Membrane Interaction of Botulinum Neurotoxin A Translocation (T) Domain

THE BELT REGION IS A REGULATORY LOOP FOR MEMBRANE INTERACTION

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The translocation of the catalytic domain through the membrane of the endosome to the cell cytoplasm is a key step of intoxication by botulinum neurotoxin (BoNT). This step is mediated by the translocation (T) domain upon endosome acidification, although the mechanism of interaction of the T domain with the membrane is still poorly understood. Using physicochemical approaches and spectroscopic methods, we studied the interaction of the BoNT/A T domain with the membrane as a function of pH. We found that the interaction with membranes does not involve major secondary or tertiary structural changes, as reported for other toxins like diphtheria toxin. The T domain becomes insoluble around its pI value and then penetrates into the membrane. At that stage, the T domain becomes able to permeabilize lipid vesicles. This occurs for pH values lower than 5.5, in agreement with the pH encountered by the toxin within endosomes. Electrostatic interactions are also important for the process. The role of the so-called belt region was investigated with four variant proteins presenting different lengths of the N-extremity of the T domain. We observed that this part of the T domain, which contains numerous negatively charged residues, limits the protein-membrane interaction. Indeed, interaction with the membrane of the protein deleted of this extremity takes place for higher pH values than for the entire T domain. Overall, the data suggest that acidification eliminates repulsive electrostatic interactions between the T domain and the membrane, allowing its penetration into the membrane without triggering detectable structural changes.

Botulinum neurotoxin (BoNT),4 which belongs to the clostridial neurotoxin family, is the causative agent of botulism. Seven serotypes designated as A to G are known (1). These toxins are considered to be one of the most potent toxins among bacterial, animal, and plant toxins, and chemical compounds (2). They are therefore considered as a major potential biological weapon. However, BoNTs have become an invaluable therapy for numerous neuromuscular diseases (3). BoNTs are synthesized as single polypeptide chains of 150 kDa and are activated by proteolytic cleavage generating two polypeptide chains linked through a disulfide bond. The light chain (LC, 50 kDa) corresponds to the catalytic domain that presents a zinc endopeptidase activity directed against core components of the neurotransmitter release apparatus (4, 5). The heavy chain (HC, 100 kDa) is composed of two functional domains: the translocation (T) domain at the N-terminal segment, and the receptor binding domain at the C-terminal segment.

The mode of intoxication by BoNTs can be divided into four steps (5, 6): (i) extracellular binding to the plasma membrane of a motoneuron junction, (ii) receptor-mediated endocytosis, (iii) membrane translocation from the endosome to the cytoplasm, and (iv) cleavage of target proteins (SNARE proteins) implicated in synaptic vesicle exocytosis. The enzymatic activity of BoNTs leads to the inhibition of acetylcholine release at the neuromuscular junction, resulting in flaccid paralysis.

Considerable insights into the mechanism of action of BoNTs have been gained over the past decade. The crystallographic structure of BoNT/A (7) and BoNT/B (8) showed a 3-domain organization, reflecting the three critical roles to be performed by the protein for cell intoxication. Catalytic activities and target proteins of the various serotypes have been identified (9–13). Recent studies have provided insights into the detailed mechanisms of toxin-receptor interactions through a double receptor model (14–17).

Nevertheless, the translocation step, a key step of the intoxication mechanism, is still poorly understood. The pH-induced translocation of BoNTs is now accepted, but the exact manner of the membrane-crossing event has not been solved. Several studies sought to address this point, mainly using lipid vesicles or planar membranes and electrophysiological measurement. It

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4 The abbreviations used are: BoNT, botulinum neurotoxin; EPA, egg phosphatidic acid; EPC, egg phosphatidylcholine; Fl, fluorescence intensity; FRET, fluorescence resonance energy transfer; HC, heavy chain; LC, light chain; SRB, sulforhodamine B; LUV, large unilamellar vesicles; T, translocation.
has been proposed that the most appropriate explanation for the role of the heavy chain is as a dual channel and chaperone activity (18, 19). In this model, the channel formed by the heavy chain participates in translocation, but the heavy chain must also function in a dynamic role to escort the light chain across the membrane.

Another interesting parameter potentially influencing the translocation process has recently been raised (20). It consists of the “belt” region of the translocation domain. The active site of BoNT/A is buried 20–24 Å deep in the protein and is partially shielded by this latter belt and the main body of the N-terminal domain of the HC (7). Although several hypotheses have been proposed concerning the role of this belt region, no experimental evidence has been described.

We studied the interaction of the isolated T domain of BoNT/A with lipid vesicles. The effects of the pH together with those of the physicochemical properties of the lipid bilayer have been investigated. We also studied the role of the belt region using “belt-region” variants. Characterization of the protein structure, when bound to lipid vesicles, and of the physicochemical parameters necessary for the interactions have been studied. Associated with vesicle permeabilization assays, these results suggest that as the pH progressively decreases, the T domain from BoNT/A binds to anionic membranes and then permeabilizes the bilayer without detectable changes in the secondary and tertiary structures. Interestingly, the belt region limits protein–membrane interaction.

EXPERIMENTAL PROCEDURES

Cloning of the T Domain of BoNT/A—A synthetic gene encoding the residues N418-S877 (named protein Tl) of the entire BoNT/A toxin cloned in the plasmid pCR-Script was purchased from Geneart (Regensburg, Germany). The restriction sites SpHl and PsTl were, respectively, introduced at the 5' and 3' ends of the sequence. Almost 50% of the native codons were modified to optimize the expression in Escherichia coli. A potential Shine-Dalgarno sequence was also mutated, and the cysteine residue at position 454 was substituted by a serine to avoid mismatches during disulfide bond formation. The sequences encoding for fragments C454/S-S877, E491-S877, and K547-S877, corresponding to the proteins Tm, Tsm, and Ts, respectively, were PCR-amplified from the plasmid containing the synthetic gene. The primers used for PCR amplification were designed to introduce an SphI and a PstI restriction site at the 5'- and 3'-ends of the fragments, respectively. These primers are GGATCCGCATGCTTATACGGAACATGGG, GGATCCCATGCCGAAAGAGCATTTAGCC-TGGATCTGTAC, and GGATCCGATGCAGGAAATACAC-CATGTTCCATCTGGCG at the 5'-end for Tm, Tsm, and Ts, respectively, and GCTTGGCTGAGATTGCTGTGGTT-GATGATGTTTTGATG at the 3'-end. After digestion of the plasmid pCR-Script-Tl and of the amplified fragments with SpHl and PsTl, each sequence was ligated in the plasmid pQE-81L (Qiagen) to give the plasmids pQE-81L-Tl, pQE-81L-Tm, pQE-81L-Tsm, and pQE-81L-Ts. The sequences were checked by DNA sequencing. These plasmids encode for recombinant proteins with a His tag at the N-terminal end.

The plasmids encoding for the triple Trp mutants of Tm, TmW460 and TmW606, were obtained by rounds of site-directed mutagenesis from the plasmid pQE-81L-Tm. Mutations W717F and W706F were first introduced, and W606F or W460F were generated to give plasmids pQE-81L-TmW460 and pQE-81L-TmW606, respectively, and sequences were checked by DNA sequencing.

Expression and Purification of Recombinant Proteins—The BoNT isoform A holotoxin was purified as previously described (21). The E. coli strain XL1Blue (Novagen, Madison, WI) was used as the host for recombinant proteins productions. Proteins expressions were performed by inoculation of Terrific Broth medium (Difco, Detroit, MI) with overnight precultures at 37 °C. Induction was started at an A600 of 0.7 by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and carried out at 37 °C for 4 h. After extraction, the T domains were purified from inclusion bodies by solubilization in 8 M urea, Tris-HCl (0.1 mM) at pH 8. Proteins were purified by immobilized nickel affinity chromatography. Samples were then subjected to three successive dialyses in either 20 mM sodium phosphate buffer at pH 8 for Tl, Tsm, and Ts, or 20 mM sodium phosphate buffer, 5 mM cysteine, 1 mM cystine, pH 8 for TI, before size exclusion chromatography. The purification buffer of the T protein was finally exchanged with NH4HCO3 on a G25SF column before lyophilization and storage at 4 °C. Yields of proteins after purification were around 10 mg per liter of bacterial culture. Coomassie Blue staining of the proteins following SDS-PAGE showed that they have been purified nearly to homogeneity (not shown). Their apparent molecular masses were consistent with values deduced from their amino acid sequences: 54.5, 50.5, 46.3, and 39.7 kDa, for TI, Tm, Tsm, and Ts, respectively.

Lipid Vesicles—Lipids were purchased from AvantiPolar Lipids (Alabaster, AL). Suspensions of large unilamellar vesicles (LUV) were prepared in either 5 or 10 mM phosphate-citrate buffer at pH 7 with egg phosphatidylcholine (EPC) for neutral vesicles, and with EPC and egg phosphatidic acid (EPA) at a 9:1 molar ratio for anionic vesicles, by reverse phase evaporation and filtration four times on 0.4-μm and six times on 0.2-μm filters (22, 23).

Experimental Buffers—Proteins were kept in 5 or 10 mM phosphate-citrate buffer (pH 7 or pH 3 for the Ts protein) at a concentration of 20–50 μM and were diluted in a range of the same buffers of various pH before spectroscopic measurements. The concentration of the protein solutions were checked by measuring the absorbance at 278 nm with an ε278 of 56,000 for TI, 53,200 for Tm, 47,600 for Tsm, and 43,400 for Ts. The pH of the diluted proteins was checked afterward.

Circular Dichroism (CD) Spectropolarimetry—CD experiments were performed on a J-815 spectropolarimeter (Jasco, Tokyo, Japan) in a thermostated cell holder at 20 °C. Proteins were diluted in 5 mM phosphate-citrate buffer at the indicated pH 1 h before measurements. The scans were recorded using a bandwidth of 2 nm and an integration time of 1 s. For near-UV and far-UV measurements, each spectrum was the average of 40 scans and 15 scans, with a scan rate of 50 and 100 nm/min, and with proteins at concentrations of 4 and 0.5 μM, respectively. Spectra in the presence of LUV were
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obtained using a lipid/protein molar ratio of 500. The spectra were corrected for the blank, were smoothed using the FFT filter (Jasco Software, Tokyo, Japan), and were treated as previously described (24).

FRET Experiments—Preparation of LUV containing dansyl has been described previously (25). Briefly, LUV were prepared from EPC, EPA, and dansyl-DHPE (57, Molecular Probes) at a 9:1:0.5 molar ratio. For each pH, aliquots of Tm (0.5 μM) were added at a lipid/protein molar ratio of 100 in the absence or presence of 200 mM NaCl, and FRET was measured (excitation wavelength: 292 nm; emission wavelength: 520 nm, bandwidth of 5 nm) after 2 h of incubation. The fluorescence intensity of the dansyl was normalized to the fluorescence of LUV in the absence of Tm.

Fluorescence Extinction of the T domain in the Presence of Brominated LUV—The experiments were carried out on a photon technology international QM-4/2005 S.E. spectrofluorimeter (PTI Inc, Ford, UK) using a 1-cm path length quartz cell, at 22 °C. A bandwidth of 5 nm was used for both excitation and emission beams. The excitation wavelength was fixed at 292 nm, and emission spectra were recorded from 310 to 370 nm. LUV containing EPC/EPA/(9–10Br)-PC (Avantipolar Lipids, Alabaster, AL) at 5:1:4 molar ratio were incubated 2 h in the presence of 1 μM protein, using a lipid/protein molar ratio of 500 in phosphate-citrate buffer at different pH values. The fluorescence extinction of Tm was evaluated using the ratio \( F/F_0 \), where \( F \) and \( F_0 \) are the fluorescence at 340 nm in the presence of LUV containing or not brominated lipids, respectively. Results represent the average of five independent measurements.

To study the role of electrostatic interactions in protein-lipid interaction, samples were then incubated one night at room temperature in the presence of 200 mM NaCl before fluorescence extinction measurements.

Fluorescence Spectroscopy in the Presence of LUV—Fluorescence measurements were performed with an FP-750 spectrofluorimeter (Jasco, Tokyo, Japan) in a thermostatted cell holder at 22 °C, using a 1-cm path length quartz cell as described previously (24, 26). Proteins at a concentration of 0.5 μM were mixed with LUV at a lipid/protein molar ratio of 1000 in a 5 mM phosphate-citrate buffer at the pH indicated, and samples were incubated 1 h at 22 °C before measurements. The maximum emission wavelength (\( \lambda_{\text{max}} \)) represents the average of three values obtained from emission spectra that were corrected for blank measurements.

LUV Leakage Assay—LUV were prepared with 5 mM phosphate-citrate, pH 7, containing 50 mM sulforhodamine B (SRB) (Molecular Probes) as previously described (27). After filtration, unincorporated dye was removed by size exclusion chromatography on a PD10 column (Amersham Biosciences) equilibrated with 5 mM phosphate-citrate, 50 mM or 200 mM NaCl, pH 7. Dye efflux was monitored by the increase in fluorescence on a Jasco FP-750 spectrofluorimeter after the addition of 9 nM Tm protein to a 1.5-ml suspension of 9 μM LUV in 5 mM phosphate-citrate buffer at different pH (excitation wavelength: 565 nm; emission wavelength: 586 nm) with stirring. SRB was selected as a fluorescent probe because of its high quantum yield independently of the pH. Fluorescence was normalized as previously described (27) with Equation 1,

\[
F_{\text{norm}} = \frac{(F_0 - F)}{(F_{\text{max}} - F_0)}
\]

where \( F_0 \) is the fluorescence level before protein addition and \( F_{\text{max}} \) the level after addition of Triton X-100 at the end of each assay. The initial rate \( (V_0) \) was deduced from the slope of the origin of the curves.

RESULTS

Characterization of the Recombinant Protein Structure and Solubility—The construction of the plasmids pQE-81L-Tl, pQE-81L-Tm (-TmW460, -TmW606), pQE-81L-Ts, and pQE-81L-Ts and the expression of the different forms of the T domain are described under “Experimental Procedures.” The structures of the recombinant proteins deduced from the structure of the entire BoNT/A are shown in Fig. 1A. The Tm protein corresponds to the activated form of the BoNT/A T domain, i.e. the T domain following proteolytic cleavage between LC and HC, before residue C454. The Tl protein starts at residue N418. It carries an N-terminal extension made of the C-terminal β-strand of LC. This element is intended to restore the β-sheet between LC and HC, in which is located the proteolytic cleavage site, and the native disulfide bond between LC and HC. In the Ts protein, the long N-terminal belt surrounding LC (C454-D546) in the native toxic is removed, compared with Tm. The Tsm protein is an intermediate form between Tm and Ts and presents a partial deletion of the N terminus of the T domain (residues C454-A490).

The folding of the proteins at pH 7 was first investigated by CD in the far- and near-UV (Fig. 1B). The far-UV spectra of Tl, Tm, and Tsm indicated a mainly α-helical content, in agreement with the crystal structure of BoNT/A (7). The spectra of Ts are less characteristic, with a loss of signal at 208 nm. Near-UV CD allows the detection of aromatic residues engaged in a rigid chiral environment, consistent with the presence of a tertiary structure. The detection of a broad signal around 280 nm for Tl, Tm, and Tsm in the near-UV, which may be attributed to the signal of Tyr and Trp, indicates that these proteins present a tertiary structure. However, at the concentration used for near-UV CD (4 μM), the Ts protein has a strong tendency to aggregate, which could explain the shape of its spectrum. This observation led us to investigate the solubility of the T proteins as a function of pH. We measured the optical density of the supernatant of centrifuged protein solutions at various pH values. As shown on Fig. 1C, the proteins are highly susceptible to pH: Tl and Tm precipitate from pH 5.5 to 3.5, and Tsm from pH 6 to 3.5. Ts is soluble at pH 4 only. The difference of solubility of the proteins above pH 5.5 can be explained by their pl, which has been estimated from their primary sequence to be 5.28, 5.09, 5.42, and 6.62 for Tl, Tm, Tsm, and Ts, respectively. The four proteins being soluble at pH 3, their folding was studied by CD at this pH. The spectra in the far- and near-UV of Tl, Tm, and Tsm are nearly identical to those obtained at pH 7 (Fig. 1B and not shown). Ts shows a similar profile as Tm (Fig. 1B, insets). These results confirm that the four proteins are folded after purification and indicate that at acidic pH, the T domain of BoNT/A does not undergo detectable conformational changes.

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The binding of the T domain to LUV was monitored by fluorescence resonance energy transfer (FRET) between the Trp residues of the proteins and a dansyl fluorescent probe linked to the phospholipid headgroups. Indeed, although the incubation of T proteins in the presence of liposomes increases their solubility, partition experiments by ultracentrifugation of the LUV (25, 28) could not be used because of some protein precipitation, whatever the pH. The T domain of BoNT/A contains four Trp residues (Fig. 1A, structure of Tl). When these residues (the donors) approach the dansyl groups (the acceptors), FRET is detected as an increase of the acceptor fluorescence. In the presence of anionic LUV, a FRET signal appears for pH values below 5.5 for Tl, Tm, and Tsm and the $F/F_0$ ratio reaches a maximum for pH values below 4 (Fig. 2). Ts shows a different behavior as the FRET is detected from pH 6, and the $F/F_0$ ratio is higher than for the other T proteins. The increase of the ratio value upon acidification reveals the proximity between the Trps and the polar headgroups of the lipid bilayer and demonstrates the interaction of the T proteins with the membrane. Interestingly, the data obtained for Ts show that deletion of the N-terminal belt of the T domain favors its interaction with the membrane. However, no conclusion can be drawn from the FRET data about the location of the Trps within the membrane, because fluorescence transfer between the donor and acceptor groups (Trp and dansyl) occurs in an nm distance range.

Finally, when the same experiments were performed in the presence of neutral LUV made of EPC, FRET is detected only for pH values under 4.5 for all four proteins (Fig. 2, inset). The $F/F_0$ ratio is about two times lower than in the presence of anionic LUV. The results indicate that charges are not necessary, but favor the interaction of the protein with the membrane. Thus, hydrophobic effects should also be involved in the binding of the T domain of BoNT/A to the membrane.

Penetration of the T Domain into the Membrane—Br is a short distance (≈10 Å) quencher of Trp fluorescence. The extinction of Trp fluorescence of a protein in the presence of LUV containing 40% phospholipids brominated at position 9–10 of the aliphatic chain reveals the insertion of the Trp into the hydrophobic core of the bilayer. As shown in Fig. 3A, the $F/F_0$ ratio at 350 nm of Tm does not significantly change at pH 7.5 and 6.5.
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and is nearly equal to 1. Around pH 5.5 and below, the $F/F_0$ ratio decreases as a function of pH and reaches 0.3 around pH 3.5. These results show that the T domain not only interacts with, but also penetrates into the anionic membrane as the pH drops. Interestingly, after the addition of 200 mM NaCl to the samples, the quenching is abolished and the $F/F_0$ ratio returns to 1, whatever the pH (Fig. 3A and not shown). However, FRET from the Tm protein to dansyl groups in the interfacial region of the membrane is not affected by the presence of 200 mM NaCl (Fig. 3B). NaCl acts as a shield for charges. Thus, the results indicate that the involvement of electrostatic interactions is more important for deep penetration in the membrane than for membrane binding. It should be noted however that Br quenching is more sensitive to distance changes than FRET.

Fluorescence of the T Proteins as a Function of pH in the Presence of LUV—The $\lambda_{max}$ of emission of a Trp excited at 292 nm reflects the polarity of its environment. In a hydrophilic environment, the $\lambda_{max}$ can reach 355 nm whereas in an apolar environment, the $\lambda_{max}$ can shift to 320–330 nm. Upon acidification, the $\lambda_{max}$ of the T proteins slightly shifts, from 337 to 334 for Tm, and from 335 to 332 for Ts (Fig. 4A). This change in the fluorescence spectra occurs between pH 5.8 and 4 and is accompanied by an increase in the intensity of Trp fluorescence (Fig. 4B). Similar results were obtained for the Tl and Tsm proteins (not shown). Although weak, we consider these changes significant because they correspond to tendencies over many data points. These variations of Trp fluorescence suggest that one or more Trp becomes less exposed to the solvent at acidic pH.

Among the four Trps of the T domain, Trps 460 and 606 are localized at the surface of the protein, whereas Trps 706 and 717 are constrained into the structure (Fig. 1A, structure of Tl). Because the localization of Trps 460 and 606 suggest a susceptibility to environmental changes, two mutant Tm proteins (TmW460 and TmW606) have been produced, in which all but Trp 460 or Trp 606 have been mutated into Phe. The secondary and tertiary structures of these mutants have been checked by CD spectroscopy. The variations of $\lambda_{max}$ measured for the two mutants in the presence of LUV as a function of pH are larger than for Tm. It shifts from 341 to 325 for TmW460 and from LUV (Fig. 3) mainly indicate that the T domain penetrates into the hydrophobic core of the membrane from pH 5.5 as the pH decreases.

Penetration of the T Domain into the Membrane Is Modulated by Its N-terminal Belt—Membrane penetration of BoNT may be detected by the permeabilization of planar bilayers (19, 29) or LUV (30). This has been shown also for the isolated T domain of other toxins (25, 28). We compared the permeabilization activity of the T proteins on anionic and neutral LUV. The release of a fluorescent dye entrapped at a self-quenching concentration within the LUV is measured by the increase of fluorescence due to dilution in the external media when the membrane is disrupted. No significant release of the dye from LUV incubated alone in experimental medium at a pH ranging from 7.5 to 3.5 is detected (data not shown). Kinetics of dye release are illustrated for Tm at several pH (Fig. 5A, inset). The apparent initial rate ($V_0$) is used to compare the release kinetics (27). As shown in Fig. 5A, addition of the T proteins to anionic LUV at neutral pH does not affect the fluorescence signal. Dye release is readily observed from pH 5.8 and below for Ts, and from pH 4.5 for Tl, Tm, and Tsm. The capacity of T proteins to induce the dye release increases as the pH decreases. Whatever the pH, the efficiency of leakage induced by Ts is higher compared with those of Tl, Tm, and Tsm. In the presence of neutral LUV, the dye release starts from pH 4.5 for Ts and from pH 4 for Tm (Fig. 5B). Neutral LUV permeabilization is much less effective whatever the pH compared with anionic vesicles. Finally, the effect of charges on the interaction is much stronger for Ts (1.3 pH unit difference between anionic and neutral LUV) than for Tm (0.5 pH unit difference). In the presence of 200 mM NaCl the permeabilization activity of Tm is also affected starting from pH values between 4.5 and less effective than in the presence of 50 mM NaCl, whatever the pH (Fig. 5A). As for the FRET results, the data of LUV permeabilization show that deletion of the N-terminal belt of the T domain promotes its interaction with the membrane. Although dispensable, electrostatic interactions facilitate the interaction of the T domain with the membrane bilayer.

Previous studies have shown that BoNT/A can form pores in membranes in the presence of 200 mM NaCl (29). To confirm...
the results obtained in our system with the T proteins, permeabilization activity of BoNT/A was tested (Fig. 5, A and C). For low salt concentration, BoNT/A presents a similar activity compared with Tm (Fig. 5A): the toxin induces liposome leakage for pH values below 5 and is more efficient than Tm at acidic pH. In the presence of 200 mM NaCl, permeabilization of LUV still occurs at acidic pH, but the kinetics of perforation of BoNT/A is decreased (Fig. 5C). Again, this suggests that electrostatic interactions are involved in the interaction of the toxin with the membrane.

The T Domain Interacts with Vesicles without Major Conformational Changes—To gain insight into possible structural changes involved in the interaction of the T domain with the membrane, the structure of the T proteins was investigated by CD in the far- and near-UV in the presence of anionic LUV as a function of pH. Spectra were recorded at pH 7, 6, 5, and 4. In the far-UV the spectra indicate a predominantly α-helical secondary structure, whatever the pH, for Tm (Fig. 6A) and for the three other T proteins (data not shown). The spectra at pH 7 and 6 are nearly identical to the ones recorded at pH 7 in solution (Figs. 6A and 1B). Slight changes of the spectra at 220 nm and 190 nm at pH 5 and 4 could be due to light scattering induced by some LUV aggregation. Alternatively, it may also indicate an increase of the α-helical content of the proteins. Although noisy in the presence of LUV, spectra in the near-UV allow the detection of a broad peak around 280 nm (Fig. 6B), similar to that found in the absence of LUV (Fig. 1B), indicating that the T proteins present a tertiary structure, whatever the pH. No significant change in the spectra is detected upon acidification.

DISCUSSION

We describe here the absence of significant detectable changes in the secondary and tertiary structures of the BoNT/A T domain during its interaction with the membrane upon acidification. As most toxins must undergo large conformational changes to convert from a water-soluble to a membrane state, these observations on BoNT/A behavior constitute an important new finding. However, the T domain of BoNT/A adopts the behavior of a membrane protein as it becomes insoluble and prone to interact with the membrane at acidic pH. This pH-dependent insolubility is found around the pI of the protein, indicating the involvement of charges in the regulation of the interaction with the membrane. Indeed, we also show that this interaction is favored in the presence of anionic membranes and inhibited in the presence of neutral membranes (Figs. 2 and 5). Interestingly, the addition of NaCl (Fig. 3), which acts as a shield for charges on the membrane and on the protein, does not prevent membrane binding (Fig. 3B) but finely regulates the insertion depth of the protein (Fig. 3A). However, it should be noted that Br quenching is more sensitive to distance changes than FRET. Moreover, NaCl does not prevent permeabilization of LUV but only slows down the process for Tm and the entire toxin. The LUV model used here is intended to probe the physicochemical properties the T domain should bear to interact with the membrane at acidic pH. It does not consider, however, other parameters involved in the interaction in the cell endosome such as membrane composition, asymmetry, and the electrochemical gradient sustained between the cis and trans sides of the membrane.

Taking into account these results, how could the T domain help the translocation of the catalytic domain through a membrane? Considering the mechanisms developed by other intracellular translocating toxins, two explanations may be given. In the first mechanism, such as the one used by the diphtheria toxin, the T domain destabilizes the integrity of the lipid bilayer (25, 31, 32). The insertion of diphtheria toxin into the membrane needs a partial destabilization of the molecule leading to a rearrangement of the tertiary
structure (molten globule state) in order to exhibit hydrophobic helices implicated in the membrane insertion of the toxin (24). In the second mechanism, such as the one used by anthrax toxin, the translocation domain forms oligomers defining a transmembrane pore (33). The delivery of the enzymatic domains of anthrax toxin (edema factor (EF) or lethal factor (LF)) is mediated by the protective antigen (PA).

FIGURE 5. Membrane permeabilization induced by the T proteins and the BoNT/A. LUV (EPC/EPA) were incubated with proteins at various pH values, and the increase in fluorescence of SRB loaded in LUV at a self-quenching concentration was recorded. A comparison of the initial rates of SRB release induced by the proteins on anionic (A) or neutral LUV (B) is shown. TI, open diamonds; Tm, closed squares; Tm in the presence of 200 mM NaCl, open squares; Tsm, open circles; or Ts, closed triangles; BoNT/A, crosses. Inset A shows the kinetics of permeabilization induced by Tm on anionic LUV at pH 7 (black), pH 5 (gray), and pH 4 (dashed black). Additions of Tm and Triton X-100 are indicated by arrows. C, kinetics of permeabilization induced by BoNT/A at pH 4 and 7 in the presence of 50 mM (black lines) or 200 mM NaCl (gray lines). Additions of BoNT/A and Triton X-100 are indicated.

FIGURE 6. Effect of pH on the structure of the T domain in the presence of membranes. Far- (A) and near-UV (B) CD spectra of the Tm protein in solution at pH 7 (blue), pH 6 (orange), pH 5.1 (green), and pH 4.1 (red) are shown.
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After internalization by endocytosis, the drop in pH results in the insertion into the membrane of preformed PA heptamers and subsequent pore formation (34). Neither structures of monomer or pre-pore oligomer showed solvent-exposed hydrophobic regions that might interact with membranes. However, a long disordered loop of the monomer, which is made of alternating hydrophobic and hydrophilic residues, was proposed to convert into a β-hairpin to form a transmembrane β-barrel (35, 36). In the case of BoNT/A, the surface of the toxin is characterized by the presence of numerous hydrophobic residues and of alternating negatively and positively charged residues localized all over the structure. The protonation of negatively charged residues at acidic pH should be sufficient to create hydrophobic stretches responsible for membrane insertion. However, further experiments will be needed to determine or exclude an eventual oligomeric state of BoNT/A as a function of pH.

Another finding of this work concerns the influence on membrane interaction of the N-terminal belt of the T domain, which embraces the catalytic domain in the native toxin. In the absence of the belt, the T domain shows a higher tendency to aggregate (Fig. 1C), suggesting the belt inhibits protein aggregation. We also show that the helical bundle of the T domain (protein Ts) interacts with the membrane very similarly to the full domain (protein Tm), except at about one pH unit higher (Figs. 2 and 5). The relative inhibition of the interaction due to the belt also involves charges. Indeed, in the presence of charged membranes, this effect is stronger than in the presence of neutral ones (Figs. 2 and 5). This suggests that charges carried by the belt residues are involved in the interaction with the membrane. Among the 93 residues constituting the belt, 5 are positively charged (4 Lys, 1 Arg), and 20 are negatively charged (12 Glu, 8 Asp). The belt also contains 34 hydrophobic residues. Thus, for pH values higher than the pI value, the belt is mainly negatively charged and participates in electrostatic repulsion with negatively charged phospholipids, leading to the inhibition of protein-membrane interaction. Insertion into the membrane is obtained only after protonation of negatively charged residues at acidic pH, when the belt mostly contains hydrophobic and neutral residues. Hence, this pH-induced neutralization of the belt negative charges controls the interaction with the membrane. Our results show that, although not directly implicated in membrane insertion, the N-terminal belt influences membrane insertion by delaying the interaction with the membrane, as the pH is not low enough.

In conclusion, as for other toxins trafficking through the endosome, the T domain of BoNT/A is sensitive to pH. However, in contrast to toxins for which translocation of the catalytic domain involves an important conformational change of the T domain like the acquisition of a molten globule state, the T domain of BoNT/A does not exhibit detectable conformational changes. The major effect of acidification is a decrease of the solubility of the T domain, which increases the propensity to bind to membranes. Indeed, all the data indicate that the T domain progressively penetrates the membrane as the pH decreases. This penetration in the membrane involves attraction by positively charged residues with the negatively charged phospholipid headgroups, and the progressive neutralization of repulsion between negatively charged residues of the protein and the phospholipids, as acidification proceeds. No notable conformational changes in the secondary or tertiary structures of the protein were detected. Interestingly, the N-terminal belt of the T domain, which surrounds the catalytic domain of the toxin, plays an important role in this phenomenon, enabling the interaction with the membrane at a pH lower than in the absence of the belt.

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