Use of Trp Mutations To Evaluate the Conformational Behavior and Membrane Insertion of A and B Chains in Whole Diphtheria Toxin

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Received May 29, 1997; Revised Manuscript Received August 28, 1997[⊗]

ABSTRACT: The structure of diphtheria toxin was examined using its Trp fluorescence. To examine the interactions of the A and B chains of the toxin independently, mutants were constructed in which Trp residues were restricted to either the A or the B chain. The conformation and stability of the mutants were very similar to those of the wild-type protein. In addition, they underwent the low-pH conformational transition and membrane insertion at about the same pH as wild-type toxin. This shows Trp do not play a critical role in these processes which are necessary for the translocation of toxin across endosomal membranes *in vivo*. There was a shift in fluorescence of the Trp mutants which showed the low-pH-induced transition increases exposure of both the A and B Trp to a more polar environment. This supports a model in which the interdomain interactions present at neutral pH break down at low pH. To evaluate the location of the A and B chains in the membrane, the fluorescence quenching of model membrane inserted toxin was measured. Comparison of the amount of quenching by lipid labeled with nitroxides localized at shallow, medium, or deep depths within the bilayer demonstrated that both the A and B chains insert deeply, but the A chain Trp are somewhat less deeply inserted. Trp on the A chain are also less exposed to lipid than on the B chain, as judged by their weaker quenching by the nitroxide-labeled lipid. This conclusion was supported by the observation that the Trp of membrane-inserted isolated A chain is more lipid-exposed than when the A chain is part of the whole toxin. Both the A and B chain Trp become less exposed to lipid after neutralizing pH. However, both chains remain inserted, with at least part of the B chain remaining deeply inserted. These results support the "partial wrapper" model in which both the A and B chains are inserted but contacts between the two chains significantly reduce the exposure of the A chain to lipid.

Diphtheria toxin (DT) is a protein secreted by *Corynebacterium diphtheriae*, the bacterium that causes the disease diphtheria. It is a single polypeptide (M_r 58K) but can be readily cleaved into two fragments, A (M_r 21K) and B (M_r 37K), which remain connected by a disulfide bond (Murphy, 1985). The A chain is the catalytic domain which can ADP-ribosylate protein synthesis elongation factor 2; the B chain contains the transmembrane (T) and receptor binding (R) domains (Choe et al., 1992). Diphtheria toxin invades the cell through receptor-mediated endocytosis. The low pH in late endosomes triggers a conformational change, inducing the exposure of hydrophobic regions within the toxin. The toxin then inserts into the membrane, and the A chain is translocated into the cytoplasm [for a review, see London (1992a) and also Montich et al. (1995)].

Among these steps, the molecular mechanism by which toxin penetrates membrane is the least understood. Several models have been proposed for the structure of the membrane-inserted toxin at low pH and the mechanism of translocation of the A chain. Boquet et al. (1976) proposed a tunnel model in which the A chain translocates through a pore formed by the B chain. This model is supported by many studies showing pore formation by the toxin (London, 1992a) and that mutations inducing a loss in cytotoxicity often involve a loss in pore formation or change in pore properties (Silverman et al., 1994; Falnes et al., 1992). The wrapper model proposes the pore is only formed after the A chain translocates out of the B chain wrapper (Misler, 1984). Photolabeling suggests models in which both the A and B chains contact the bilayer (Hu & Holmes, 1984; Zalman & Wisnieski, 1984; Montecucco et al., 1985). Finally, the cleft (Papini, 1987) and partial wrapper (Zhao & London, 1988a) models propose that the hydrophilic surface of the A chain is protected from the lipid bilayer by the B chain, and that only the more hydrophobic surface of the A chain contacts lipid. The proposal that A chain membrane insertion is triggered by its unfolding in the partial wrapper model (Zhao & London, 1988a) is supported by the blocking effects of artificial internal A chain disulfides on translocation (Falnes et al., 1994). In all of these models, transmembrane helices formed by the T domain portion of the B chain at low pH presumably play a critical role in the insertion and translocation process (Silverman et al., 1994; Mindell et al., 1994b; Zhan et al., 1995; Oh et al., 1996).

There is a further change in toxin conformation after exposure of toxin to neutral pH. This conformational change affects the disposition of both A and B chains within the bilayer (Montecucco et al., 1985; Jiang et al., 1991a,b). A chain only refolds upon release from the membrane by reduction of the disulfide bonds.

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[⊗] Abstract published in *Advance ACS Abstracts,* December 1, 1997.

S0006-2960(97)01281-6 CCC: $14.00

To distinguish among translocation models, and to understand how this globular protein folds within a lipid bilayer, it is necessary to obtain more detail on toxin structure under different conditions. In this report, this problem has been approached by creating toxin mutants in which the fluorescent Trp residues are restricted to either the A or the B chain. Using these mutants, we find that the A and B chains both insert deeply into the lipid bilayer.

EXPERIMENTAL PROCEDURES

Materials. Wild-type diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canada), and purified as previously described (Jiang et al., 1991a). The free (i.e., lacking bound ApUp, see below) monomer form was used for most experiments. The isolated A chain was purified as previously described (Zhao & London, 1988a). Wild-type toxin concentration was determined by absorbance at 280 nm (Blewitt et al., 1985). Isolated A chain and Trp mutant toxin concentrations were determined by absorbance or estimated by fluorescence intensity using wild type as a standard and assuming wild type and mutants have different fluorescence due to different extinction coefficients but have similar quantum yields (estimated $\epsilon_{280} = 39\,580$ for DT_{Btrp}; $\epsilon_{280} = 34\,020$ for DT_{Atrp}; $\epsilon_{280} = 50\,700$ for wild type from Trp and Tyr extinction). Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), 1-palmitoyl-2-(5- or 12-doxyl)stearoyl-PC (5- or 12SLPC), and 1,2-dioleoyl-*sn*-glycero-3-[4-*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium]-2,2,6,6-tetramethylpiperidine-1-oxyl (TempoPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Oligonucleotides for mutagenesis were purchased from Oligos Etc. (Wilsonville, OR).

Cloning DT into pFLAG and Site-Directed Mutagenesis. A ptacDT-E148S plasmid containing DNA coding for diphtheria toxin, with the E148S substitution to ablate enzyme activity (Barbieri & Collier, 1987), was obtained from the lab of R. John Collier (Harvard Medical School). An *Eco*RI fragment containing the DT gene was cloned into pBluescript/KS+(Stratagene) (Sambrook et al., 1989a). 5′ *Hin*dIII and 3′ *Eco*RI restriction sites were introduced around the coding sequence by polymerase chain reaction using the *Eco*RI fragment, a 30 base oligonucleotide (5′ ttctt**aagctt**ggcgctgatgatgttgttg 3′) designed to introduce a *Hin*dIII site (boldface) immediately upstream of the DT coding sequence, and a 21 base oligonucleotide (5′ ccgagctc**gaattc**tgctcta 3′) complementary to the region around the *Eco*RI site (boldface) downstream from the DT coding sequence (Sambrook et al., 1989b). After the *Hin*dIII/*Eco*RI fragment containing the toxin was released by digestion and isolated by agarose gel electrophoresis, it was ligated into the *Hin*dIII/*Eco*RI site of the pFLAG-1 expression vector purchased from IBI (now Scientific Imaging Systems Division, Eastman Kodak, New Haven, CT). Transformants of *E. coli* XL-1 Blue were selected on plates containing ampicillin.

This plasmid allows production of both single-stranded DNA (ssDNA) and FLAG-tagged fusion proteins from *E. coli*. The final vector (pFLAG-DT) contains the toxin with an N-terminal extension consisting of the OmpA signal sequence followed by an extended FLAG peptide (DYKDDDDKL) attached to the N-terminal of the wild-type toxin.

Mutagenesis involved sequential mutation of single Trp residues in the A or B chain to Phe. Mutagenesis was carried out by the method of McClary et al. (1989), which is a modification of that of Kunkel et al. (1987). pFLAG-DT was used to transform *E. coli* BW 313 (dut^-, ung^-). Then the culture was infected with M13K07 helper phage, and uracil-containing single-stranded (ss) pFLAG-DT DNA isolated from the phage was released from the bacterial cells. The uracil-containing ss DNA was used for oligonucleotide-primed double-stranded DNA synthesis and transfected into *E. coli* DH5-α essentially as described by Kunkel et al. (1987). The identification of colonies containing the desired mutations was performed by DNA sequencing (Sambrook et al., 1989b).

Protein Expression and Purification. To obtain mutated DT, the pFLAG-DT vector coding for the appropriate mutant toxin was transformed into *E. coli* strain SG21173. Twenty-five milliliters of an overnight culture in Luria Broth (LB) was used to inoculate 1 L of expression media [20 g of bactopeptone and 2.84 g of Na_2HPO_4 autoclaved after dissolving in 900 mL of distilled-deionized water was mixed with 100 mL of 10× MOPS−PIPES buffer (Neidhardt et al., 1974) with PIPES replacing Tricine], 25 mL of 20% (w/v) glucose, 1 mL of 0.05% (w/v) thiamine, and 50−70 mg of ampicillin. Toxin expression was induced by addition of 1.5 M isopropyl *β-D*-thiogalactopyranoside (IPTG)/water to a concentration of 1.5 mM when the optical density of the culture at 600 nm reached 0.6−0.8. The cells were harvested 2−4 h after induction by centrifugation at 12000*g* for 10 min. The pellet was resuspended in 50 mL of 10 mM Tris-HCl, pH 8.0, followed by the addition of 50 mL of 40% (w/v) sucrose, 60 mM Tris-HCl, and 5 mM EDTA, pH 7.8. Mutant protein was then released from the periplasmic space by osmotic shock. After recentrifugation, the pellet was rapidly resuspended in 50 mL of water titrated to pH 7. After centrifugation at 5000*g* for 15 min at 4 °C, the supernatant was harvested and then applied to a 30 mL 3 cm diameter Q Sepharose Fast Flow anion exchange column (Pharmacia Biotech, Piscataway, NJ). The column was eluted with a 600 mL of a 0−0.6 M NaCl gradient containing 15 mM Tris-HCl, 1 mM EDTA, pH 8. Fractions containing mutant toxin were identified by a dot-blot using commercial horse antitoxin polyclonal antibodies (Connaught Laboratories) and concentrated to 20−40 mL with an Amicon stirrer cell using a PM10 ultrafilter (Amicon, Beverly, MA).

A 2−3 mL M1 anti-FLAG affinity column (IBI) was used for further purification of FLAG-tagged mutant toxins. A 2 mL column was first washed with 10 mL of 0.1 M glycine, pH 3. Then the column was washed with about 20 mL of TBS buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7−8) with 5 mM $CaCl_2$ (TBS+Ca^{2+}). The sample was diluted by addition of one-tenth volume of 10× TBS+Ca^{2+} or predialyzed against TBS to which 5 mM $CaCl_2$ was subsequently added. Two milliliter aliquots were loaded on the column, incubating for 15−30 min between additions. The column was extensively rinsed with TBS−Ca^{2+} (a minimum of 20−40 mL, sometimes with an additional 5 mL between aliquots loaded). Finally, the column was eluted with 2 mL of TBS followed by TBS with 5 mM EDTA; 1 mL fractions were collected. The purified toxin was dialyzed extensively against phosphate-buffered saline [PBS, pH 7.5; Sambrook et al. (1989c)] and was stored at 4 °C.

Occasionally, a mutant toxin was further purified on a 3 mL DEAE-Sephadex anion exchange column eluted with 150 mL of a 10−200 mM K^+ phosphate, pH 7, gradient. All

purification buffers contained a protease inhibitor cocktail: 0.5 mg/mL leupeptin, 1 mM Na_2EDTA, 0.7 mg/mL pepstatin, and 1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride] (Boehringer Mannheim, Indianapolis, IN). Purified protein was dialyzed against PBS buffer.

The purity of the isolated toxins was analyzed by SDS gel electrophoresis using precast gradient gels on a Phast-system apparatus (Pharmacia) (Jiang et al., 1991a). Protein was detected by silver or Coomassie blue staining. By loading toxin at a number of dilutions, we could estimate the purity was approximately 95% or better.

Fluorescence Measurements. Fluorescence was measured at room temperature with a Spex 212 Fluorolog spectrofluorometer operating in the ratio mode. Unless otherwise noted, measurements were made in a semi-micro cuvette (excitation path length, 10 mm; emission path length, 4 mm). The excitation and emission slit widths used were 2.5 and 5 mm, respectively. Trp fluorescence was measured with excitation at 280 nm, and emission at 330 and 350 nm, or emission spectra were taken. In all cases, background intensities and spectra from samples lacking protein have been subtracted to obtain the reported values.

Fluorescence Quenching Assay of ApUp Binding. Samples containing 7 μg of wild-type DT, 12 μg of DT_{Atrp} mutant, or 6.5 μg of DT_{Btrp} mutant in 0.7 mL of PBS were placed in a semimicro cuvette. A micro stirring bar was used to mix the solution. Six 1 μL aliquots of water were added, measuring the fluorescence after each addition as a control to check that intensity was stable. Then 1 μL aliquots of 25 mM ApUp were added, reading the fluorescence after each addition. Samples were stirred for about 30 s to 1min between readings.

Protease Assay of ApUp Binding. The blockage of proteolytic digestion by bound ApUp was used to assay ApUp association with the toxin (Zhao & London, 1988b). Samples containing 0.11 mg/mL free monomer toxin in 50 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.2, were prepared with or without 25 μM ApUp and samples with 0.15 mg/mL DT_{Btrp} with or without 1 mM ApUp. Samples were then incubated with 4 ng/μL trypsin for 0, 30, or 60 min before stopping the reaction by addition of SDS loading buffer, and analyzed on 10−15% Phastsystem reducing SDS gels (Jiang et al., 1991a).

Effect of Guanidinium Chloride on Toxin Stability. Samples containing 7 μg of free monomer wild-type toxin, 12 μg of DT_{Atrp}, or 6.5 μg of DT_{Btrp} in 0.7 mL of PBS were prepared and stirred with a micro stir bar. After addition of 9−90 μL aliquots of 8 M guanidinium chloride, they were mixed for about 1 min, and then fluorescence was measured.

Effect of pH on Fluorescence. Samples were prepared with 5 μg of wild-type toxin, 2 μg of DT_{Btrp}, or 5.8 μg of DT_{Atrp} in 0.64 mL of PBS, pH 8. The fluorescence of each sample was measured after aliquots of acid were added. Samples were titrated with 0.8 M acetic acid to pH 4.7, and then with 0.9 M HCl. Background fluorescence values for acid-titrated buffer were subtracted.

Incorporation of Toxin into Model Membranes. Toxin was incorporated in model membranes as described previously (Jiang et al., 1991a). Samples lacking quencher contained 30% DOPG (dioleoylphosphatidylglycerol) and 70% DOPC (mol/mol). In samples containing quencher, the composition was 30% DOPG/30% quencher PC/40% nonquenching PC. Lipids dissolved in organic solvent were mixed and dried under N_2. The mixture was then redissolved with 100−200 μL of chloroform and redried. Then the lipids were redissolved in 10 μL of ethanol. Vesicles were formed by dilution with buffer under the appropriate conditions for each experiment (see below).

Fluorescence Quenching vs pH. Lipid samples containing DOPG/DOPC and DOPG/12SLPC/DOPC were prepared as described above. To form vesicles, 0.7 mL of PBS, pH 7.5, containing 4.2 μg of free monomer wild-type toxin, 0.64 mL of PBS with 4.8 μg of DT_{Btrp} mutant, or 0.7 mL of PBS with 7.6 μg of DT_{Atrp} mutant was added. The final lipid concentration was 180−200 μM. Sample pH was titrated as described above. Fluorescence intensity was measured at 330 nm.

Dependence of Fluorescence on Lipid Concentration. A stock solution of 30% DOPG/70% DOPC vesicles was prepared essentially as described above and diluting with 500 mM sodium acetate, 150 mM NaCl, pH 4.4, to give a final lipid concentration of 200 μM. Then 200 μL solutions with lipid diluted to 0, 20, 40, 80, 100, and 200 μM were prepared by dilution with the same buffer. To each was added 400 μL of 6.4 μg/mL wild-type toxin, DT_{Atrp}, or DT_{Btrp} in PBS to each tube to yield a final pH of 4.5. Fluorescence emission in each sample at 330 nm was then measured.

Fluorescence Quenching Studies of Trp Depth. Lipid mixtures containing DOPG/DOPC with or without 30% 12SLPC, 5SLPC, or TempoPC were prepared as described above. Lipids were mixed, dried under N_2, redissolved in 100−200 μL of $CHCl_3$, and dried under N_2. After dissolving in 3−5 μL of ethanol, 213 μL of 500 mM sodium acetate, 150 mM NaCl, pH 4.4, was added. Then 6.4 μg/mL protein in 417 μL of PBS, pH 7.5, was added giving a final pH 4.5. To reverse pH, an aliquot of saturated Na_2HPO_4 was added to each sample to reach pH 6.7. To make pH 3.5 samples, 140 μL of 500 mM acetic acid, 150 mM NaCl was added to the lipid, and then 500 μL of 5.5 μg/mL A chain dissolved in PBS, 7.5, was added. The final lipid concentration was 200 μM. Duplicate samples were prepared. In blanks, PBS buffer without protein was used.

RESULTS

Properties of Wild-Type Toxin and Trp Mutants at Neutral pH: Fluorescence Properties. There are five naturally occurring Trp in DT, two in the A chain (residues 50 and 153) and three in the B chain (206, 281, and residue 398). Trp to Phe mutations were introduced into the toxin so that Trp was restricted to only within the A chain (DT_{Atrp}) or the B chain (DT_{Btrp}). These molecules were used to separately monitor the conformational behavior of the A and B chains while they were part of whole DT.

Both the wild-type toxin and the mutants exhibit strong Trp fluorescence at neutral pH. Trp on the B chain have fluorescence at more blue-shifted wavelengths (and thus are in a somewhat less polar environment) than those on the A chain, as judged by λ_{max} (Figure 1). It was also observed that the Trp fluorescence of isolated A chain was even more red-shifted than that in DT_{Atrp}. This suggests the Trp of the A chain become more exposed to solution upon removal of the B chain.[1]

Properties of Wild-Type Toxin and Trp Mutants at Neutral pH: Ligand Binding. The mutants and wild-type toxin were compared to determine whether removal of Trp influences

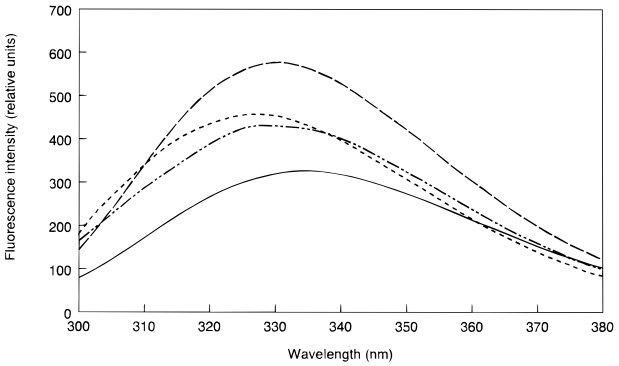

FIGURE 1: Spectra of wild-type DT, DT Trp mutants, and isolated A chain at neutral pH. Spectra of 10 μg/mL free monomer wild-type toxin (—), 17 μg/mL DT_{Atrp} (— ·· —); 9.3 μg/mL DT_{Btrp} (---), and 4 μg/mL isolated A chain (— —) in PBS are shown normalized to approximately the same protein concentration.

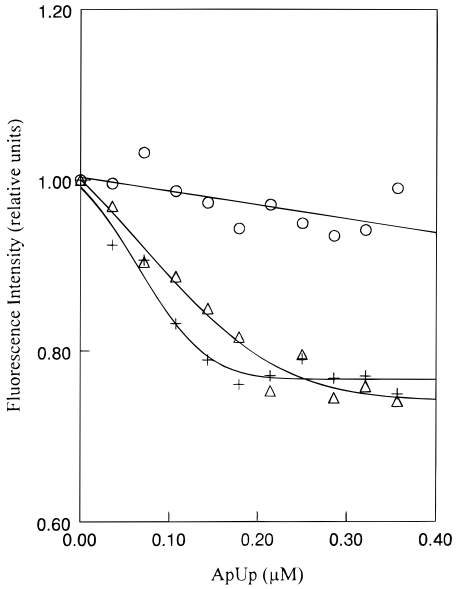

FIGURE 2: Effect of ApUp concentration upon the fluorescence of wild-type DT and Trp mutants. Samples were prepared as described under Experimental Procedures. Fluorescence intensity is shown for free monomer 0.17 μM wild type (+), 0.16 μM DT_{Btrp} (○), and 0.29 μM DT_{Atrp} (△).

toxin conformation. Ligand binding was one method used to probe toxin conformation. The dinucleotide ligand ApUp is found naturally bound to diphtheria toxin molecules (Carroll et al., 1986). Its function, if any, is unknown but may involve protease protection (Zhao & London, 1988b). The binding site for ApUp is at the substrate (NAD^+) binding site on the A chain, but involves interaction with the B chain as well (Bennett et al., 1994), and its binding to toxin is very tight only when the toxin is in the native conformation (Collins & Collier, 1984).

ApUp binding to toxin was evaluated by fluorescence and proteolysis. It was found the Trp fluorescence of (originally ApUp-free) DT is quenched upon ApUp binding, with saturation occurring at a ApUp to toxin ratio of about 1. Similar quenching was also observed when ApUp was titrated into DT_{Atrp} mutant (Figure 2). No or weak quenching was observed with DT_{Btrp}. The latter result is not surprising

[1] Notice the fluorescence of the mutants does not add up to that in the wild-type toxin. This may reflect a weak self-quenching in wild-type protein or simply small errors in protein concentration.

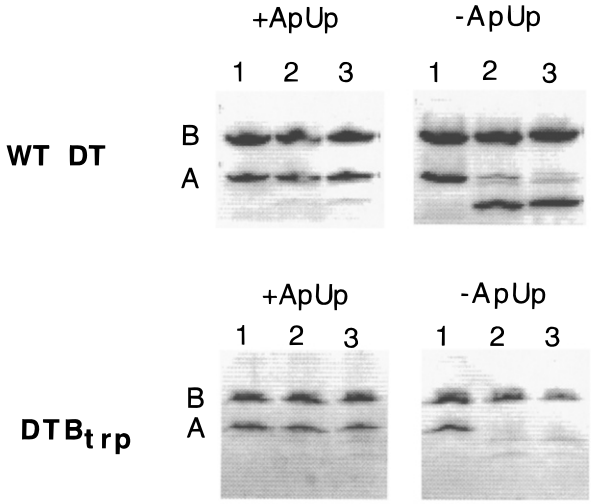

FIGURE 3: Effect of ApUp upon sensitivity of wild-type DT and Trp mutants to proteolysis. Lane 1, 0 min digestion. Lane 2, 30 min digestion. Lane 3, 60 min digestion. Upper gels, 1.9 μM free monomer wild-type DT with or without 25 μM ApUp. Lower gels, 2.6 μM DT_{Btrp} with or without 1 mM ApUp. The position of the B chain and A chain is shown on the left. Similar results were obtained with DT_{Atrp} (not shown).

as quenching is most likely to involve the Trp of the A chain (Trp 50 and Trp 153) which are relatively nearby the active site (and can affect catalytic function when mutated) (Wilson et al., 1994), rather than quenching of the more distant Trp of the B chain (Choe et al., 1992).

To confirm ApUp binds to DT_{Btrp}, its effect on the toxin's proteolytic sensitivity was examined. We previously demonstrated that ApUp binding prevents trypsin attack on Lys 39 (Zhao & London, 1988b). This is due to a local conformational change upon binding of a molecule in the substrate binding site (Bell & Eisenberg, 1997). Figure 3 shows that low concentrations of ApUp are able to prevent trypsinolysis not only for wild-type DT and but also for DT_{Btrp}. Therefore, both mutants have the ability to bind tightly ApUp, and upon binding both are likely to undergo the same conformational change in the loop containing Lys 39 as does wild-type toxin.

Sensitivity of Wild-Type Toxin and Trp Mutants to Denaturation. The stability of the native conformation of wild-type DT and the mutants was also examined by comparing their sensitivity to denaturation by guanidinium chloride. This unfolding can be followed by the shift in Trp fluorescence emission wavelengths, which can be easily followed by the ratio of the emission at 330 nm to that at 350 nm ($F_{330/350}$) (Zhao & London, 1986). Figure 4 shows that the unfolding of both the wild-type and mutant toxins have midpoints close to a guanidinium chloride concentration of 1 M at neutral pH. The midpoint for DT_{Atrp} unfolding occurs at a slightly lower concentration (about 0.75 M) than that for DT_{Btrp} (about 1.2 M). The average of these values is close to that for wild-type toxin. The difference between the two mutants suggests A chain denaturation occurs at a slightly lower guanidinium chloride concentration than the B chain. [A mutant (G1C) containing a full complement of five Trp denatured at the same concentration as wild-type toxin. This indicates the FLAG peptide at the N terminus does not greatly perturb folding stability (data not shown).]

The thermal stability of the toxin and mutants was also compared using fluorescence. In all cases, denaturation was

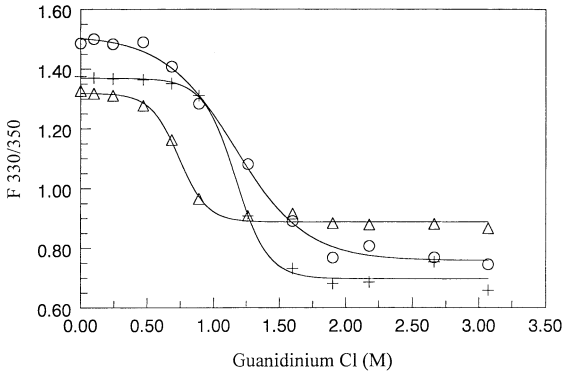

FIGURE 4: Effect of guanidinium chloride on the fluorescence of wild-type DT and Trp mutants. Samples in Figure 1 were titrated with 8 M guanidinium chloride. The ratio of fluorescent intensity at 330 nm to that at 350 nm is shown for free monomer wild-type DT (+), DT_{Btrp} (o), and DT_{Atrp} (Δ).

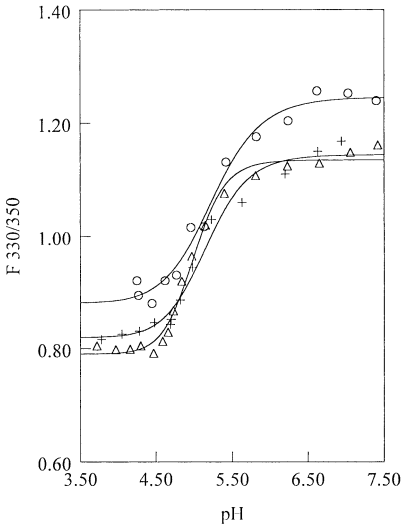

FIGURE 5: Effect of pH on the fluorescence of wild-type DT and Trp mutants. The ratio of fluorescence intensity at 330 nm to that at 350 nm is shown for wild-type DT (+), DT_{Btrp} (○), and DT_{Atrp} (△). Experimental details are given under Experimental Procedures.

observed only above 45 °C, although the midpoint of thermal unfolding for all the mutants occurred at a 5−10 °C lower temperature than for wild-type toxin, indicating the mutations affect thermal stability to some degree (not shown).

Comparison of the pH Transitions of Wild-Type Toxin and Trp Mutants. The low-pH-induced conformational change in DT is the critical step triggering its translocation across the endosomal membrane. Therefore, the effect of pH on the conformation of wild type and mutant toxins was compared (Figure 5).[2] As observed previously, in wild-type DT a transition can be observed at pH 5 (Blewitt et al., 1985). Below pH 5, the Trp are in a more polar environment, as judged by the red shift in their emission. The mutants show similar changes in fluorescence close to pH 5. The B chain Trp remain in a slightly more hydrophobic environment than those of the A chain even at low pH. The observation that the Trp of both the A and B chains are apparently more exposed to solution at low pH is in agreement with antibody studies showing sites on both the A and B chains become more antibody accessible after incubation at low pH (Tortorella et al., 1995a).

Membrane Insertion of Wild-Type Toxin and Trp Mutants at Low pH. DT inserts into model membranes upon exposure

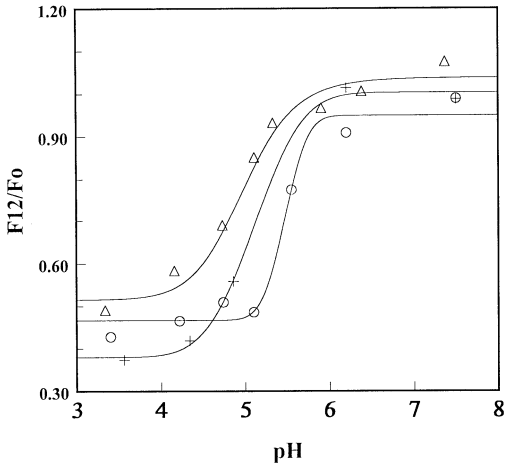

FIGURE 6: Fluorescence quenching assay of the effect of pH on membrane insertion of wild-type DT and Trp mutants. F_{12}/F_o is the ratio of the fluorescence emission intensity at 330 nm in the presence of PG/PC model membrane vesicles containing 12SLPC to that in the presence of vesicles without 12SLPC. Samples contained wild-type DT (+), DT_{Btrp} (○), or DT_{Atrp} (△). Other details are given under Experimental Procedures.

to a pH of 5 or below (Chung & London, 1988). This binding and insertion can be studied by measuring the Trp fluorescence of toxin bound to model membrane vesicles containing a lipid carrying a deeply placed nitroxide or brominated group. Upon binding and insertion of the toxin, there is strong quenching of Trp fluorescence (Chung & London, 1988; Jiang et al., 1991a) as detected by the decrease in the ratio of fluorescence in the presence of vesicles containing the nitroxide (F_{12}) to that in the presence of vesicles lacking the nitroxide (F_o).

The quenching of the Trp mutants due to insertion into the nitroxide-labeled vesicles as a function of pH is shown in Figure 6. There is no quenching above pH 5, indicating the mutants and wild-type toxin do not insert into the model membrane vesicles under those conditions. In contrast, wild-type toxin and both Trp mutants insert below about pH 5. DT_{Atrp} inserts at a slightly lower pH than DT_{Btrp}, consistent with previous cross-linking studies showing the A chain inserts at a slightly lower pH than the B chain (Montecucco et al., 1985; Papini et al., 1987).

Fluorescence was used to evaluate the lipid binding strength of the Trp mutants compared to wild type at low pH. At pH 4.5, wild type, DT_{Atrp}, and DT_{Btrp} are about half bound at 20 μM phospholipid, and totally bound at about 100 μM (Figure 7).

Location of A and B Chains Relative to the Lipid Bilayer at Low pH. Because the DT_{Atrp} and DT_{Btrp} mutants behave very similarly to wild-type toxin, their fluorescence behavior can provide information about the relative locations of the A and B chains in membranes. Previous studies showed when toxin inserts into the bilayer there is a small blue shift in fluorescence (Chung & London, 1988), and slight blue shift upon membrane insertion was also observed in DT_{Atrp} but not DT_{Btrp} fluorescence (not shown). However, wave-

[2] These values are slightly lower than other experiments because they were made with a different spectrofluorometer (Spex τ2) with a detection efficiency (mainly due to different gratings) with a slightly different wavelength dependence. This does not affect the conclusions of this experiment because only the change in emission ratio is important.

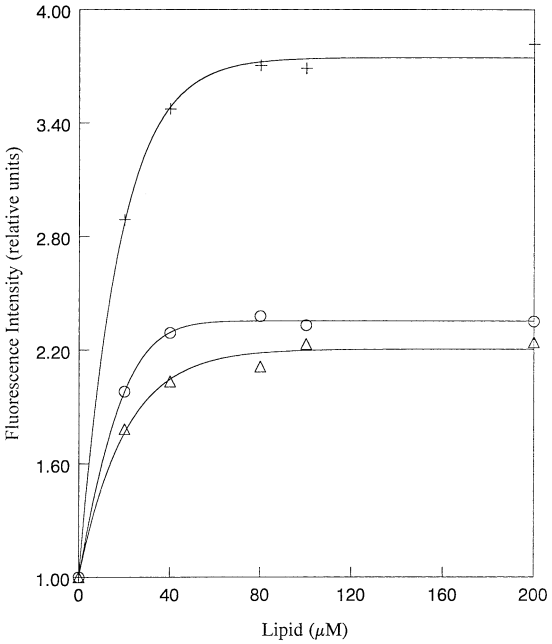

FIGURE 7: Fluorescence assay of binding of WT toxin and Trp mutants to model membranes. The fluorescence intensity at 330 nm was measured at different concentrations of 30% DOPC/70%DOPC model membrane vesicles. Samples contained wild-type 0.11 μM DT (+), DT_{Btrp} (○), or DT_{Atrp} (△). Other details are given under Experimental Procedures.

length shifts are insufficient to determine Trp locations within the bilayer. Instead, to examine Trp location in membranes, their quenching by phospholipids carrying a nitroxide quencher at a shallow, medium, or deep depth in the membrane was compared. The relative strength of quenching as a function of quencher depth allows estimation of the relative depth of the Trp residues.

Table 1 shows the ratio of fluorescence in the presence and absence of nitroxide (spin)-label quencher (F/F_o). A smaller F/F_o value indicates stronger quenching. The fluorescence of model membrane-inserted wild-type DT, DT_{Atrp}, and DT_{Btrp} were all quenched most strongly by the 12SLPC, which has the nitroxide located closest to the center of the bilayer, and were quenched the least by TempoPC, which has the nitroxide attached to the polar headgroup. This result indicates that when toxin inserts in the membrane, both the A and B chains are deeply inserted. The difference between 12SLPC and TempoPC quenching is smallest for DT_{Atrp}, suggesting a shallower location for its Trp than those of DT_{Btrp}. This is also shown by the observation DT_{Btrp} is more strongly quenched by the deep quencher 12SLPC than DT_{Atrp}, whereas quenching of DT_{Btrp} and DT_{Atrp} by the shallow quencher TempoPC is similar.

Because nitroxide quenching is a short-range process, the overall level of quenching reflects the degree of exposure of Trp to lipid as well as Trp depth (London & Feigenson, 1981). The fact that quenching of DT_{Atrp} was less than that of wild-type toxin or DT_{Btrp} for all three quenchers suggests that A chain Trp are relatively less exposed to the lipid bilayer than those of the B chain. This conclusion is supported by the quenching of membrane-inserted **isolated** A chain. The quenching values obtained (F/F_o = 0.35 for 12SLPC, 0.51 for 5SLPC, and 0.45 for TempoPC) indicate significantly stronger quenching than for DT_{Atrp}.[3]

Table 1: Fluorescence Quenching of Trp in Diphtheria Toxin Mutants

	F/F_o[a]		
protein	12 SLPC	5SLPC	TempoPC
	Low pH		
DT(wt)	0.55	0.63	0.72
DT_{Atrp}	0.67	0.70	0.73
DT_{Btrp}	0.53	0.60	0.68
	After pH Neutralization		
DT(wt)	0.76	0.79	0.88
DT_{Atrp}	0.79	0.75	0.79
DT_{Btrp}	0.70	0.71	0.80
	(F/F_o After pH Neutralization) − (F/F_o at Low pH)		
DT(wt)	0.21	0.16	0.16
DT_{Atrp}	0.12	0.06	0.06
DT_{Btrp}	0.18	0.12	0.13

[a] F/F_o is the ratio of fluorescence in samples containing model membrane vesicles with quencher to that in samples with model membrane vesicles lacking quencher, generally determined in these experiments from the average of four measurements. The average standard deviation was ±0.02 at low pH, and ±0.03 after pH neutralization. No significant difference in quenching was detected in control experiments with excitation at 295 nm instead of 280 nm. It should be noted that, in principle, the precise depth of the Trp residues can be determined from quenching experiments via the parallax analysis (Chattopadhyay & London, 1987; Abrams & London, 1993). We did not apply this analysis to our experiments because the mutants have more than one Trp residue. We considered that the calculated depth would be too crude an average to be of much use.

At higher temperatures (>30 °C), previous studies have shown membrane-inserted toxin undergoes a conformational change involving the A chain (Jiang et al., 1991a). However, when the fluorescence of the mutants was measured at higher temperatures, very little difference in quenching was observed (within 3%), indicating the conformational change at high temperature does not greatly affect the location of the A and B chain Trp (data not shown).

Location of A and B Chains Relative to the Lipid Bilayer After Reversal of pH to Neutral. The exposure of membrane-inserted toxin to neutral pH occurs when the toxin becomes exposed to the cytoplasm (London, 1992a), and reversing the pH to neutral induces changes in toxin that has been inserted into model membranes at low pH (Montecucco et al., 1985; Jiang et al., 1991a). To see how neutralization of pH affects the location of A and B chain Trp in membrane inserted toxin, quenching was measured after reversing the pH to neutral.[4]

Both mutants and wild-type toxin are quenched less by all the quenchers after pH neutralization than at pH 4.5 (Table 1, middle). In all cases, the loss of quenching by the deep 12SLPC probe is more than that by the other quenchers (Table 1, bottom), so it appears Trp locate slightly more shallowly after pH neutralization.

[3] The observation of more quenching by shallow and deep quencher than by the intermediate quencher may suggest that one Trp inserts at a shallow location in the bilayer and the other at a deeper location. Alternately, the A chain may insert in a mixture of conformations with different Trp depths. These experiments had to be done at pH 3.5, because the A chain requires a lower pH in order to insert efficiently. Control experiments on DT_{Atrp} at pH 3.5 show similar maximal quenching levels to those at pH 4.5 (data not shown).

[4] Toxin does not dissociate from the bilayer after pH neutralization under our conditions (Hu & Holmes, 1984; Jiang et al., 1991a). Centrifugation experiments performed under our conditions to duplicate this result confirmed this for both the wild type and mutants (data not shown).

For DT_{Btrp}, Trp are still quenched more strongly by the deep 12SLPC quencher than the shallow TempoPC after pH neutralization, suggesting a portion of the B chain remains deeply inserted. The Trp residues of DT_{Atrp} seem to take a slightly shallower location after pH neutralization relative to those of the B chain, as they are quenched similarly by the 12SLPC and TempoPC quenchers.

DISCUSSION

Trp Are Not Required for Maintenance of the Native Conformation, the Conformational Change at Low pH, or Membrane Insertion. The Trp mutants show the same or very similar behavior as the wild-type DT as judged by a number of methods. Trp were not required for formation of the native conformation or ApUp binding. In addition, mutation of Trp did not greatly affect the low-pH conformational change or membrane insertion. Thus, there is no individual Trp residue that plays a critical role in the overall membrane insertion process. (Since the mutants also contain the FLAG peptide, the similarity of mutant and wild-type behavior means this peptide must also not perturb toxin behavior significantly.)

Disruption of Close Interchain Interactions at Low pH. At low pH, diphtheria toxin undergoes a very large conformational change involving a partial unfolding that results in molten globule-like behavior (London, 1992b). Some details of what happens to each domain at low pH have been obtained from previous studies. Experiments on isolated domains have shown there can be conformational changes in all three domains at low pH which result in the exposure of hydrophobic groups (Zhao & London, 1988a; Zhan et al., 1994; Esbensen et al., 1994). In addition, as inferred from crystallographic data, and observed experimentally from the pH dependence of antibody binding (Tortorella et al., 1995a), after exposure to low pH interdomain interactions are disrupted, accompanied by increased exposure of individual domains to solution. However, whether this loss of interdomain interactions occurred at low pH or after pH neutralization was not certain. The observation in this study that both A and B chain Trp become more exposed at low pH indicates that the breakdown of interdomain interactions occurs at low pH.

A and B Chain Interaction with Membranes at Low pH. Based on the results of the quenching studies, the relative locations of the Trp residues on both the A and B chains were determined both at low pH and after pH neutralization. These studies show both the A and B chains must insert deeply at low pH. Quenching also indicates that both A and B chains remain inserted after neutralization of pH. The lesser overall quenching suggests lesser exposure of Trp to lipid. At least partially, this seems to reflect a movement of Trp out of the bilayer core, as illustrated by the observation that the A chain becomes shallower after pH neutralization. However, quenching shows a portion of the B chain remains deeply inserted. This conclusion is supported by previous studies with hydrophobic photolabels (Montecucco et al., 1985).

The indications from quenching that the B chain Trp are more exposed to lipid than those of the A chain, and that the B chain shields the A chain Trp from the bilayer, represent significant evidence for the "cleft" or "partial wrapper" model for the membrane-inserted toxin (Papini et al., 1987; Zhao & London, 1988a). In this model, the A chain is partly protected from lipid contact by the B chain (Figure 8). Thus, it appears the B chain has a significant effect on the manner of A chain interaction with lipid. This is supported by the observation that the isolated A chain requires a lower pH and higher lipid concentration to bind to lipid bilayers than when in whole toxin (Zhao & London, 1988a).

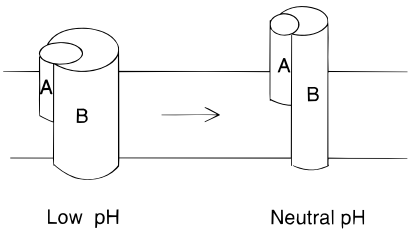

FIGURE 8: Schematic diagram of DT structure in model membranes. *Approximate* locations of the A and B chains are shown as judged from Trp location.

Location of Trp Residues in Membrane-Inserted Diphtheria Toxin. The results of this study suggest the Trp in both the A and B chains are deeply inserted in the bilayer. There is very little additional information on the location of the Trp in diphtheria toxin in the membrane-inserted state. Since the crystal structure is of the soluble form of the toxin, it provides few clues about Trp depth in membranes. The most information is available about the T domain of the toxin. However, most T domain studies have concentrated on helices 8 and 9, whereas the Trp in the T domain are in helices 1 and 5. Helix 1 is somewhat amphipathic, with the Trp 206 on the nonpolar face of the helix. This helix is in the N-terminal region of the B chain, which may translocate across the membrane together with the A chain (Madshus et al., 1994). Therefore, it would not be unreasonable for it to form some sort of membrane-inserted structure. It has been shown that the polar side of this helix is unlikely to contact lipid (Madshus, 1994). However, maintenance of the hydrophobicity of the nonpolar face appears to be necessary for function (vanderSpek et al., 1994). Therefore, it is conceivable that this face, including Trp 206, is in contact with the lipid bilayer.

Trp 281 is even more likely to be deeply inserted, because it is in helix 5, which is highly hydrophobic. Attempts to determine whether the helix 5/6 pair translocates across the bilayer the same way that has been proposed for helices 8 and 9 have been made by examining whether using mutagenesis to reverse the charge on Asp 295 in the loop between helices 5 and 6 inhibits toxin action, specifically pore properties. Such an inhibition would be possible because this loop must translocate across the membrane if helices 5 and 6 are part of transmembrane structures, and charge reversal could disrupt translocation, and thus normal pore formation. Conductivity studies in model membranes have indicated that the mutation of Asp 295 to Lys has little effect on pore properties (Mindell, 1994a), although a large effect has been observed in cells (Falnes, 1992). Thus, the degree of insertion of helix 5 into the membrane remains unclear.

The location of the Trp 398 in the R domain is also unknown. It is even uncertain whether the R domain penetrates membranes *in vivo*. However, the lack of proteolysis of cell membrane bound R domain after exposure

to low pH has been interpreted in terms of its membrane penetration (Moskaug et al., 1991). Also, the R domain does become hydrophobic at low pH (Esbensen et al., 1993), and has several β-sheets with a potential to form transmembrane structures (Tortorella et al., 1995b). Finally, immunochemical analysis suggests R domain penetration of the membrane can occur in model membranes (Tortorella et al., 1995b). Therefore, it is possible that Trp 398 penetrates the lipid bilayer.

There is also no information about the location of the Trp (residues 50 and 153) in the A chain. The previous observations that the A chain does become hydrophobic at low pH (Zhao & London, 1988a) and comes into contact with the bilayer do suggest that it is reasonable that these Trp could be inserted into the membrane, although since they are not part of hydrophobic sequences they would be expected to have limited contact with the lipid bilayer, at least in whole toxin.

Studies on DT with Single Fluorescent Sites. Studies with protein having single Trp would clearly yield even more detailed information than the mutants used in this study. We did construct single Trp mutants of DT but found that they could not be expressed at high enough levels to be useful for fluorescence studies. We found even DT_{Atrp} and DT_{Btrp} are not expressed as well as DT mutants with a full complement of Trp residues. Presumably, the number of mutations (4 or greater) that must be introduced in order to obtain single Trp residues at desired positions is even more deleterious to high expression levels. The most practical strategy may be to study isolated domains of the toxin, and/or use Cys mutations (Mindell et al., 1994a,b; Zhan et al., 1995; Oh et al., 1996), in which smaller numbers of mutations are necessary in order to obtain single fluorescent sites. This is the aim of our future studies.

ACKNOWLEDGMENT

We thank Nancy Ulbrandt for her assistance in developing the expression and isolation protocols, and the lab of Dr. R. John Collier for the gift of the DT-containing plasmid and helpful advice.

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BI971281Z