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Comparison of the pH-induced conformational change of different clostridial neurotoxins☆

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Abstract

Clostridial neurotoxins are internalized inside acidic compartments, wherefrom the catalytic chain translocates across the membrane into the cytosol in a low pH-driven process, reaching its proteolytic substrates. The pH range in which the structural rearrangement of clostridial neurotoxins takes place was determined by 8-anilinonaphthalene-1-sulfonate and tryptophan fluorescence measurements. Half conformational change was attained at pH 4.55, 4.50, 4.40, 4.60, 4.40, and 4.40 for tetanus neurotoxin and botulinum neurotoxin serotypes /A, /B, /C, /E, and /F, respectively. This similarity indicates the key residues for the conformation transition are strongly conserved. Acidic liposomes support the conformational rearrangement shifting the effect versus higher pH values, whereas zwitterionic liposomes do not. The disulfide bridge linking the light and the heavy chains together needs to be oxidized to allow toxin membrane insertion, indicating that in vivo its reduction follows exposure to the cytosol after penetration of the endosomal membrane.

Keywords: Botulinum neurotoxins; Tetanus neurotoxin; Conformational change; ANS; Tryptophan fluorescence

The bacterial genus *Clostridium* comprises anaerobic, gram-positive bacteria, some of which are pathogenic because they release powerful protein toxins. Tetanus neurotoxin (TeNT) is released by *Clostridium tetani*, whilst seven different serotypes of botulinum neurotoxins (BoNT/A to G) are produced by different species of *Clostridia* [1–3]. TeNT causes the spastic paralysis of tetanus by inhibiting neurotransmitter release from the inhibitory interneurons of the spinal cord, which ensure balanced skeletal muscle contraction [2]. On the contrary, the seven BoNTs inhibit the release of acetylcholine from peripheral nerve terminals and cause the flaccid paralysis of botulism [2]. BoNT/A, BoNT/B, and BoNT/E are the three serotypes which account for nearly all cases of human botulism.

Recently, botulinum neurotoxins have been the focus of an intense research mainly because of their growing use in human therapeutics and pharmaco-cosmetics [4–6]. The large majority of the clinical work has been carried out with BoNT/A, but recently BoNT/B has been approved for the use in humans [5,7] and additional serotypes have been tested [8–10].

The clostridial neurotoxins are A–B toxins, with an enzymatically active part connected to a binding and membrane translocation part. They are synthesized as a single-chain 150 kDa protein and subsequently nicked at a single exposed loop to give a 100 kDa heavy chain (H) and a 50 kDa light chain (L), held together by an interchain disulfide bond. The mechanism of cell intoxication by A–B toxins can be conveniently divided into four steps [11]. Clostridial neurotoxins bind specifically to presynaptic nerve terminals and are then endocytosed inside intracellular compartments whose lumen become acidic following the operation of a vacuolar-type

☆ *Abbreviations:* TeNT, tetanus neurotoxin; BoNT, botulinum neurotoxin; ANS, 8-anilinonaphthalene-1-sulfonate; DML, 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine; DMPA, 1,2-dimiristoyl-*sn*-glycero-3-phosphate.

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(H^+)-ATPase pump which is specifically inhibited by bafilomycin A1 and concanamycin A [12–16]. Low pH triggers a conformational change which enables both the H and the L chains to penetrate the lipid bilayer and to enter into contact with the hydrocarbon chains of membrane lipids forming a *trans*-membrane ion channel [17,18]. The H chain is mainly responsible for such an activity, but it is clear that also the L chain changes conformation at low pH and that it crosses the membrane to reach the cytosol.

The L chains of TeNT and BoNT/B, /D, /F, and /G are metalloproteases which cleave specifically VAMP/synaptobrevin, whereas SNAP-25 is proteolyzed by the L chains of BoNT/A, /C, and /E, and syntaxin is cleaved by the BoNT/C L chain [19,20]. The three protein substrates of the clostridial neurotoxins form a heterotrimeric complex which mediates the exocytosis of vesicles [21,22]; their proteolysis prevents the formation of, or destabilizes, the exocytotic complex and therefore the release of vesicle content is blocked. In the case of nerves this causes a persistent synaptic paralysis [8].

The crystallographic structures of BoNT/A and BoNT/B show the presence of three domains: (a) the L chain, (b) the first half of the H chain termed H_N mainly responsible for membrane translocation, and (c) the second half of the H chain termed H_C, mainly responsible for membrane binding [23,24]. The primary sequence of the eight clostridial neurotoxins shows regions of high similarity, particularly within the L chains, the second part of the H_N domain and the first part of the H_C domain [25,26]. The remaining parts are less similar, which might determine differences in the structural behavior of the toxins with respect to lowering pH. This could in turn affect the sensitivity of nerves to the various serotypes and therefore partially account for their specific toxicities [27,28]. Indeed, very recently [16] have reported that concanamycin A, which prevents acidification of intracellular compartments, distinctly affects BoNT/A and BoNT/E in their intoxication of spinal cord motoneurons, raising the possibility of a difference in the sensitivity to low pH of the two neurotoxins.

Here, we have compared TeNT and BoNT/A, /B, /C, /E, and /F with respect to their low pH-driven conformation change which was monitored both with the fluorescent probe 8-anilinonaphthalene-1-sulfonate (ANS) and by following the change in fluorescence of the toxins' tryptophan residues. The influence of both neutral and negatively charged liposomes and the role of the interchain disulfide bond on such conformational transition were also tested here.

Materials and methods

Chemicals and toxins. All chemicals were purchased from Sigma. TeNT and BoNT serotypes /A, /B, /C, /E, and /F were purified according to [29]. TeNT was reduced by a 30 min incubation at 37 °C in 100 mM Na_2HPO_4/HCl, pH 7.4, 1 mM Na_2EDTA, and 10 mM DTT as described [30].

Preparation of liposomes. A suitable amount of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine monohydrate (DML) or of DML and 1,2-dimyristoyl-*sn*-glycero-3-phosphate mono sodium salt (DMPA) in the ratio 4:1 was dissolved in chloroform and methanol in the ratio 3:1 and dried in a glass vial under flux of nitrogen. The solvents were further removed by applying vacuum for three hours. The lipids were dissolved in double-distilled water in order to obtain a 1.6 M concentration and ultrasonically dispersed for 45 min at 25 °C in an Ultrasonic bath sonicator (Falc), yielding a clear solution. The liposomes were further diluted in the desired buffer to a final concentration of 0.4 mM in the assays.

Fluorimetric measurements. Spectra were recorded at 25 °C in a Perkin–Elmer LS50B Spectrometer at a scan speed of 800 nm min^{-1} with excitation slit of 15 nm and emission slit of 10 nm. Samples were kept at 25 °C for 10 min before measurements. Fluorescence intensities were estimated by averaging five readings. Blanks were mathematically subtracted from spectra. Measurements were either taken in 100 mM ammonium acetate/HCl, pH 4.0–5.0, 100 mM NaCl, or in 100 mM Mops/NaOH, pH 6.5, 100 mM NaCl or in 100 mM Hepes/NaOH, pH 7.5, 100 mM NaCl with addition of liposomes when required. Protein concentration was determined spectrophotometrically in a Life Science UV/Vis Spectrophotometer DU 530 (Beckman).

The change in fluorescence of the hydrophobicity marker ANS magnesium salt was determined by adding the toxin to the desired buffer containing 50 μM ANS and following emission between 400 and 650 nm. The excitation wavelength was 380 nm. For assays above pH 5.0, toxins were used at a concentration of 0.8 μM instead of 0.6 μM and the results were corrected mathematically. Quantum yield was assessed as the area under spectra by integration of curves.

The intrinsic tryptophan fluorescence of TeNT at a protein concentration of 1.5 μM was followed between 310 and 390 nm with an excitation wavelength of 290 nm.

Results and discussion

ANS fluorescence of clostridial neurotoxins as a function of pH

ANS is a lipophilic fluorescent dye capable of adsorbing onto hydrophobic patches present on the protein surface thereby changing its fluorescent properties [31–33]. ANS has been used extensively to monitor conformational changes of proteins [34]. A rise in fluorescent emission indicates that hydrophobic patches become exposed to the aqueous solvent and accessible to the dye, whereas a blue shift of emission maxima indicates that the ANS-binding sites are less solvent-accessible.

It is well established that clostridial neurotoxins, similarly to other A–B type bacterial toxins, change conformation with lowering pH and become capable of inserting into lipid bilayers with the formation of transmembrane ion channels. This is essential for nerve intoxication to take place and it is possible that differences among the eight neurotoxins with respect to their behavior with lowering pH may contribute to their different toxicities, as suggested by recent experiments [16]. However, a detailed comparison of the acidic pH-driven conformational change for the eight clostridial

neurotoxins is not available. Some of the eight known neurotoxins are available in limited amounts in highly purified form and this has limited our comparison here to six of them.

ANS was employed to determine the range of acidic pH values in which clostridial neurotoxins change their conformation. Fig. 1A shows the fluorescence spectra of ANS in the presence of TeNT at three different pH values which are representative of what was found with the other clostridial neurotoxins. Such spectra were subsequently integrated to give the quantum yield of ANS incubated with the various clostridial neurotoxins at the various pH values tested here. Fig. 1B shows the quantum yield versus pH plot obtained with TeNT, wherefrom the pH at which half of the change had taken place was determined: such a figure is given in Table 1 for all neurotoxins tested here. Judging from the shape of this curve the low pH-driven structural change is very sharp and it occurs in a very narrow range. This holds true for all the proteins assayed here; moreover the half-change pH values are comprised within 0.2 pH units for all toxins, indicating that the clostridial neurotoxins behave very similarly with respect to lowering pH. Though the key residues implicated in such conformation transitions have not been identified, from these data it can be extrapolated that they are highly conserved among the eight neurotoxins. Therefore, the fact that BoNT/A and BoNT/E are different with respect to their sensitivity to the effect of the V-ATPase proton pump inhibitor concanamycin A [16] is unlikely to be due to a difference in their acid-triggered conformational change.

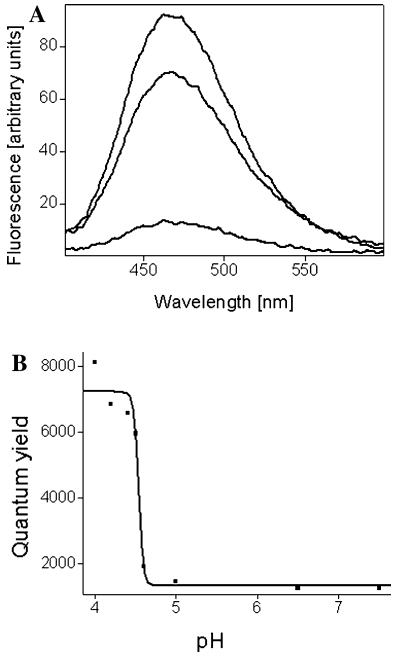

Fig. 1. ANS fluorescence of TeNT in absence of artificial membranes. (A) Fluorescence curves for pH 4.0, 4.4, and 6.5. (B) Quantum yield of fluorescence measurements. The flex of the curve is at pH 4.55, representing the point at which half of conformational change has occurred.

Table 1
pH values for TeNT and BoNTs at which half of conformational rearrangement has taken place as determined from quantum yield

	pH
TeNT	4.55
BoNT A	4.50
BoNT B	4.40
BoNT C	4.60
BoNT E	4.40
BoNT F	4.40

ANS fluorescence of clostridial neurotoxins as a function of pH in the presence of liposomes

TeNT and BoNTs internalized inside acidic nerve terminal vesicles necessarily have to cross a lipid bilayer in order to intoxicate the nerve terminal. In vitro, they were shown to penetrate the lipid bilayer at low pH [35–37] forming transmembrane ion channels [38–40], raising the possibility that their low pH-driven conformational change is affected by the composition of the lipid bilayer. Due to limitations in the amount of available purified protein, and according to the data presented in Table 1, this possibility was tested only for TeNT and BoNT/A. Fig. 2 shows the data obtained with ANS added to TeNT

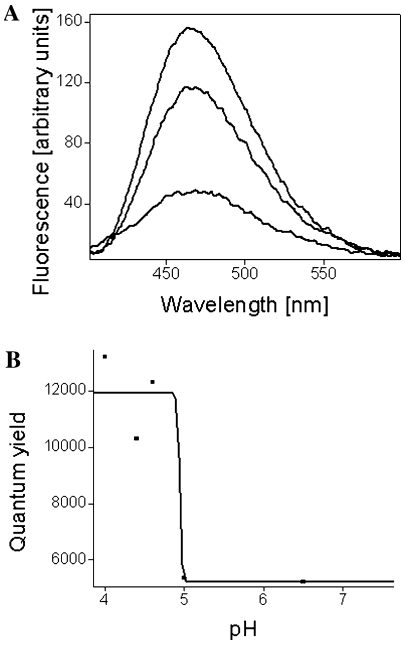

Fig. 2. ANS fluorescence of TeNT in presence of negatively charged artificial membranes of DML and DMPA. (A) Fluorescence curves for pH 4.0, 4.4, and 6.5. (B) Quantum yield of fluorescence measurements. The value at which half conformation transition has taken place is pH 4.95. Rise of the pH value found with respect to samples lacking artificial membranes points out that negatively charged lipids support conformational rearrangement.

in the presence of negatively charged liposomes. ANS does not interact with liposomes in such a way as to generate a high background blurring the protein-specific emission. The pH value at which half of the change takes place is shifted to higher values in the presence of acidic liposomes: 4.95 and 4.60 for TeNT and BoNT/A, respectively. On the contrary, for TeNT and BoNT/A interacting with neutral liposomes of dimyristoyl-lecithin the half-change pH values were 4.30 and 4.20, respectively. This finding is not surprising in the light of the fact that these proteins have mildly acidic isoelectric points (5.80 for TeNT and 5.50 for BoNT/A) and thus become increasingly positively charged as the pH of the solution is lowered within the present range of interest. The compartment which mediates nerve entry of clostridial neurotoxins was identified as endocytosed synaptic vesicles for TeNT at the CNS level [41], but it is not known for BoNTs. In any case, presynaptic nerve terminal binding is followed by endocytosis and the presynaptic membrane is enriched in negatively charged lipids [42,43]. The shift versus higher pH values brought about by acidic liposomes are particularly interesting in the light of the fact that pH values above 5 have been recorded in endosomal compartments and that only endosomal compartments, marked by the small GTP-binding protein Rab5a which regulates fusion of endocytic vesicles to early endosomes, have been detected in hippocampal nerve terminals [44]. The toxin structural change induced by low pH monitored here by ANS clearly begins already at pH 6 and a substantial fraction of the toxin molecules appears to have acquired hydrophobicity at pH values likely to be present within acidic compartments of nerve terminals.

Tryptophan fluorescence of clostridial neurotoxins

ANS is an "external" probe of protein conformational change and therefore it is advisable to control the results obtained with an "internal" protein reporter such as the intrinsic fluorescence of tryptophan residues. This latter approach also provides a response which highlights the protein conformational changes, as the fluorescence intensity rises when this amino acid becomes exposed to the aqueous solvent. A blue shift of emission maxima is characteristic of insertion of the lateral chains of tryptophan residues into a hydrophobic environment. Its drawback is the limited sensitivity.

An analysis of the tryptophan fluorescence emission of TeNT at different pH values provided a pH-driven transition closely similar to that obtained with ANS (not shown), and the analysis was not extended to the other toxins given the high amount of protein required.

This technique was used to investigate the role of the interchain disulfide bond in the low pH-driven conformational transition. It was shown before that this disulfide bond is essential to the neurotoxicity of both TeNT and BoNT, and that disulfide reduction does not affect neurotoxin membrane binding [30,45]. On the other hand, disulfide reduction is a pre-requisite for the display of the metalloproteolytic activity of these neurotoxins [17,46]. These data suggest that the interchain disulfide bridge may play a role in the toxin structural change under consideration. This bond is stable at low pH and therefore might act as a sort of hinge. The comparison of Figs. 3A and B shows that reduction of the interchain disulfide bond of TeNT does not change its fluorescence spectrum at neutral pH. In the case of oxidized TeNT (Fig. 3A) lowering the pH of the solution brings about an increase in quantum yield and a red shift consistent with the exposure of tryptophans to the water solvent. In the presence of acidic liposomes the quantum yield at pH 7.5 was slightly higher than in absence of negatively charged artificial membranes, which may be taken as an indication of a small structural change induced by lipids resulting in an increased exposure of tryptophans. In the presence of acidic liposomes, the fluorescence increase brought about by lowering the pH of the solution was weaker than without, suggesting a penetration of TeNT into the lipid bilayer with exposure of some tryptophan residues to the hydrocarbon chains of lipids, as expected. The blue shift of emission maxima confirms this interpretation.

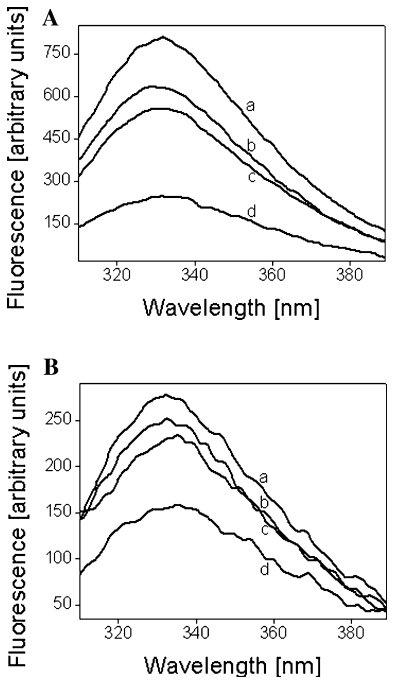

Fig. 3. Intrinsic tryptophan fluorescence of TeNT. (A) Tryptophan fluorescence of oxidized TeNT at pH 7.5 (c) and 4.2 (a) without liposomes and pH 7.5 (b) and 4.2 (d) with addition of negatively charged bilayers of DML and DMPA. Low fluorescence intensity in the case of acidic, liposome-containing samples points towards membrane insertion of the neurotoxin. (B) Tryptophan fluorescence of reduced TeNT at pH 7.5 (a) and 4.2 (b) without liposomes and pH 7.5 (d) and 4.2 (c) with addition of negatively charged bilayers of DML and DMPA.

When measurements were performed with reduced TeNT (Fig. 3B) in the absence of negatively charged artificial bilayers the fluorescence intensity at pH 4.2 was very weak, probably due to aggregation of the protein. This assumption is further corroborated by the fact that no blue shift of emission maxima could be observed. In the presence of liposomes, the fluorescence at pH 4.2 was similar to that at neutral pH thus indicating no penetration of the toxin into the lipid bilayer, possibly because self-aggregation is predominant in the case of the reduced TeNT at low pH.

Conclusions

Taken together, these data indicate that in clostridial neurotoxins acidic pH induces a sharp structural transition followed by insertion into the lipid bilayer in a process dependent on the integrity of the interchain disulfide bridge. These findings suggest that the reduction of this disulfide bond takes place after the penetration of the toxin into the limiting membrane of the acidic endocytic compartment and not in its lumen. This is in agreement with the recent statement that the reducing activity of a cytosolic thioredoxin is required for the membrane translocation of diphtheria toxin [47]. Very recently, the acid structure of BoNT/B has been determined by lowering the pH of crystals obtained under slightly acidic conditions [48]. No major rearrangement of the polypeptide chain was determined to the contrary of what was found in the case of some membrane fusogenic viral proteins. This was rather surprising in the light of the fact that BoNT/B does interact with the fatty acid portion of lipids and that it forms transmembrane ion channels. However, it is not incompatible with the present results as the low pH-acquired hydrophobicity of the clostridial neurotoxin demonstrated here may be the result of limited changes of the position of single residues, which may take place rather independently of structural changes of the polypeptide chains.

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