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Spectroscopic analysis of low pH and lipid-induced structural changes in type A botulinum neurotoxin relevant to membrane channel formation and translocation

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Abstract

Botulinum neurotoxin (BoNT) is an extremely toxic protein to animals and humans. In its mode of action, one of its subunits mediates its translocation by integrating itself into the membrane bilayer. We have examined the membrane channel activity of type A BoNT (BoNT/A) and its heavy (H) chain in planar lipid membrane under various pH conditions to understand the possible role of the channel activity in the translocation of the BoNT/A light (L) chain under physiological conditions. Only BoNT/A H chain, and not the BoNT/A, exhibited membrane channel activity for translocation of ions. The H chain-induced increase in conductance did not require a pH gradient across the lipid membrane, although it was enhanced by a pH gradient. To understand the molecular basis of the membrane channel activity and the translocation of the L chain, the secondary structure of BoNT/A and its H and L chains were analyzed using circular dichroism (CD) and Fourier-transform infrared (FT-IR) spectroscopy at different pH values. BoNT/A showed no structural alternation upon acidifying the buffer pH. However, an increase in β-sheet content of BoNT/A H chain at low pH was noted when examined by FT-IR. The L chain structure significantly changed with decrease in pH, and the change was mostly reversible. In addition, the neurotoxin and its subunit chains induced a partially reversible aggregation of liposomes at low pH, which indicated their integration into the lipid bilayer. Temperature-induced denaturation studies of BoNT/A H chain indicated major structural reorganization upon its interaction with membrane, especially at low pH.

Keywords: Botulinum; Circular dichroism; FT-IR; Membrane channel; Neurotoxin; Translocation; Spectroscopy

1. Introduction

Botulinum neurotoxins (BoNTs) are the most toxic substances known to mankind, with strong potential as biological warfare agents; they are lethal to animals within 30 min. Seven serotypes of BoNT are produced by different strains of *Clostridium botulinum* in the form of a single polypeptide chain. The single chain is nicked endogenously or exogenously by proteases into a 50-kDa light (L) chain and a 100-kDa heavy (H) chain, linked through a disulfide bond [1,2].

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The mode of BoNT action is not well understood at the molecular level, especially in terms of its internalization by endocytosis and translocation across the endosomal membrane into the cytoplasm. Based on some experimental evidence and analogies with other dichain toxins, such as diphtheria, cholera and *Pseudomonas* exotoxin A, a working model has been proposed [2,3]. According to this model, the C-terminus half of the BoNT H chain binds to the presynaptic membrane, presumably through gangliosides and a protein receptor. Upon binding, BoNT is internalized into the neuronal cell through endocytosis [1,4,5]. Inside the cell, the pH of the endosome is lowered to 5–6, which leads to the formation of a membrane transporter by the N-terminal half of the H chain. This transporter helps to translocate the whole or a part of BoNT into the cytoplasm. At least the 50-kDa BoNT L chain is transported into the cytoplasm from acidic endocytotic vesicles. Although the transporter is often referred to as the 'H chain channel', we refer to the H chain-based structure that facilitates the L chain transport as the L-chain transporter (LCT), and reserve the term 'channel' for experimentally detected ion channels. The induction of other small molecule permeability is referred to as (small molecules such as calcein) transport.

An understanding of the molecular mechanism of membrane channel or small-molecule transport activity of the neurotoxin could provide knowledge of the translocation process of the neurotoxin and its toxic subunit into the cell. Membrane channel formation by a water-soluble protein is an intriguing phenomenon, because for water solubility, hydrophilic domains are needed on the surface of a protein, whereas for membrane channel formation, adequate hydrophobic segments are required for interaction with a non-polar membrane bilayer. Sequence analysis has revealed a 23-residue stretch in the N-terminal domain of BoNT H chain that would have a propensity to form an amphipathic helix bundle in a hydrophobic environment [6], and the synthetic peptide of the same sequence has been shown to form channels in phospholipid bilayers [7]. However, the diameter of such channels is approximately 2.5 Å, definitely not sufficient for the translocation of a 50-kDa L chain.

Hydrophobicity calculations for BoNT/A have revealed two adjacent peptide segments (H-183–201 and H-205–240, where H refers to the H chain), with strong hydrophobicity compatible for interaction with a lipid bilayer [8]. However, one–two hydrophobic segments of limited length would not be adequate for the membrane channel formation [7]. To explain adequate interaction of BoNT with lipid bilayers, hydrophobic moment characteristics of BoNT/A were analyzed [9–11]. Several polypeptide segments were amphiphilic and could form integral membrane segments or surface segments in conjunction with lipid bilayers. Surface and/or membrane segments were identified in both L and H chains of the neurotoxin. However, it is not clearly understood how these segments are organized to form membrane channels, nor whether there is any relationship between ion channels observed in lipid membranes and the light chain translocation mechanism.

There have been several reports of interactions of clostridial neurotoxins with artificial membranes that lead to the formation of ion-conducting pores, which is generally an acid-dependent process [6,12–15]. These studies were either accomplished with planar lipid bilayers for channel conductance measurement, or were carried out with lipid vesicles for ion permeability. The acid dependence of neurotoxin translocation in intact cells has been inferred from studies in which agents that neutralize or dissipate pH gradients in acidic compartments and block the acidification of the endosomes have been shown to block neurotoxin action [16–19].

Low pH is required for the strong channel formation activity of BoNT and its H chains [12,20]. A pH of 5.0 or lower induces the channel formation. Low pH is likely to affect not only the surface charges of the neurotoxin, but also its conformation [1]. Extensive research work has been carried out with diphtheria toxin to examine low pH-induced structural changes to correlate its membrane channel activity and translocation. However, no information is available on the structural response of BoNT or its subunits to low pH.

In this article, we describe the membrane channel activity of BoNT/A and its H chain in planar lipid membrane under various pH conditions to

elicit the quantitative difference in the channel-forming activity of these proteins. Only BoNT/A H chain, and not the BoNT/A, exhibited ion transport activity (channel). The H chain-induced increase in conductance did not require a pH gradient across the lipid membrane, although it was enhanced by a pH gradient. The secondary structure of BoNT/A and its H and L chains was analyzed using circular dichroism (CD) and Fourier-transform infrared (FT-IR) spectroscopy at different pH values. BoNT/A showed no structural alternation upon acidifying the buffer pH. However, an increase in β-sheet content of the BoNT/A H chain was noted at low pH using FT-IR. The L chain structure significantly changed with decrease in the pH, and such a change was mostly reversible. In addition, the neurotoxin and its subunit chains induced a partially reversible aggregation of liposomes at low pH, which indicated their integration into the lipid bilayer.

2. Material and methods

2.1. Preparation of BoNT/A and its L and H chains

BoNT/A was isolated following the method described in detail by Fu et al. [21]. In order to obtain L and H chains, the pure neurotoxin was applied to a QAE Sephadex A-50 column (2.5×40 cm) for chain separation [22]. Following overnight incubation with 0.1 M DTT and 2 M urea containing borate buffer (pH 8.4), the L chain was first eluted with 0.01 M DTT and 2 M urea containing buffer, and the H chain was then eluted with the same buffer (0.01 M DTT, 2 M urea) containing 0.2 M NaCl. Both L and H chains were pooled after analyzing the fractions on SDS-PAGE for their purity.

2.2. Membrane channel activity in planar lipid bilayer experiments

Diphytanoylphosphatidycholine (DPhPC, Avanti Polar Lipids, Birmingham, AL) was first dissolved in HPLC-grade chloroform and was dried under a nitrogen stream to a thin lipid film, which was resuspended in squalene (Sigma Chemical Co, St Louis, MO) to a final concentration of 20 mg ml^{-1}. Planar lipid bilayers were formed by painting the lipid solution across the orifice, ~100 μm in diameter, of a 250-μl polyethylene pipette tip [23]. The membrane bilayers formed from the DPhPC were found to be more stable for our experiments than those from typical diacylphosphatidyl choline. The salt solution, containing 1 M KCl, 5 mM $CaCl_2$ and 0.1 mM EDTA, was buffered at pH 4.7 with 5 mM dimethylglutaric acid (DMG; Sigma Chemical Co, St Louis, MO) or with HEPES buffer, pH 7.4. A 100-mV square-wave potential was applied across the bilayer and the current was relayed to a digitizing oscilloscope (Nicolet, Madison, WI) and then to a strip chart recorder. The sign of the potential was recorded according to the potential applied to the *trans* compartment. The bilayer was considered optimally thinned when its resistance approached 50 GΩ and its capacitance, 250 pF. After the membrane was stabilized, heavy chain was added to the front compartment (*cis* side) and mixed into the solution. The *trans* compartment was alkalinized (pH>7.5) by the addition of 0.85% volume of 1 M KOH.

2.3. Effect of BoNT/A and its L and H chains on liposomes aggregation

In this set of experiments, the liposomes were prepared as previously described [24]. In order to monitor the degree of liposome aggregation, the optical density (OD) of liposome suspensions was monitored at 465 nm, a wavelength that is away from the protein absorption spectral region (320–240 nm). The OD was expected to increase upon aggregation of liposomes because large particles scatter more light. The OD was recorded using a UV/Vis spectrophotometer (Uvikon model 9410, Kontron Instruments, San Diego, CA) at room temperature (25 °C). A 1-ml aliquot of liposome suspension with OD of approximately 0.05 at 465 nm was used for each experiment. The liposome suspension was placed in a 1-cm-pathlength quartz cuvette and OD was recorded for 4 min. The solution was then acidified using 2 M HCl (12–15 μl) to pH 4.0 and the OD signal was recorded for another 4 min. The suspension was again

titrated with 2 M NaOH (14–17 μl) back to pH 7.5 and the OD recording was continued for an additional 4 min. The OD after every 4-min incubation period was considered to be the steady-state signal and was used to obtain data for histograms. To investigate the effect of BoNT/A and its H/L subunits on liposome aggregation, 0.1 μM of each protein was separately premixed with liposomes before recording the low pH-induced light scattering changes.

2.4. Circular dichroism spectroscopy

The structures of BoNT/A and its L and H chains were analyzed by CD spectroscopy using a Jasco model J-715 spectropolarimeter equipped with a Peltier temperature controller (model PTC-348W). Samples for CD recording in the far UV spectral region were prepared in 50 mM citrate (pH 4.0) or 50 mM citrate–phosphate (pH 7.0) buffer to a final concentration of 0.5–1.0 mg ml^{-1}. CD spectra were recorded at room temperature (~25 °C) using a 0.1-mm-pathlength quartz cell at a spectral resolution of 0.1 nm. Five spectral scans were averaged to obtain a spectrum for each sample. The scan speed was set at 20 nm min^{-1} with a response time of 8 s. To obtain the actual spectrum of a protein sample, the spectrum recorded for the buffer used for protein solution was mathematically subtracted, and the resulting spectrum was smoothed using a seven-point, third-order smoothing algorithm.

Near-UV CD spectra were recorded between 250 and 320 nm on a Jasco J500 upgrade instrument using a 1-cm-pathlength quartz cuvette at a spectral resolution of 0.2 nm with ~0.6–1.0 mg ml^{-1} protein solution either in 50 mM citrate-phosphate buffer, pH 7.0, or in 50 mM citrate buffer, pH 4.0. The scan speed used was 20 nm min^{-1} with a time constant of 4 s. The spectral bandwidth was set at 1 nm, and five scans were recorded at room temperature (25 °C), averaged and smoothed. Actual protein spectra were obtained by subtracting CD signals of buffers used for protein solutions.

2.5. FT-IR spectroscopy

The secondary structure of the heavy chain was also examined by FT-IR spectroscopy using a Nicolet 8210 FT-IR spectrometer with a zinc selenide attenuated total reflectance (ATR) accessory. The spectral recording conditions were similar to those described by Fu et al. [25,26]. The H chain (~0.5 mg ml^{-1}) in different pH buffers (pH 4.0 or 7.0) was introduced to the ATR compartment and the spectra were immediately recorded. The protein spectra at pH 4.0 and 7.0 were obtained by subtracting the respective buffer (pH 4.0 or 7.0) spectra. The secondary structure estimation was carried out by curve-fitting analysis after identifying spectral band positions with Fourier self deconvolution and second-order derivatization, as described by Fu et al. [25,26].

3. Results

3.1. Effect of pH on the channel formation of the BoNT/A and its subunits

In order to investigate the pH effect on the channel activity of BoNT/A, reduced BoNT/A (10 μg ml^{-1}), or its H chain (5 μg ml^{-1}) was added to the front of two chambers divided by a 100-μm-diameter thin lipid bilayer. The pH conditions on the two sides of the bilayer were varied and a measurement of the conductance across the lipid bilayer was carried out as an index of the channel activity of the neurotoxin and its H chain subunit. The L chain of BoNT does not exhibit voltage-gated membrane channel activity [12].

Addition of BoNT/A H chain to one side of the DPhPC membrane separating solutions at a symmetric pH of 7.4 did not induce any significant channel activity, except for a few single-channels that open and close within seconds (Fig. 1). The membrane conductance (G) at a given time was obtained from the relation $G=I/V$, where V is the voltage applied to the *cis* compartment and I is the resulting current. The single channel conductance of the BoNT/A H chain was estimated to be ~5 pS under this symmetric, neutral pH (7.4) condition.

At a symmetric pH of 4.7, shortly after (1.6 min) the H chain addition, active single-channel activity began (indicated by the noisy trace) (Fig. 2), which was not observed under symmetric pH 7.4 conditions (Fig. 1). Following this initial

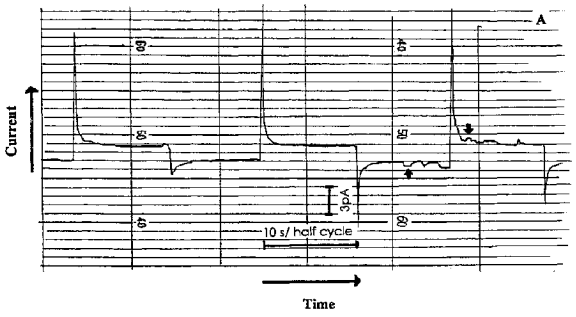

Fig. 1. Single channel currents in a planar lipid bilayer caused by addition of BoNT/A H chain (final concentration 5 μg/ml) at pH 7.4. A 100-mV square-wave potential was applied to a thin DPhPC/squalene bilayer in 0.1 M KCl, 5 mM $CaCl_2$, 0.1 mM EDTA at room temperature (25 °C). The arrow indicates single channel activity; 3-pA and 10-s bars indicate the current and time scales, respectively.

channel opening, more channels simultaneously opened (indicated by an abrupt drop within the negative half-cycle), which then led to a steady rise in the macroscopic membrane conductance (indicated by the downward trace in the negative-potential half-cycles) (Fig. 2). From the initial to the macro-opening, the rate of increase in channel activity (to reflect the rate of peptide integration in the membrane) is estimated as 7.5 pS s^{-1}.

When H chain was placed in an asymmetric pH condition (*cis* side 4.7, *trans* alkaline), a rapid increase in the macroscopic membrane conductance was observed following a short time lag after H chain addition (Fig. 3). A rate of increase in channel activity was estimated as 51 pS s^{-1} within the first 10 s.

In Figs. 2 and 3, active channel activity was also observed within the positive half-cycles. However, the channel closing frequency appears to be greater than the opening frequency at the positive potential. Such observations are illustrated more clearly in Fig. 3, as indicated by the asymmetric trace. This phenomenon indicates the voltage-gated channel property of BoNT/A H chain channels, which has also been reported by other researchers [12]. In our membrane bilayer system, membrane conductance was increased when the low pH side was made electrically positive.

The reduced form of BoNT/A was also tested for its channel activity. At symmetric low pH (4.7)

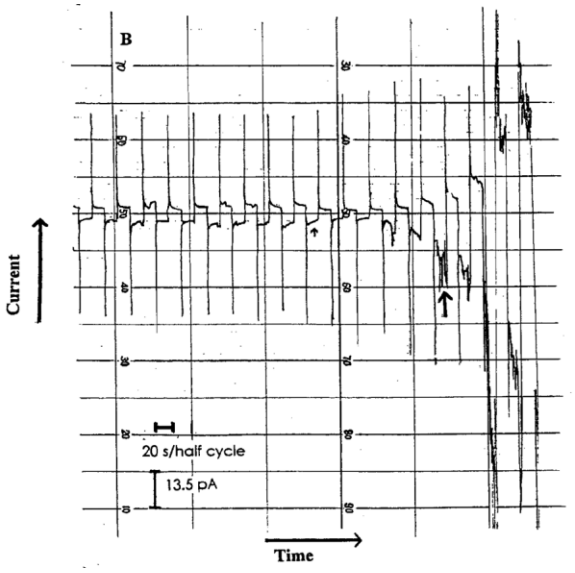

Fig. 2. Channel conductance of a thin DPhPC/squalene bilayer in the presence of 5 μg/ml of H chain with symmetric pH of 4.7. The short arrow indicates the time of H chain addition, and the long arrow indicates the region of the trace with active channel activity. All other experimental conditions were the same as in Fig. 1.

condition, 10 μg ml^{-1} reduced BoNT/A (treated with 15 mM DTT) either did not exhibit any channel activity or at the maximum evoked 7.5 pS

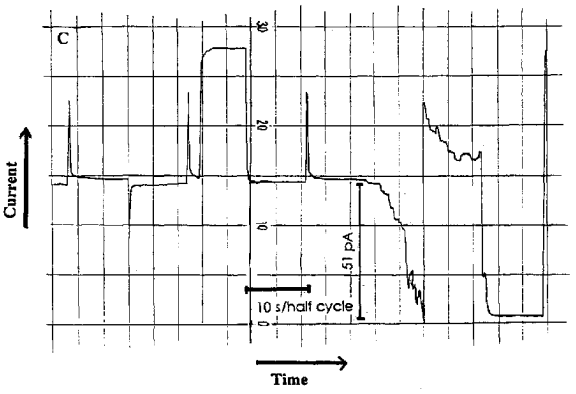

Fig. 3. Channel conductance of DPhPC/squalene bilayer in the presence of 5 μg/ml H chain at an asymmetric (*cis* 4.7/*trans* alkalized) pH condition. The sample was added before the chart shown here, and other experimental conditions were the same as in Fig. 1.

of conductance over a 25-min period (data not shown). When attempts were made to induce the asymmetric conditions, a higher channel activity was triggered, but the membrane bilayer repeatedly collapsed, suggesting that the reduced protein might destabilize the membrane. A similar observation was made earlier by Blaustein et al. [20], who reported that the membrane channel conductance was noisy when the holo-neurotoxin (reduced) rather than H chain was used. Whether or not this observation could be due to possible interference with the membrane channel activity by the presence of the L chain in the holo-neurotoxin needs to be further investigated.

3.2. Effect of pH on structure of BoNT/A, H and L chains

The conformations of BoNT/A and its subunits were examined with far- and near-UV CD spectroscopy to derive structural information, both at the secondary and tertiary structural level, under low and neutral pH conditions to address the question of molecular responses of these proteins to pH, which could provide a molecular basis for their membrane channel activity and translocation.

3.2.1. Alteration in the secondary structure of H and L chains upon acidification

It has been suggested and observed from the functional activity assays that low pH triggers the membrane channel activity (vide supra), which might be responsible for the translocation of the light chain into the cytosol. The effect of pH on the secondary structure of dichain BoNT/A and its subunits was monitored using far-UV CD. The spectrum recorded for BoNT/A at pH 7.4 (spectrum not shown) showed maximum negative signals at 210 ($-11443°$ cm^2 dmol^{-1}) and 218 nm ($-11220°$ cm^2 dmol^{-1}), and a large positive signal at 193 nm (16761° cm^2 dmol^{-1}), indicating a relatively high α-helical content. Applying a secondary structure estimation method that employs the least-squares combination of standard protein spectra [27] revealed 36.6 ± 1.6% α-helix, 24.9 ± 2.6% β-sheet, 12.4 ± 1.1% turns and 26.2 ± 0.7% random coil of BoNT/A (Table 1). Changing the pH to 4.0 did not seem to alter the secondary structure significantly, except for a slight increase in the β-sheet content (Table 1), which was within the experimental error of estimation.

Table 1
Secondary structure estimation of BoNT/A, and its subunits, H and L chains under different pH conditions

	Secondary structure (%)			
	α-helix	β-sheet	Turn	Random coil
BoNT/A				
pH 7.4	36.6 ± 1.6	24.9 ± 2.7	12.4 ± 1.1	26.1 ± 0.7
pH 4.0	35.2 ± 1.6	28.1 ± 2.8	12.2 ± 0.8	24.6 ± 0.5
HC				
pH 7.4	32.6 ± 1.8	21.5 ± 3.8	17.5 ± 1.1	28.5 ± 2.1
pH 4.0	33.6 ± 3.3	21.4 ± 6.7	16.1 ± 2.3	28.9 ± 3.1
LC				
pH 7.4	44.8 ± 3.1	22.2 ± 5.2	11.8 ± 1.8	21.3 ± 1.8
pH 4.0	31.2 ± 3.1	32.8 ± 8	18.8 ± 10.1	23.8 ± 5.3
pH 7.4[a]	48.8 ± 4.7	16.1 ± 2.1	17.0 ± 2.1	18.2 ± 0.3

Results were obtained by analyzing the CD spectra using the JASCO secondary structure estimation program.

[a] Denotes the buffer condition was back titrated from pH 4.0 to pH 7.4.

The far-UV CD spectra were also recorded for the BoNT/A H chain under the same conditions as for BoNT/A. A double minimum signal at 210 and 218 nm (-10074 and $-10218°$ cm^2 dmol^{-1}, respectively, spectrum not shown) was also present in the H-chain spectrum at neutral pH. A positive spectral band was observed at ~193 nm (12947° cm^2 dmol^{-1}). Similar to the BoNT/A, the CD spectral shape of BoNT/A H chain basically remained unaltered upon lowering the pH (spectrum not shown). Application of the spectral analysis program [27] revealed no structural difference in BoNT/A H chain at two pH values (pH 7.4 and 4.0; Table 1).

In contrast to insignificant structural alternations observed in BoNT/A and H chain with a decrease in pH, the far-UV CD spectrum of the L chain appeared to indicate drastic structural differences under the two different pH conditions (Fig. 4). The double-well spectral feature of BoNT/A L chain with minima at 218 and 210 nm observed at pH 7.4 changed dramatically to a single minimum centered at 225 nm. The intensity of the CD signal also decreased substantially (33, 57 and 51% change at 218, 210 and 193 nm, respectively).

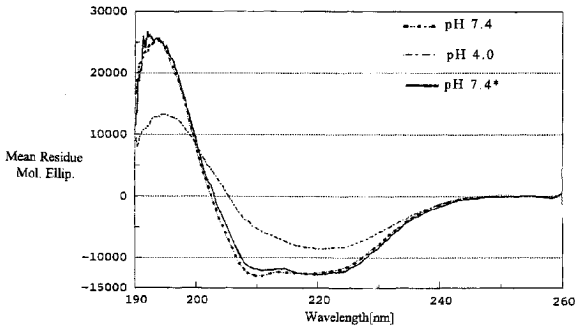

Fig. 4. Far-UV CD spectra of BoNT/A L chain were recorded under different pH conditions: 0.5–1 mg/ml of BoNT/A in a 0.1-mm-pathlength cuvette was scanned from 180 to 260 nm at a scan speed set at 20 nm/min with 8-s response time at room temperature. The spectra presented are the average spectrum from five scans, and the spectral resolution was 0.1 nm.

Changing the pH of BoNT/A L chain, which was previously exposed to pH 4.0 buffer, back to the pH 7.4 buffer fully restored the far-UV CD spectrum to the original BoNT/A L chain spectrum recorded under pH 7.4 buffer conditions (Fig. 4). The double minimum spectral feature of BoNT/A L chain, similar to BoNT/A and H chain, suggested that the L chain was also rich in α-helical polypeptide folding.

Upon exposure to the acidic condition, the changes in the spectral shape and intensity of BoNT/A L chain CD signal suggested a significant decrease in the α-helical content. However, the remnant positive signal between 190 and 200 nm ($\sim$13000° cm^2 dmol^{-1} at the peak) indicated the presence of a substantial amount of the ordered structure under the low pH condition. Secondary structure estimation of BoNT/A L chain at pH 4.0 revealed 31.2$\pm$3.1% α-helix, 32.8$\pm$8% β-sheets, 18.8$\pm$10.1% β-turns and 23.8$\pm$5.3% random coils. (Table 1). Analysis of the reversibility of the low pH-induced structural changes in the L chain revealed a very high degree of secondary structure restoration (44.7$\pm$3.1 vs. 47.2$\pm$4.7 α-helix, 22.1$\pm$5.2 vs. 21.1$\pm$7.1 β-sheet, 11.8$\pm$1.8 vs. 15$\pm$2.1 turns and 21.3$\pm$1.8 vs. 18.2$\pm$0.3 random coils; Table 1).

The effect of pH on the secondary structure of the H chain was also investigated using the FT-IR/ATR technique. Fig. 5 shows the IR spectra of the H chain in the amide III spectral region under two pH conditions. At pH 7.0, two major peaks featured at $\sim$1235 and 1315 cm^{-1}, representing bands corresponding to β-sheets and α-helix, respectively. The small shoulder at $\sim$1280 cm^{-1} corresponds to unordered structure [25]. When the H chain was exposed to acidic solution, the band at 1235 cm^{-1} maintained similar intensity, although it was shifted slightly. A shoulder at $\sim$1260 cm^{-1} appeared, and a general decrease in spectral intensity was observed above 1265 cm^{-1}. Most strikingly, the band at 1324 cm^{-1} corresponding to the α-helical structure of H chain was drastically reduced under the low pH conditions. Estimation of secondary structure by curve-fitting analysis [25,26] revealed a decrease in α-helical content (32 vs. 23%) and increase in β-sheets (32 vs. 45%) after acid exposure of the BoNT/A H chain. The content of other structures (β-turns and random coils) showed only relatively small changes (36 vs. 32%).

3.2.2. Low pH effects on the Tyr topography of BoNT/A H chain

Near-UV CD signals of aromatic amino acid residues are sensitive to topographical and envi-

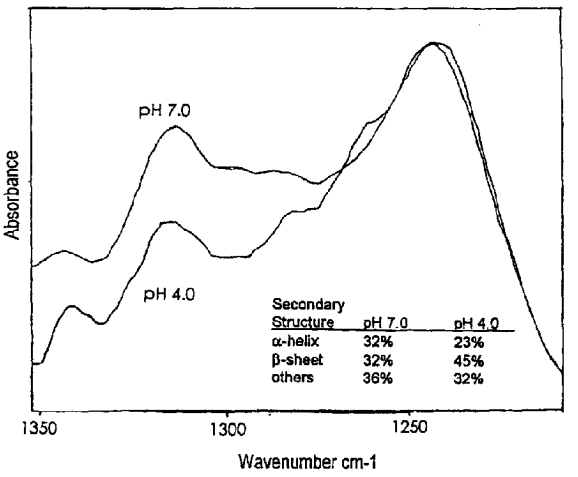

Fig. 5. FT-IR spectrum of BoNTA H chain in the amide III spectral region under neutral and acidic conditions: 0.5 mg/ml of H chain was applied to a 60° horizontal zinc selenide ATR crystal and 256 scans at 2-cm^{-1} resolution were collected at room temperature (25 °C). The average was smoothed and the buffer background was subtracted.

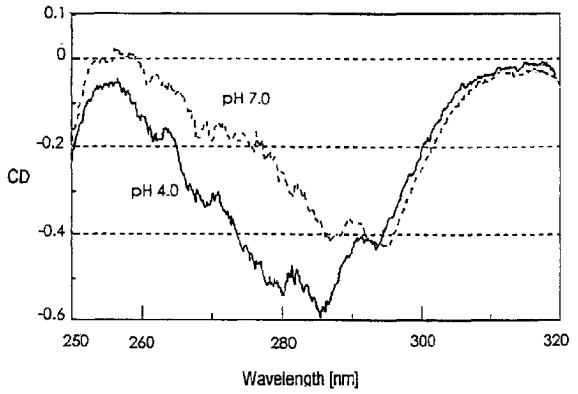

Fig. 6. Near-UV CD spectra of BoNT/A H chain at pH 7.0 and 4.0. Spectra were recorded between 250 and 320 nm at 0.2-nm spectral resolution with a scan speed of 20 nm/min and time constant of 4 s.

ronmental changes in their vicinity [28–31]. At pH 7.0, the H chain spectrum showed two distinctive bands at 295 and 285 nm (Fig. 6), which could be assigned to the ^1L_b vibronic state of tryptophan and the ^1L_a state of tyrosine, respectively [29]. A relatively featureless spectrum was observed from 280 to 255 nm, with a small shoulder at 270 nm. Upon exposure of the H chain to an acidic environment (pH 4.0), the overall optical activity increased (~35–50%) at 285 and 280 nm, whereas the band at 295 nm remained unchanged (Fig. 6). Relatively more prominent bands at 264 and 270 nm, known to arise from Phe residues, were observed under pH 4.0 conditions (Fig. 6).

3.2.3. Heavy chain induces the liposome aggregation at low pH

An attempt was made to investigate the impact of liposomes on the folding pattern of the BoNT/A H chain, especially under acidic conditions. However, addition of the H chain to liposome suspension under acidic conditions resulted in a turbid solution that was unsuitable for CD spectral recording for secondary structure analysis. Temperature-induced unfolding of BoNT/A H chain was investigated by monitoring CD signal changes at 222 nm in the presence and absence of liposomes at pH 7.4 and 4.0. The H chain in the absence of the liposomes showed clear optical activity under both acidic and neutral conditions (vide supra). Heating of the H chain at pH 7.4 appeared to precipitate it quickly once the temperature reached approximately 50 °C (Fig. 7). Under low pH conditions, temperature-induced unfolding exhibited a relatively prolonged transition, which appeared to have at least one intermediate state during the transition (Fig. 7). Temperature-induced unfolding of the H chain, when mixed with liposomes under pH 7.4 conditions, exhibited initiation of the unfolding transition at 30 °C, which reached a steady-state plateau at 45 °C, with clear steady-state intermediates at 37–39 and 41–42 °C. When the pH of the H chain/liposomes mixture was decreased, it resulted in a turbid solution with a total loss of the CD signal. The H chain-mediated enhancement of aggregation (turbidity) was specifically observed only under low pH conditions, which appeared to correlate well with its functional and structural responses analyzed in this study.

In order to investigate further the H chain-induced aggregation of liposomes under different conditions, change in the turbidity was monitored

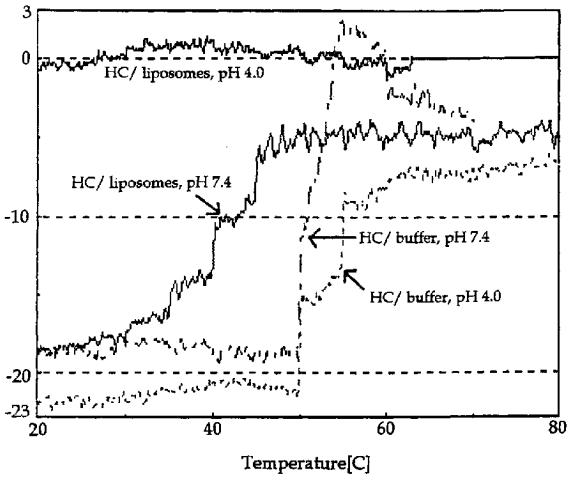

Fig. 7. Temperature-induced unfolding of the BoNT/A H chain (HC) in the presence and absence of liposomes under neutral and acidic pH values: 0.26 mg/ml of the HC was dissolved in pH 7.4 buffer, which was subsequently acidified by adding 3.4 μl of 2 M HCl to pH 4.0. The same concentration of the HC was mixed with liposome suspension ($OD_{465}=0.185$) for the liposome effect experiments, and the pH was adjusted by adding 3.4 μl of 2 M HCl.

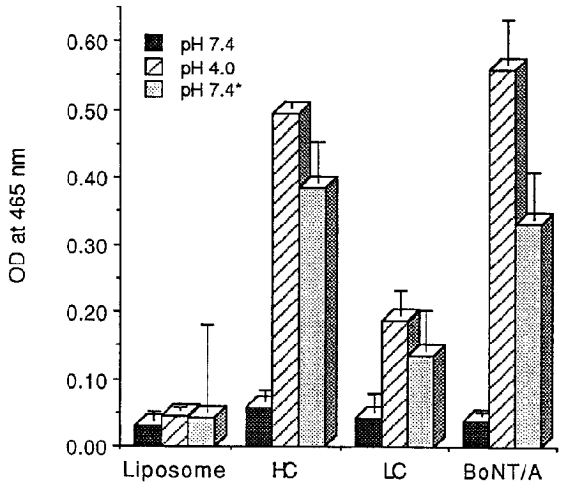

Fig. 8. Degree of liposome aggregation induced by BoNT/A, H chain (HC) and L chain (LC), as measured by optical density (OD) at 465 nm. pH* denotes that the pH has been readjusted from 4.0 back to 7.4 using 2 M NaOH.

by recording changes in OD, as a measure of light scattering, using a UV/Vis spectrophotometer. Steady-state changes in light scattering of the liposome solution, either without or with added protein, were monitored at 465 nm for 4 min at three pH levels, and final OD readings are plotted in Fig. 8.

As shown in Fig. 8, the OD of the liposome suspension in the absence of any added protein was not affected by lowering the pH from 7.4 to 4.0 (the OD only increased from 0.03 to 0.045) (Table 2; Fig. 8), indicating that alteration of the external pH in the absence of BoNT/A or its L and H chains did not induce liposome aggregation. Addition of H chain to the pH 7.4 solution containing liposomes did not induce any change in the OD (Fig. 8). However, a drastic increase in OD occurred after acidifying the solution (OD increased from 0.058 to 0.50). Titration of the low-pH sample back to pH 7.4 partially reduced the OD to 0.39 (a 22% reduction; Table 2). When a similar experiment was performed with BoNT/A L chain, lowering of the pH evoked an OD increase from 0.044 to 0.19, and the back titration to pH 7.4 resulted in the reduction of the OD to 0.137, showing a reversibility of 27% (Fig. 8; Table 2). Lowering the pH of BoNT/A in the presence of liposomes resulted in the largest increase in OD (from 0.039 to 0.56). Back titration of the BoNT/A/liposome mixture reduced the light scattering by approximately 41% (Fig. 8; Table 2).

4. Discussion

Translocation of the BoNT or its L chain across endosomal membrane to the cytosol is a critical step in the mode of BoNT action. Because BoNT is a water-soluble protein, its passage through a non-polar lipid bilayer membrane must require structural adaptation so that peptide segments within the protein having membrane interactive properties (amphiphilic and hydrophobic) could rearrange themselves for integration in the endosomal membrane. One major factor that is known to trigger such structural responses is the low pH occurring physiologically during the endocytosis process. The response of BoNT and its active subunits to low pH and the subsequent behavior of the proteins with planar lipid bilayers and liposomes have been analyzed in this study.

Table 2
Degree of aggregation of liposomes induced by BoNT/A, H chain (HC) and L chain (LC) as monitored by the OD reading at 465 nm under different pH conditions

	Optical density at 465 nm			Reversibility
	pH 7.4	pH 4.0	pH 7.4[a]	(%)
Liposomes	0.03±0.01	0.045±0.00	0.04±0.01	22
BoNT/A	0.04±0.00	0.56±0.06	0.33±0.07	41
HC	0.06±0.02	0.50±0.01	0.39±0.06	22
LC	0.04±0.03	0.19±0.04	0.14±0.06	27

[a] Denotes the buffer back titrated from pH 4.0 to pH 7.4.

The BoNT/A H chain ion-conducting channel in the planar lipid bilayer (Figs. 1–3) [1] is voltage-gated, [2] has low conductance (5 pS) at near-neutral pH values, and [3] has increased propensity of formation under low pH conditions. Based on these observations, low pH seems to stimulate H chain channel activity, and may play a role in inducing the H chain insertion into the lipid bilayer. The role of pH gradient in the membrane channel activity is not very clear as yet. However, in view of the 'voltage-gated' nature of the channel, the pH gradient might play the role of a 'driving force'. In this model, while the applied voltage could trigger a specific structure of H chain membrane channel to an open state, the pH gradient could provide an ionic gradient, allowing stabilization to the open channel state. Such a situation could sustain a deeper opening of the channel, resulting in faster ionic conduction indicated by higher conductance in our experiments (Fig. 3). It is possible that the deepened opening of the membrane channel could be responsible for the translocation of L chain. Hoch et al. [12] demonstrated that under similar conditions BoNT/B H chain membrane channels could translocate cations with the size as large as 12–16 Å. However, in previous studies the symmetric low pH condition was not examined for ion channel activity of BoNT H chain. Our experimental results under symmetric low pH conditions (Fig. 2) suggest that structural changes in BoNT/A H chain introduced by the low pH are not sufficient to induce substantial ion transport. These results therefore suggest the possibility of a new role of the low pH on the *cis* side of the membrane.

Others have also shown that low pH is required for the strong channel formation activity of BoNT and its H chains [12,32]. A pH of 5.0 or lower induces the channel formation. Two possible effects of a low pH are (i) neutralization of negative charges of the protein, and/or (ii) conformational changes in the polypeptide folding due to modulation of backbone hydrogen bonds, either of which could allow exposure of hydrophobic domains and integration of the neurotoxin into the membrane bilayer. It has been observed that low pH increases the surface hydrophobicity of tetanus neurotoxin (TeNT), as demonstrated by labeled Triton X-100 binding [32]. Similar observations were made for other dichain toxins, such as *Pseudomonas* exotoxin A [33].

The L chain of BoNT, which is the pharmacologically active subunit, is translocated from the endosome to the cytosol to exhibit the toxic activity. How the L chain retains its biological activity after adaptation to low pH and translocation across the membrane is not clearly understood. Based on the observation of the ion-conducting channels formed by BoNT/A and other similar toxins at low pH [6,12–14], it is hypothesized that the L chain undergoes total unfolding and directly traverses the membrane through this ion pore [12] into the cytosol.

The unfolding hypothesis is necessary to explain how a protein the size of the L chain can pass through a pore that has been demonstrated to be only 12–16 Å in diameter [12]. Our experimental results clearly indicate low pH-induced structural changes in BoNT/A L chain as monitored by CD spectroscopy (Fig. 4). The structure reflects a refolded state at pH 4.0. Although our results do not match with results obtained with TeNT L chain [34], where no secondary structure change was observed at pH 5.0, the β-sheets structure promoted below pH 3.0 is similar to our observations at pH 4.0 (Table 1). Most significantly, we have observed that the structural change induced by the low pH in BoNT/A L chain is almost totally reversible, a finding that explains retention of its biological activity as a zinc protease against SNAP-25 at near-neutral pH under both in vivo and in vitro conditions [35]. It has also been reported that the zinc protease activity of BoNT/A L chain pre-exposed to pH 4.7 is identical to the L chain maintained at pH 7.0 [36].

Because the L chain is not unfolded; *it is rather refolded* under low pH conditions, it is reasonable to consider an alternative mechanism for L chain transport through the H chain ion channel. The L chain might undergo a structural change at low pH, leading to a partially unfolded state, where part of the L chain can interact with the H chain and another part with the lipid bilayer. Such a L/H/lipid multimeric transient complex may only exist at low luminal pH. Once the L chain reaches the cytoplasmic face of the membrane, the com-

plex dissembles in response to the change in pH (back to physiological), where the L chain regains its natural conformation. The fact that L chain is actually capable of interacting with lipid membrane as observed in liposome permeability experiments [37,38] affords evidence in support of such a proposed mechanism. Furthermore, low pH is known to increase L chain interaction with lipids [39] and its surface hydrophobicity [36].

In contrast to the L chain, the secondary structure of H chain as monitored by CD does not change with pH. However, a decrease in the α-helix content is detected by the FT-IR/ATR technique. The discrepancy in results from the two techniques could be either due to higher sensitivity of the FT-IR technique in the amide III region, or to the differential interaction of the protein at two pH values with the ATR crystal. FT-IR spectroscopy is known to be relatively more sensitive to structural change [40,41]. We therefore believe that, irrespective of the cause of sensitivity of the FT-IR, this technique is more likely to elucidate differences in BoNT/A H chain at two different pH values. Results from X-ray crystallographic data are at quite a degree of variance from the estimation of secondary structure of BoNT/A in solution with optical spectroscopy [42]. Therefore, it is not possible to have a reliable independent method to verify estimation of the secondary structure. However, it has recently been reported that the hydrophobic transmembrane segment (650–681), which is predicted to be the channel-forming segment [7,9], is actually a 95-Å straight α-helix according to X-ray crystallography [43]. Taking the thickness of the lipid bilayer (32 Å) into consideration, it is reasonable to predict that this fragment undergoes a conformational change to prevent exposing itself to the hydrophilic (endoplasmic or cytosolic) environment and to accommodate itself within the lipid bilayer. Whether such a change could be triggered by low pH or a lipid environment remains to be elucidated. Our near-UV CD data clearly show a change in the aromatic side-chain arrangement of H chain under acidic (pH 4.0) conditions. A substantial increase in the CD intensity at 285 and 280 nm, possibly arising from Tyr residues, suggests that the microenvironment around Tyr residues becomes more asymmetric or that the domain containing Tyr residues becomes more rigid under low pH conditions. There are 49 Tyr residues in the H chain and six of them are distributed in the hydrophobic region of the H chain. Y636, Y648 and Y684 are located in the vicinity of the channel-forming region of the BoNT/A H chain, and are therefore likely to be sensitive to any conformational change induced at low pH.

Liposome aggregation experiments reveal that BoNT/A and its individual subunits can induce vesicle aggregation at low pH. Low pH does not cause any significant aggregation of liposomes in the absence of BoNT proteins, suggesting a critical role for proteins in this process. Protein-induced aggregation of lipid vesicles could occur either through charge screening of lipid head groups by the protein or through the association of bound proteins. Proteins could also induce fusion of lipid vesicles by the same mechanisms, leading to larger vesicles that scatter more light. Aggregation of vesicles either through charge screening or through protein association could be reversible, whereas lipid bilayer fusion would probably not be reversible. BoNT/A was the most effective reagent in inducing aggregation and nearly half was reversible, suggesting that the aggregation mediated by the holotoxin does not involve membrane fusion to a large extent. The H chain is nearly as effective in inducing liposome aggregation as the holotoxin, whereas the L chain was only 38% as effective as the H chain (Fig. 8). The sizes of these proteins differ, which is likely to account for some of the differences in their ability to induce liposome aggregation. Reversibility of both L and H chain-mediated aggregation is 22–26%, which is quite significant, but considerably less than the reversibility of holotoxin induced aggregation.

A significant portion of the aggregation reversibility with pH can be explained if we assume that at least part of the aggregation is directly mediated by the oligomeric association of the proteins. Perhaps the intact BoNT/A protein, which does not seem to insert itself as effectively in the membrane as its L and H chains (based on their ability to permeabilize liposomes for calcein release [37,38]), adsorbs to the surface of the liposomes and enhances liposome aggregation

through self-association. It is also possible that BoNT/A adsorbed to the surface could screen surface charges, allowing liposomes by themselves to associate and aggregate. A similar mechanism has been proposed for lipid vesicle aggregation mediated by myelin basic protein [44].

While BoNT/A H chain induced nearly the same level of liposome aggregation as the holotoxin, its reversibility was approximately half that of BoNT/A (Fig. 8). The low reversibility could be explained by either irreversible self-association of the H chain and liposomes, or H chain-induced membrane fusion, which would obviously be an irreversible process. Evidence of aggregation of the H chain was also observed in temperature-induced unfolding experiments. At pH 4.0, even in the absence of liposomes, H chain unfolding exhibited intermediate states (Fig. 7), which could arise from the non-cooperative unfolding generally observed in oligomeric structures [45]. A drastic difference in the unfolding pattern in the presence of liposomes, even at pH 7.4, is indicative not only of the existence of oligomeric structures, but also of considerable alteration in the polypeptide folding, perhaps arising from binding at the surface of liposomes. Further investigation is needed to dissect the contributions of the different mechanisms in the observed aggregation of liposomes induced by BoNT/A and its active subunit fragments.

The salient features of this study can be summarized as follows. (1) The H chain forms ion channels in planar bilayer membranes. (2) H/L chains induce aggregation/fusion of lipid vesicles at low pH that is believed to occur via L/H chain-induced lipid destabilization. (3) Both H and L chain structures are altered at acidic pH, suggesting a conformational change in the protein to adapt to the membrane insertion process. (4) The structural reversibility of conformational changes in the light chain induced by low pH enable it to readapt to the active toxic structure in the cytosol after exposure to low pH in the endosomes.

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