. It was observed that the release of calcein was 58% with BoNT/A HC, as against 23% with the holotoxin (2.5 fold higher for HC) [12]. Similarly, our results from the liposomal ion release assay also show a threefold increase in the release of K^+ ions with BoNT/A HC and are in concurrence with the earlier observations of ion channel formation by BoNT/A HC at acidic pH. The channel formed was dependent on the presence of a pH gradient (acidic-liposomal bath buffer and neutral-inside liposomal vesicle), consistent with the requirements for channel formation put forth by Montal *et al* [26]. Thus, low pH likely triggers conformational changes in BoNT/A HC moiety resulting in the exposure of hydrophobic residues suitable for membrane insertion and channel formation. While BoNT/A HC exhibits channel activity at low pH, the nicked form of non-toxic variant of BoNT/A-DrBoNT, which has an intact channel forming domain, showed only 7% release of K^+ ions at pH 4.5 [27]. A review of the LC translocation process as described by Montal's group reveals that HC channel is occluded by LC during the initial stages of translocation where the channel conductance is low followed by an increase in conductance after LC leaves the transmembrane pore and reaches the cytosol. This channel occlusion by LC might be the reason for lower release of K^+ ions from liposomes [27]. To examine this phenomenon in our experimental setup, we examined whether L-chain could impair the K^+ release induced by H-chain, but we did not observe any difference or influence of L-chain on the cation release by H-chain. In the case of the di-chain protein, the lower % of release of K^+ ions could be due to the holistic structural conformation of the protein, which renders it less amenable to channel formation or due to the occlusion by light chain when it is still tethered to the H-chain. However, we cannot expect to see the same effects by L-chain added to liposomes treated with heavy chain since the protein is physically separated from the H-chain. The structural conformation of DrBoNT and the protein dynamics might be less amenable to channel formation when the two proteins are associated by the disulfide bonds, which could be quite distinct when the two subunits are separate, similar to that seen with Tetanus neurotoxin [28]. Hence, we could not see any influence of light chain on the channel formed by heavy chain.

In parallel to studying the low pH effects on endosome membrane channel formation by HC, we examined the fate of LC in the translocation process by monitoring the structural changes associated with low pH exposure. Though the secondary structural analysis did not show differences in the % of secondary structural elements with pH changes, the tertiary structural unfolding pattern structure, as revealed by the thermal denaturation studies showed a slight increase in ellipticity with temperature at low pH (Fig. 2a). The protein did not denature even up to 90 °C, indicating structural flexibility in the protein at low pH. In another experiment, Fu *et al* showed hydrophobic interaction of LC with lipid groups at low pH and also solvent exposure of the protein hydrophobic groups as ascertained by 1-Anilino-8-Naphthalene Sulphonate (ANS) binding experiments (data not shown) [12]. Taken together, the difference in tertiary structure, but intact secondary structure with solvent-accessible hydrophobic protein patches is a characteristic of molten globule state [11]. Extrapolating the results of the structural information of LC observed from our study to the inferences from earlier research, it could be indicated that at acidic pH, LC likely acquires a flexible molten globule behavior to translocate through the HC membrane channel in to the cytosol. Otherwise, it would be unimaginable for a large protein of 50 kDa to pass through a narrow channel of 15 Å in diameter [19]. However, the flexible structure acquired by LC was reversible as the re-exposure of the protein to neutral pH from acidic pH restored the structural features as seen with the similarities in the spectra at pH 7.2 and pH 4.5 to 7.2 (Fig. 1a). This observation is consistent with the physiological condition where LC remains functionally active in the cytosol to carry out its endopeptidase activity. Thus, with the flexible structure acquired by LC, it could escape through the narrow translocation pore and could also regain the structure after low pH exposure for subsequent SNARE cleavage at neutral pH condition [11]. Underlying this structural resilience is the unique conformational state adopted by LC at the neutral pH. In the work by Kukreja *et al*, it was inferred that based on the T_m values associated with secondary and tertiary structural unfolding, light chain bears a molten-globule state in which the enzymatic activity is retained up to 60% at 50 °C [29].

The pH effect on the structural behavior of BoNT also sheds light on the contribution of LC and HC to the structural make-up of toxin. The thermal unfolding patterns of BoNT proteins revealed that there was 18.5% difference in the mean residue ellipticities between HC and LC combination and BoNT holotoxin at their T_m at pH 7.2 (for HC and LC, the average of their mean residue weights at 222 nm was found to be -11.7 ± 0.2 deg cm^2/dmol whereas the mean residue ellipticity value for toxin at 222 nm was -14.3 ± 1.3deg cm^2/dmol). This implies that the structure of toxin undergoes some conformational changes upon the association of LC and HC. Also, considering the thermal denaturation curve for toxin unfolding at pH 4.5, a dual transition pattern was observed, the first transition characterized by an increase in ellipticity with T_m at 57.0 ± 0.3 °C, followed by a decrease in ellipticity in the second transition phase with a T_m at 71.0 ± 1.0 °C (Fig. 2c). It might be tempting to associate the dual transitions in toxin to the contributions from LC and HC. Notwithstanding this possibility, the T_m for these proteins do not match the dual melting temperatures associated with toxin and also toxin showed a higher T_m compared to LC and HC (dual T_m for toxin at pH 4.5–57 °C and 71 °C, T_m for LC and HC unfolding were 44.4 °C and 60.4 °C, respectively). The average of the mean residue ellipticities at the T_m for LC and HC unfolding at pH 4.5 was -6.9 ± 0.06 deg cm^2/dmol, while for holotoxin, the mean residue weights corresponding to the two T_m were -19.1 ± 0.31 deg cm^2/dmol and -13.1 ± 0.91 deg cm^2/dmol. Also, though LC showed a slight increase in ellipticity around 44 °C it cannot be strictly considered a transition, whereas in the case of toxin, a sharp transition pattern was apparent (T_m -57 ± 0.3 °C). Thus, the results indicate the influence of the LC and HC in the structure of toxin, which show distinct unfolding patterns upon their separation. Further, the higher T_m associated with toxin unfolding compared to that of its subunits strongly suggests tighter binding and structural interdependence between the LC and HC when

they exist in toxin form. In effect, the strong association between the subunits might be the reason for toxin being less susceptible to low pH changes.

5. Conclusions

pH-based structural studies on BoNT proteins suggest low pH-induced membrane association of BoNT/HC in the presence of liposomes, with a reduction in α-helical structure. Further, the higher release of K^+ ions at pH 4.5 from HC–liposome mixture unambiguously supports the occurrence of membrane channel activity by HC at low pH. LC, on the other hand exhibits a highly flexible structure at low pH during its translocation through the HC membrane channel. Further, LC shows structural resilience with low to neutral pH transition, the condition being physiologically consistent with its endopeptidase activity in the cytosol after low pH exposure. In addition, comparative thermal unfolding analysis of BoNT proteins indicates that HC and LC, in part act semi-independently of each other and the toxin's conformation is influenced by strong associations and stabilizing interactions between the two subunits.

Author contributions

B.R.S. conceived the idea and G.C. designed and carried out the experiments and analyzed the data. R.K helped with data recording and interpretation and discussed experimental modifications and manuscript preparation. D.G designed and performed the CD experiments involving liposomes. G.C. and B.R.S. wrote the manuscript. S.C. offered comments on data interpretation and manuscript preparation.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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