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Effects of Mutations in Proline 345 on Insertion of Diphtheria Toxin into Model Membranes

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Abstract. Translocation of the catalytic domain of diphtheria toxin (DT) across the endosomal membrane to the cytoplasm of mammalian cells requires the low-pH-dependent insertion of a hydrophobic helical hairpin (TH8-TH9) that is buried within the T domain of the native protein. Mutations of Pro345, which terminates helix TH8, have been reported to block toxicity for Vero cells. We found that mutant toxins in which Pro345 had been replaced by Cys, Glu, or Gly were profoundly defective at low pH in forming channels in planar phospholipid bilayers and in permeabilizing phospholipid vesicles to entrapped fluorophores. Experiments with isolated T domain containing a polarity-sensitive fluorophore attached to Cys at position 332 suggest that the P345E mutation blocks membrane insertion. None of the Pro345 mutations shifted the pH-dependence of binding in solution of the hydrophobic fluorophore, 2-*p*-toluidinyl-naphthalene 7-sulfonate. The results indicate that proline at position 345 is required for the T domain to insert into phospholipid bilayers or to adopt a functional conformation within the bilayer.

Key words: Diphtheria toxin — Proline — Mutagenesis — Membrane insertion — Transmembrane domain — Site-specific labeling

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Introduction

Although many bacterial toxins are known to act by enzymically modifying intracellular substrates, there is no toxin for which we understand the mechanism of membrane translocation. Diphtheria toxin (DT) has been extensively studied as a model for exploring this process (London et al., 1993; Falnes et al., 1995; Oh et al., 1996). DT is a single-chain protein containing three structural domains: the catalytic (C), transmembrane (T), and the receptor binding (R) domains (Choe et al., 1992). Before or after binding to cells, the toxin is proteolytically processed into two disulfide-linked fragments: fragment A, which corresponds to the C domain, and fragment B, which is composed of domains T and R. DT binds to its receptor, undergoes receptor-mediated endocytosis and is trafficked to the endosome. There, acidic conditions induce the T domain to insert into the endosomal membrane, promoting translocation of the C domain to the cytosol. The disulfide linking the C and T domains is reduced, releasing the C domain into the cytosol, where it catalyzes the ADP-ribosylation of elongation factor-2. This inhibits protein synthesis and causes death of the cell.

The T domain consists of ten α-helices, arranged in three layers (Choe et al., 1992). The innermost layer is composed of a long, buried hydrophobic helical hairpin, consisting of helices TH8 and TH9, bridged by an acidic loop (TL5). There is now considerable evidence that this hairpin plays a central role in translocation. Both DT and the isolated T domain form channels in artificial bilayers under acidic conditions, and replacement of either of two acidic residues in the TL5 loop (Glu349 and Asp352) with Lys or Arg strongly inhibits channel formation, translocation, and toxicity (O'Keefe et al., 1992; Mindell et al., 1994*b;* Kaul et al., 1996). Evidence from

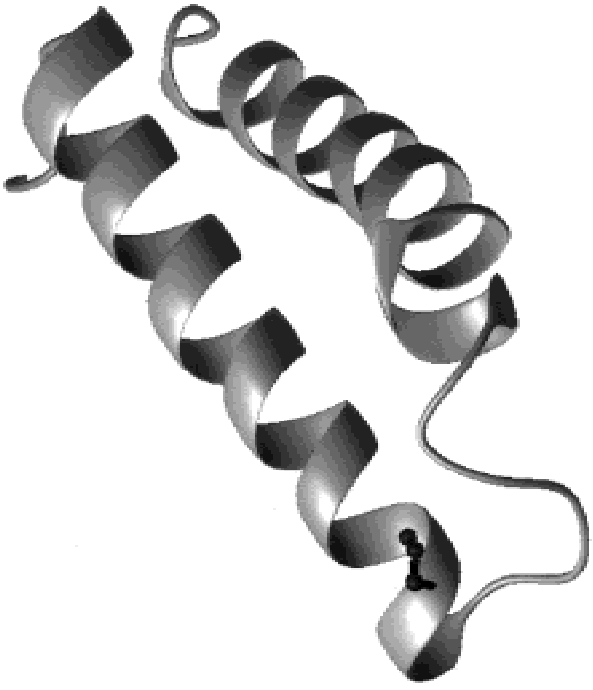

Fig. 1. Ribbon diagram of the TH8-TH9 hairpin in native DT (residues 321 to 378) with the Pro345 sidechain highlighted. This image was generated with the molecular display program MOLMOL (Koradi, Billeter & Wuthrich, 1996) using the diphtheria toxin coordinates of M. J. Bennet and D. Eisenberg (1994).

single-channel conductance experiments indicates that the TH8/TH9 hairpin undergoes insertion and suggests that the acidic loop is positioned near the aqueous solution on the opposite face of the membrane (Mindell et al., 1994*a*, 1994*c;* Oh, et al., 1996; Silverman, 1994; Zhan et al., 1994, 1995). Results of disulfide clamping experiments support the notion that channel formation depends on movement of adjacent helices away from the TH8/TH9 hairpin (Zhan et al., 1994). Finally, use of electron paramagnetic or fluorescent reporter groups attached to selected sites on the T domain has given information about the conformation of the membrane inserted T domain (Zhan et al., 1995; Wang et al., 1997; Oh, et al., 1996). No model of the inserted hairpin that is consistent with all of the data has yet emerged, however.

Johnson et al. (1993) reported that replacement of Pro345 with either Glu or Gly caused a dramatic decrease in the translocation activity and cytotoxicity of DT (Johnson et al., 1993). Pro345 lies at the end of the TH8 α-helix, where it may serve as an α helix-breaker (*see* Fig. 1). To probe the basis of the functional defects caused by these mutations, we prepared mutant forms of DT or T domain in which Pro345 was replaced by Cys, Glu or Gly. Our results show that mutations in Pro345 disrupt the ability of the T domain to insert into model bilayers or to adopt functional conformations in the membrane.

Materials and Methods

Site-Directed Mutagenesis

Site-directed mutagenesis was performed with the Oligonucleotide Directed in vitro Mutagenesis System, Version 2.1 (Amersham), according to the manufacturer's instructions. Phage M13mp18::DT vector containing the entire diphtheria toxin gene sequence (with the E148S substitution to attenuate its toxicity) was constructed by inserting the *Bam*HI-*Eco*RI fragment containing the DT sequence from pDO1 (O'Keefe et al., 1992) into M13mp18. The double mutant T-domain 332C/345E was constructed by mutagenic PCR using a template containing the 332C mutation (Zhan et al., 1995). All mutations were screened and verified by DNA sequencing using Sequenase 2.0 (United States Biochemicals). All other DNA manipulations were performed as described by Ausubel and Brent (1987). T4 DNA ligase and restriction enzymes were from New England Biolabs.

Mutant DT Expression and Purification

A Nsi I-Sph I DNA fragment containing the Pro345 mutations from double stranded M13mp18::DT was subcloned into pWHS105 (Shen et al., 1994) for over-expression and purification. pWHS105 is a derivative of pET-15b (Novagen) coding for full-length WT DT, lacking a signal sequence, but possessing an N-terminal hexa-histidine affinity tag and a short linker, which do not affect toxicity. To avoid confusion, the numbering of residues is the same as in native DT (Greenfield et al., 1983). The expression and purification procedures were as described in the pET System Manual from Novagen and as previously reported (Zhan et al., 1995). Mutant DTs were expressed in the cytoplasm of *E. coli* BL21(DE3) (Studier & Mofat, 1986) and purified to homogeneity, as judged by the absence of additional bands on nonreducing 7.5% SDS/polyacrylamide gels. Mutant DT proteins were stored in 20 mM Tris-HCl, pH 8.0, at −70°C. For 345C, no obvious dimers were observed, even after storage for one week at 4°C in the absence of reducing agent. The identities of the expressed DTs were confirmed by western immunoblotting. Protein concentrations were determined with Protein Assay Reagent (Pierce). Nicking of the purified mutant DTs was achieved by mild digestion with Protease Arg-C (Sigma) (1% w/w).

T Domain Expression and Characterization

Wild-type T domain, the 332C mutant, and the double 332C/345E mutant were purified as described (Zhan et al., 1995). T domain protein concentrations were determined spectrophotometrically, using a value of the extinction coefficient at 280 nm, 1.85 ml^{-1} mg cm^{-1}, determined by correlating the absorbance at this wavelength and with the quantity of protein as measured by amino acid analysis. Circular dichroism spectra were obtained on a Jasco J-710 spectropolarimeter at 25°C. A cuvette with a path length of 0.1 cm was used for the far-UV

region (200–260 nm). Samples were scanned three times at a rate of 40 nm/min in 20 mM HEPES buffer, pH 7.6, and the scans were averaged.

Cys-Specific Labeling

Residue 332C on the T domain was labeled with N,N′-dimethyl-N-(iodoacetyl)-N′-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ethylenediamine (IANBD) (Molecular Probes) as follows. Immediately before reaction, the proteins (T-WT, T-332C, or T-332C/345E), which were stored in 1 mM DTT, 20 mM Tris-HCl, pH 8.0, were exchanged into 20 mM Tris-HCl buffer, pH 8.0, on a G-25 size-exclusion PD10 column (Pharmacia). IANBD dissolved in DMSO was slowly added to a 35-fold molar excess of reagent. The final concentration of DMSO in the sample solution was less than 1%. The reaction mixture was incubated at 4°C in the dark for 18 h. After incubation, free IANBD was removed on a PD10 column, equilibrated with 20 mM Tris-HCl, pH 7.6, 140 mM NaCl. Fluorescence emission spectra for NBD labeled T domains were recorded at room temperature on a Perkin Elmer luminescence spectrometer LS 50B at an excitation wavelength of 485 nm.

TNS-Dependent Sensing of Protein Unfolding

The protocol was essentially the same as that described (Koehler & Collier, 1991). Mutant or WT DT (330 nM) was incubated with 150 μM TNS for 20 min at room temperature in 150 mM NaCl, 1 mM EDTA, containing 100 mM buffer (Tris-HCl for pH 8.0 and 7.0; 2-(N-morpholino) ethane sulfonic acid (MES) for pH 6.5 and 6.0; sodium acetate buffer for pH 5.5, 5.25, 5.0, 4.75 and 4.5). The fluorescence emission was recorded (excitation 366 nm, emission 440 nm), with constant stirring at 25°C in an Aminco SLM SPF-500C spectrofluorometer equipped with a thermostated cuvette chamber.

Cell Culture and Protein Synthesis Assays

The ability of the wild-type or mutant toxins to inhibit protein synthesis was measured in Vero cells (African Green Monkey Kidney, ATCC CCL81), as previously described (Kaul et al., 1996). Acid precipitable radiolabel was measured and expressed as a percentage of incorporation by control cells.

Channel Measurements

The method of measuring channel formation and properties in planar phospholipid bilayer membranes is essentially the same as described (Silverman, 1994). The solution on the *cis* side was 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, 30 mM MES, pH 4.8, and for the *trans* side was 1 M KCl, 2 mM $CaCl_2$, 1 mM EDTA, 50 mM HEPES, pH 6.8. After the formation of the membrane, a known concentration of WT or mutant DT was added to the *cis* solution, which was stirred with a miniature magnetic stir-bar, and known voltages were applied across the membrane. Voltages are those of the *cis* compartment; the potential of the *trans* compartment is taken as zero. Membranes were voltage clamped using an EPC-7 patch-clamp amplifier (List Medical Systems, Darmstadt, Germany) and current was monitored on a chart recorder.

Small Unilamellar Vesicles

Mixtures of 20% DOPG (w/w) and 80% PC (w/w) in $CHCl_3$ (20 mg for fluorophore leakage assays or 10 mg for NBD-labeled T-domain studies) were dried under Ar or N_2 and then further dried under vacuum for 3 hr. The dried lipid film was hydrated in 2 ml 12.5 mM ANTS, 45 mM DPX, 45 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 (for liposome leakage assays) or 1 ml buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, 140 mM NaCl, pH 7.6 (for NBD experiments) with vortexing. Samples were then sonicated on ice in a Branson Model 350 Sonifier until nearly optically clear. Care was taken to avoid heating the lipid suspension during the sonication. After sonication, free ANTS and DPX fluorophores were separated from the lipid vesicles by chromatography on a Sephadex G-50 column equilibrated with 10 mM Tris-HCl, 0.1 mM EDTA, 140 mM NaCl, pH 7.5. The phospholipid content of the liposomes was measured by inorganic phosphate analysis (McClare, 1971).

Monitoring of Fluorophore Leakage from Phospholipid Vesicles

Insertion of mutant DT into the model membranes was measured by a fluorophore leakage assay as described (Mel & Stroud, 1993). DPX quenches the fluorophore ANTS when coentrapped within liposomes, and leakage of ANTS and DPX from the liposomes into the buffer leads to the dilution of the fluorophore, dequenching of ANTS, and an increase in fluorescence. WT or mutant DT was added to liposomes (50 μM final phospholipid concentration in 10 mM Tris-HCl, 140 mM NaCl and 0.1 mM EDTA, pH 7.5) containing a mixture of ANTS and DPX, and the fluorescence of the mixture was monitored (excitation 360 nm, emission 550 nm) with continuous stirring at 25°C. After determining baseline fluorophore leakage, 0.5 M sodium acetate (pH 4.3) buffer was added to bring the mixture to pH 5.0 and the volume to 2 ml. Triton X-100 (5 μl of a 5% w/v solution) was added at the end of the experiment to lyse the liposomes, effecting complete release of the fluorophores.

Abbreviations

ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DOPG, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol; DPX, *p*-xylylenebis (pyridinium bromide); DT, diphtheria toxin; EDTA, ethylenediaminetetraacetic acid; IANBD, N,N′-dimethyl-N-(iodoacetyl)-N′-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ethylenediamine; MES, 2-(N-morpholino) ethane sulfonic acid; PC, phosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SUV, small unilamellar vesicles; TCA, trichloroacetic acid; T domain, transmembrane domain; TNS, 2-*p* toluidinyl-naphthalene 6-sulfonate; Tris, tris(hydrooxymethyl) aminomethane; WT DT, ''pseudo wild-type'' DT, containing the active-site mutation, E148S, to attenuate toxicity in accord with NIH guidelines.[1]

[1] In compliance with the National Institutes of Health recombinant DNA guidelines, all studies utilized an attenuated form of DT carrying the active site mutation, E148S (Barbieri & Collier, 1987), designated WT DT in this paper.

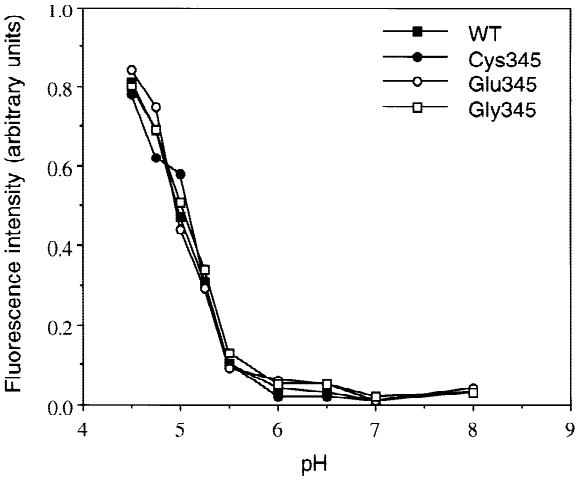

Fig. 2. Effect of pH on the fluorescence of TNS in the presence of WT and mutant forms of DT. Proteins were diluted to 330 nM in 150 μM TNS in 150 mM NaCl, 1 mM EDTA-containing buffer at pH 8.0, 7.0, 6.5, 6.0, 5.5, 5.25, 5.0, 4.75, or 4.5 and incubated for 20 min at room temperature. The fluorescence was determined at 25°C (excitation 366 nm, emission 440 nm). The data shown are the direct readout values from the fluorimeter.

Results

Preparation of Mutant Forms of DT

We replaced Pro345 in WT DT with Glu, Gly and Cys by site-directed mutagenesis. Cys was selected instead of Ala because its side chain is of intermediate bulk and is relatively hydrophobic (Kyte & Doolittle, 1982; Dunten, Sahin-Toth & Kaback, 1993). The mutant proteins were expressed cytoplasmically in *E. coli* and purified from the soluble fraction by Ni^{++}-chelate affinity chromatography, followed by anion exchange chromatography. All three proteins were reactive with DT antiserum. The yield of the 345C and 345G mutants was similar to that of WT DT (~2.5 mg/L culture), and the mutant proteins were stable at 4°C for up to two weeks in 20 mM Tris-HCl, pH 8.0. The 345G mutant was much less stable, and its yield was only ~20% that of WT. This low yield could reflect off-pathway folding events in *E. coli* or increased sensitivity to proteases. All three mutations virtually eliminated the toxic activity of DT; residual activity was ≪1% of WT, as determined by the ability to inhibit protein synthesis in Vero cells in 24-hr assays (*data not shown*). These results confirm the findings of Johnson et al. (1993).

pH-Dependent Binding of TNS

The fluorophore TNS exhibits a much higher quantum yield in apolar environments than in aqueous environments and has been employed as a probe of hydrophobic sites on DT as the protein undergoes conformational changes under acidic conditions (Collins & Collier, 1987). As illustrated in Fig. 2, the pH-dependence profiles of TNS binding by the three 345 mutant proteins were similar to that of WT DT, suggesting that the mutations do not cause major perturbations in structure at either neutral or acidic pH.

Channel Activity Assays

At low pH, DT inserts into membranes and forms voltage-gated, ion-conducting channels in artificial lipid bilayers, an activity attributable to the T domain. Channel formation provides a simple means of analyzing the capacity for membrane insertion, and single-channel conductance may be used to examine the structure of the DT channel. The mutant DTs were tested for ability to form conductive channels in asolectin bilayers under conditions that mimic those in the endosome (pH 4.8 on the *cis* side or the DT-containing chamber: pH 6.8 on the *trans* side). Current recordings were analyzed both for the ability of the toxin to form channels (defined as the amount of protein required to produce single channels within a given time) and for the current carried by individual channels (channel conductance).

The most striking difference between all of the Pro345 mutant toxins and WT DT was in their channel-forming abilities. Whereas only 1 ng of WT toxin was necessary to produce one or two channels, several μg (over 1000-fold more) of the mutant toxins were required for the same level of activity. A good illustration of this difference was the following experiment, performed on a single membrane: with the voltage held at +60 mV, the addition of 20 μg of mutant toxins (successive addition of 5 μg 345C, 5 μg 345G, 5 μg 345E, and 5 μg of un-nicked 345G) resulted in a current of 17.5 pA after ~14 min; whereas, in less than 4 min after the subsequent addition of 0.5 μg of WT DT, the current rose to 2000 pA. Thus, all of the Pro345 mutants were profoundly deficient in channel-forming ability.

Single channels from each mutant could be observed. The conductances of the mutant channels, 23 and 30 pS for 345C, 23 pS for 345E and 30 pS for 345G, were similar to that of wild type channels (30 pS). DT channels flicker between an open state and a short lived closed state (Mindell et al., 1994*c*), and this flickering pattern was not the same for all of the mutant channels. For the WT and 345G channel, most of the time during this flickering was spent in the open state; thus 345G appears to maintain the properties of the wild-type channel. For the 345E channel, roughly half the flickering time was spent in the open state and half in the closed

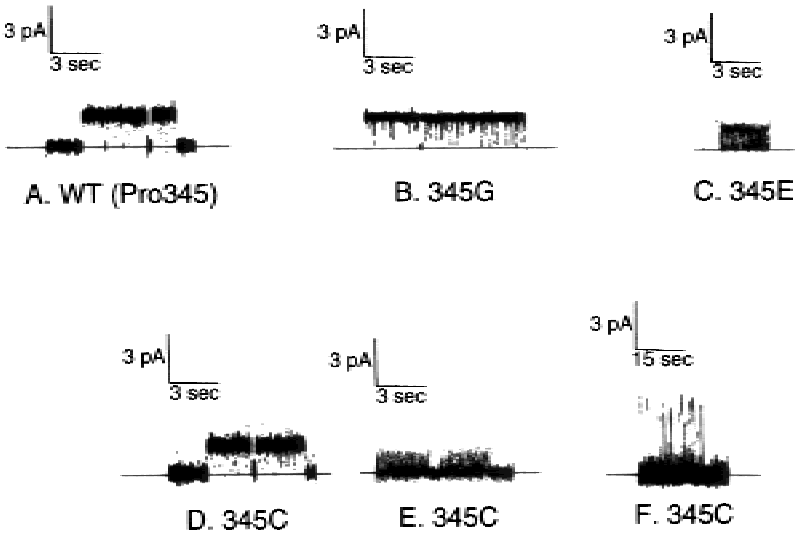

Fig. 3. Records of single channels formed by 345G, 345E, 345C, and WT DT in planar lipid bilayer membranes. In all records, the voltage across the membrane was held at +60 mV, and the thin horizontal line marks the closed level of the channel ($I = 0$). Note that the conductance and flickering behavior of the 345G channel (*B*) is essentially the same as that of the wild-type channel (*A*). The conductance of the 345E channel (*C*) is slightly smaller than that of the wild-type channel, and it spends roughly equal amounts of time in the flicker on and flicker closed states. Three types of channels are formed by 345C. In *D*, the channel is similar to that of wild-type; in *E*, the channel conductance is slightly smaller, and the channel spends most of its time in the flicker closed state; in *F*, we see brief openings to a conductance state about 2.5-fold larger than that of wild-type channels. All three of these channel types appeared in a single membrane.

Membranes were formed from asolectin (Lecithin type IIS from Sigma) as previously described (Silverman et al., 1994). The amounts of protein added to the chamber to obtain these records were 0.5 μg, 3 μg, 2.5 μg and 0.01 μg for 345G, 345C, 345E, and WT DT, respectively. Note the much smaller concentration of wild-type protein required to obtain single-channel activity. In fact, the difference in concentrations is even larger, since with the mutants, we had to wait several minutes before single channels appeared, whereas with wild-type, the first channel (which is shown in the record) appeared within seconds, and subsequently more channels entered the membrane.

state. For 345C we saw two different types of channel behavior. In one, the flickering pattern was similar to that of WT; in the other, most of the flickering time was spent in the closed state. In addition, we also saw brief openings of about 80 ps (Fig. 3).

Liposomal Leakage Studies

Effects of the Pro345 mutations on the ability of the toxin to permeabilize small unilamellar vesicles were also examined. Permeabilization of the vesicle membranes at low pH was measured by release of entrapped ANTS/DPX fluorophores (Mel & Stroud, 1993). As illustrated in Fig. 4, the extent of release over 5 min at pH 5.0 was dramatically reduced with 345C and 345E DT and approached undetectable levels with the 345G mutant. All three mutants displayed a large (>100-fold) reduction in the initial rate of fluorophore release in comparison with WT DT.

Effect of Glu345 on Membrane Insertion by T Domain

To probe the effects of the Pro345 mutations in greater detail, we prepared isolated T domain in which a unique Cys had been introduced at position 332 as an attachment site for a fluorescent reporter group. It had been determined in earlier studies that acidification in the presence of vesicles caused this residue to move into a lipid-exposed surface site at a depth of ~15 Å within the bilayer (Zhan et al., 1995; Oh et al., 1996). Wild-type, 332C, and double mutant 332C/345E T-domain proteins were prepared. All three proteins were stable, and their CD spectra were nearly identical, indicating that the mutations did not cause large perturbations in structure (Fig. 5).

The unique cysteine (332C) in T-332C and T-332C/345E was labeled with the fluorophore IANBD, as monitored by absorption at 481 nm. As expected, no labeling was observed in wild-type T domain. Insertion of an NBD-labeled chain into the phospholipid vesicle bilayer results in a large enhancement of fluorescence of the NBD group (Maduke & Roise, 1993). When we titrated small unilamellar vesicles into a solution of NBD-labeled T-332C at pH 5.0, the fluorescence increased as the concentration of lipids increased, until a plateau was reached at ~100 μg lipids per ml (Fig. 6*A*), while the λmax was blue-shifted by several nm over the same range of lipid concentrations (Fig. 6*B*). These results support the notion that the 332C residue is inserted into the hydrophobic core of the membrane. As shown in the Table, both NBD-labeled T-332C and T-332C/345E showed a λmax of 524–525 nm at pH 7.6 in the presence or absence of a saturating concentration of lipids (250 μg/ml). The presence of lipids caused little or no change in the intensity of 332-NBD fluorescence under these conditions. If in the presence of lipid, however, the pH was shifted from 7.6 to 5.0, there was a blue-shift in the

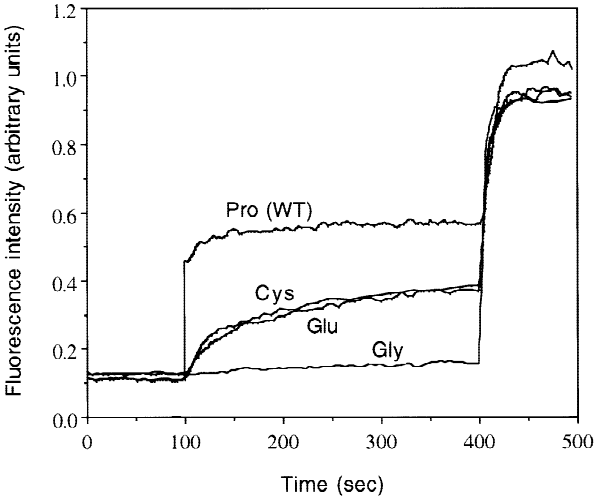

Fig. 4. Fluorophore (ANTS) leakage from the liposomes at pH 5.0. The assay had a final DT concentration of 62.5 nM and a final phospholipid concentration of 50 μM (molar ratio 1:800). Each of the DT samples was mixed with ANTS/DPX-containing liposomes at neutral pH for 100 sec; the pH was then lowered to 5.0. Detergent was added to lyse the liposomes for 100% fluorophore release at 400 sec. The initial rate of fluorophore leakage increased with increasing WT DT concentration within the range of 15.6 nM to 62.5 nM (*data not shown*). The upper-limit DT concentration was applied to maximize the sensitivity of the analysis. Fluorescence was not recorded during the time required for sample additions (~3 sec).

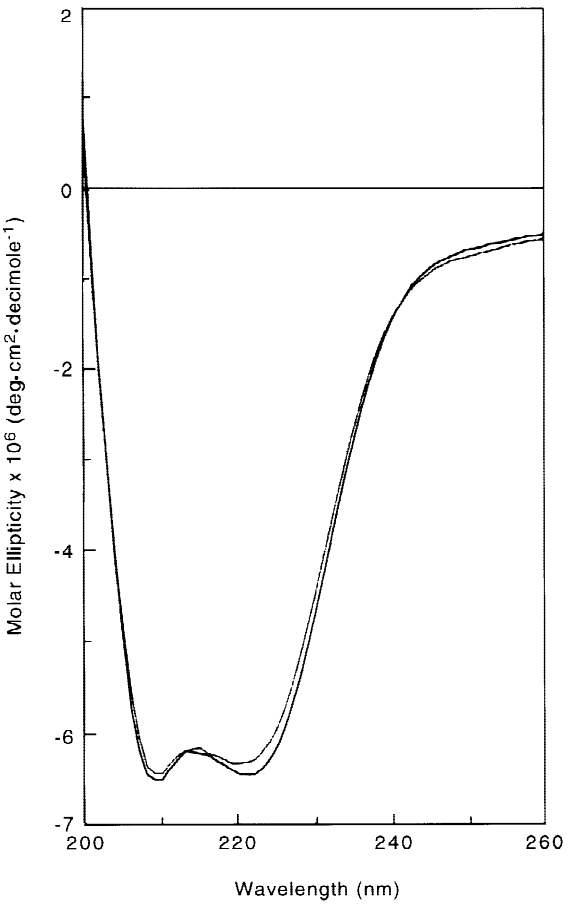

Fig. 5. Far UV CD spectra of wild-type T-domain (Pro345) (solid line) and the double mutant T-322C/345E (dotted line). Protein concentrations of 6.7 μM were used. The spectrum for T-332C (*not shown*) is substantially similar to those in this figure.

λmax and a strong increase in fluorescence intensity with T-332C-NBD. With the T-332C/345E-NBD mutant, in sharp contrast, there was no change in either λmax or fluorescence intensity. At pH 5.0 in the absence of lipids, both the wild-type and mutant proteins behaved similarly: there was no shift in λmax, and a strong decrease occurred in fluorescence intensity, due to aggregation of the proteins. These results indicate that the 345E mutation prevents the NBD reporter group from inserting into the hydrophobic interior of the bilayer, suggesting that the T domain does not undergo membrane insertion.

Discussion

While it is clear that the TH8-TH9 hairpin is involved in membrane insertion and pore formation by DT, the conformation adopted by the hairpin within the bilayer and its relationship to pore structure and the process of translocation remain elusive. We have shown here that replacement of Pro345 with Cys, Glu, or Gly causes a profound alteration in the interaction of DT with artificial phospholipid bilayers. This is evidenced by: (i) drastic reduction in channel-forming activity by the toxin in planar bilayers (an indicator of insertion in a transmembrane mode); (ii) loss of ability of the toxin to permeabilize liposomes to entrapped fluorophores; and (iii) inability of an NBD reporter group attached to 332C of isolated T domain to interact in the normal fashion with the hydrophobic interior of the bilayer. These results as a whole suggest that DT containing a mutation at position 345 is unable to undergo insertion into the bilayer. Alternatively, the mutant toxins may insert but be unable to adopt its normal, functional conformation (or may do so with low probability, relative to other accessible conformations).

As a consequence of its bulky ring and tertiary nitrogen, proline acts as an alpha-helical structure breaker. However, when glycine, another helix-breaker, was substituted for Pro345, the resulting protein was almost completely inactive in the channel formation and liposome leakage assays. The fact that the 345G mutant toxin was unstable (as indicated by its lower yield) im-

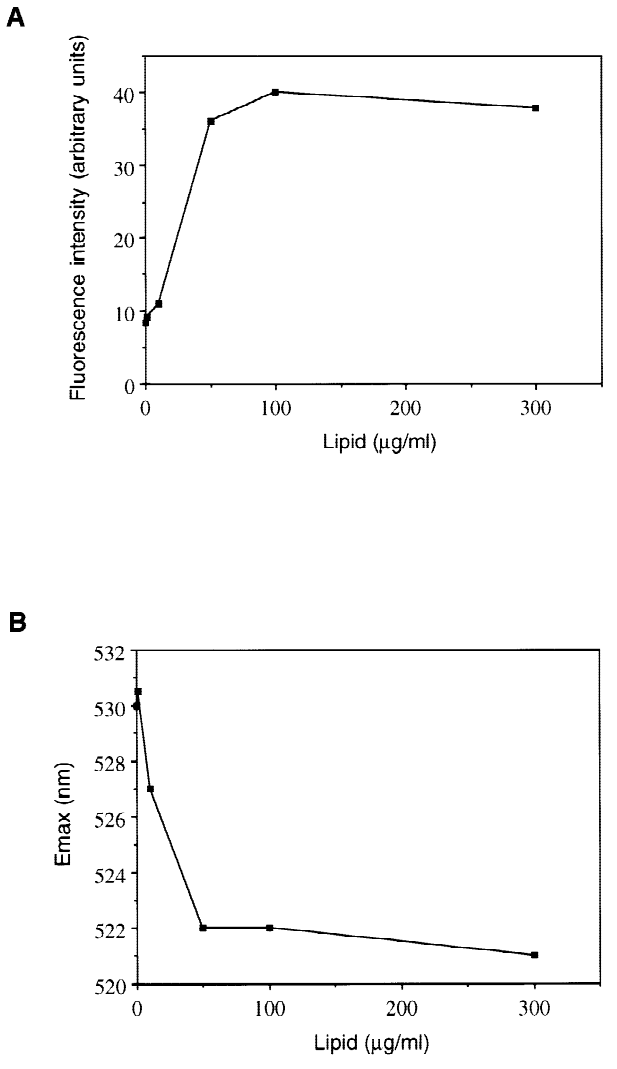

Fig. 6. Fluorescence of T-322C-NBD at pH 5.0 as a function of SUV concentration. Increasing concentrations of SUV (0.8:0.2 mixture of DOPC/DOPG) and 35 μg/ml T-332C-NBD were incubated for 10 min before bringing the pH to 5.0 with 0.8 M NaOAc. After 5 min, the fluorescence intensities (arbitrary units) (*A*) and emission maxima (*B*) were recorded. This figure represents a different data set than that shown in the Table.

plies that the conformation of the native protein was altered, although no change was seen in the pH-dependent conformational rearrangement, as detected by TNS binding. The fact that 345G failed to substitute for Pro345 suggests that this residue plays a specific structural role in the conformation of the helices.

The proline residue in a peptide bond manifests two important conformational characteristics: (i) the Φ angle is constrained to values of −68 or −75 (ring puckered up or down) and (ii) the peptide bond exhibits both *cis* and *trans* isomers (Yaron & Naider, 1993). *Cis/trans* isomerization of prolyl peptide bonds constitutes an important aspect of the folding of many proteins (Schmid, 1993), and the native conformation requires the ''correct'' prolyl isomer, which may be stabilized by structural constraints. Proline is the only residue for which the *cis* peptide bond is energetically accessible. In the crystal structure of the T domain (Bennett & Eisenberg, 1994) Pro345 is in the *trans* configuration. The rate of isomerization is pH-dependent (Steinberg et al., 1960), and given that the membrane insertion by DT requires a conformational transition, it is conceivable that proline(s) may be involved in this process, as proposed by Deleers et al., (1983). Specifically, Pro345 may play an important role in determining the conformation of the TH8-TH9 hairpin within the membrane. Incorrect orientation of the helices resulting from the replacement of Pro345 could create an energy barrier for membrane penetration.

The information generated from the single channel analysis is intriguing. The fact that the single-channel behavior of 345G was similar to that of wild-type DT, while the behaviors of 345E and 345C were different, suggests that after membrane insertion, α-helix disruption at residue 345 is necessary for normal channel function. In other words, though Gly at 345 interfered with the membrane insertion process, it has the capacity to replace Pro, restoring normal function after the insertional event. While the relative liposome permeabilization activities of the 345C and 345E proteins were considerably better than that of the 345G (Fig. 4), the single-channel properties were altered. These observations

Table 1. Effects of lipid and pH on fluorescence of NBD in T-332C-NBD and T-332C/345E-NBD

		T-332C-NBD		T-332C/345E-NBD	
		pH 7.6	pH 5.0	pH 7.6	pH 5.0
Protein alone	λmax	524.8 ± 0.7	527.1 ± 1.7	523.9 ± 0.9	527.4 ± 1.2
	Intensity	21.8 ± 1.2	7.4 ± 0.3	29.0 ± 0.3	9.9 ± 0.2
Protein + lipids	λmax	525.0 ± 0.3	518.9 ± 0.6	525.0 ± 1	523.5 ± 1.3
	Intensity	22.0 ± 0.8	39.9 ± 0.8	31.2 ± 0.5	28.4 ± 1.2

The concentrations of T-332C-NBD and T-332C/345E-NBD were kept constant at 35 μg ml^{-1}; lipids were added to 250 μg ml^{-1} where indicated. The data shown are the average of 4 measurements ± the SD.

imply that Pro345 may play a role in maintaining different conformations of the helices before and after insertion into the membrane, possibly achieved by the unique *trans/cis* isomerization property of proline. It is interesting to note that in the lactose permease of *E. coli*, a transmembrane protein, replacement of Pro31 (located at the interface with the membrane) with glycine does not perturb activity. Other replacements, including Ala, Cys, and Leu, significantly or completely inactivate the protein, however (Consler, Tsolas & Kaback, 1991; Sahin-Toth et al., 1994).

Regardless of the details of the mechanism by which Pro345 alters interactions with the membrane, the findings presented here indicate that the translocation defect in the Pro345 mutants, initially observed by Johnson et al. (1993), is likely due to a disruption of normal low-pH-triggered interactions of the T domain with the bilayer *per se.* Since the substitution of Pro345 leads to a major loss of toxicity of DT, mutations at this site may have practical applications in developing a new vaccine against diphtheria.

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References

Ausubel, F., Brent, R. 1987. Current Protocols in Molecular Biology. John Wiley and Sons, New York

Barbieri, J.T., Collier, R.J. 1987. Expression of a mutant, full-length form of diphtheria toxin in *Escherichia coli*. *Infect. Immun.* **55:**1647–1651

Bennett, M.J., Eisenberg, D. 1994. Refined structure of monomeric diphtheria toxin at 2.3 Å resolution. *Protein Science* **3:**1464–1475

Choe, S., Bennett, M.J., Fujii, G., Curmi, P.M., Kantardjieff, K.A., Collier, R.J., Eisenberg, D. 1992. The crystal structure of diphtheria toxin. *Nature* **357:**216–22

Collins, C.M., Collier, R.J. 1987. Effects of acidic pH on the conformation and activity of diphtheria toxin. *In:* Membrane-mediated cytoxicity. B. Bonavida and R.J. Collier, editors. pp. 41–52. Alan R. Liss, New York

Consler, T.G., Tsolas, O., Kaback, H.R. 1991. Role of proline residues in the structure and function of a membrane transport protein. *Biochemistry* **30:**1291–1297

DeLeers, M., Beugnier, N., Falmagne, P., Cabiaux, V., Ruysshcaert, J.M. 1983. Localization in diphtheria toxin fragment B of a region that induces pore formation in planar lipid bilayers at low pH. *FEBS Lett.* **160:**82–86

Dunten, R.L., Sahin-Toth, M., Kaback, H.R. 1993. Cysteine scanning mutagenesis of putative helix XI in the lactose permease of *Escherichia coli*. *Biochemistry* **32:**12644–12650

Falnes, P.Ø., Wiedlocha, A., Rapak, A., Olsnes, S. 1995. Farnesylation of CaaX-tagged diphtheria toxin A-fragment as a measure of transfer to the cytosol. *Biochemistry* **34:**11152–11159

Greenfield, L., Bjorn, M.J., Horn, G., Fong, D., Buck, G.A., Collier, R.J., Kaplan, D.A. 1983. Nucleotide sequence of the structural gene for diphtheria toxin carried by corynebacteriophage beta. *Proc. Natl. Acad. Sci. USA* **80:**6853–6857

Johnson, V.G., Nicholls, P.J., Habig, W.H., Youle, R.J. 1993. The role of proline 345 in diphtheria toxin translocation. *J. Biol. Chem.* **268:**3514–3519

Kaul, P., Silverman, J., Shen, W.H., Blanke, S.R., Huynh, P.D., Finkelstein, A., Collier, R.J. 1996. Roles of Glu 349 and Asp 352 in membrane insertion and translocation by diphtheria toxin. *Protein Sci.* **5:**687–692

Koehler, T.M., Collier, R.J. 1991. Anthrax toxin protective antigen: low-pH-induced hydrophobicity and channel formation in liposomes. *Mol. Microbiol.* **5:**1501–1506

Koradi, R., Billeter, M., Wuthrich, K. 1996. MOLMOL: a program for display and analysis of macromolecular structures. *J. Molec. Graphics* **14:**29–32

Kyte, J., Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157:**105–132

London, E., Ulbrandt, N.D., Tortorella, D., Jiang, J.X., Abrams, F.S. 1993. Insights into membrane protein folding and translocation from the behavior of bacterial toxins: models for membrane translocation. *Society of General Physiologists Series* **48:**45–61

Maduke, M., Roise, D. 1993. Import of a mitochondrial presequence into protein-free phospholipid vesicles. *Science* **260:**364–366

McClare, C.W. 1971. An accurate and convenient organic phosphorous assay. *Analyt. Biochem.* **39:**527–530

Mel, S.F., Stroud, R.M. 1993. Colicin Ia inserts into negatively charged membranes at low pH with tertiary but little secondary structural change. *Biochemistry* **32:**2082–2089

Mindell, J.A., Silverman, J.A., Collier, R.J., Finkelstein, A. 1994*a*. Structure function relationships in diphtheria toxin channels: II. A residue responsible for the channel's dependence on trans pH. *J. Membrane Biol.* **137:**29–44

Mindell, J.A., Silverman, J.A., Collier, R.J., Finkelstein, A. 1994*b*. Structure-function relationships in diphtheria toxin channels: III. Residues which affect the *cis* pH dependence of channel conductance. *J. Membrane Biol.* **137:**45–57

Mindell, J.A., Zhan, H., Huynh, P.D., Collier, R.J., Finkelstein, A. 1994c. Reaction of diphtheria toxin channels with sulfhydryl-specific reagents: observation of chemical reactions at the single molecule level. *Proc. Natl. Acad. Sci. USA* **91:**5272–5276

Oh, K.J., Zhan, H., Cui, C., Hideg, K., Collier, R.J., Hubbell, W.J. 1996. Organization of diphtheria toxin T domain in bilayers: a site-directed spin labeling study. *Science* **273:**810–812

O'Keefe, D.O., Cabiaux, V., Choe, S., Eisenberg, D., Collier, R.J. 1992. pH-dependent insertion of proteins into membranes: B-chain mutation of diphtheria toxin that inhibits membrane translocation, Glu-349-Lys. *Proc. Natl. Acad. Sci. USA* **89:**6202–6206

Sahin-Toth, M., Persson, B., Schweiger, J., Cohan, P., Kaback, H.R. 1994. Cysteine scanning mutagenesis of the N-terminal 32 amino acid residues in the lactose permease of *Escherichia coli*. *Protein Science* **3:**240–247

Schmid, F.X. 1993. Prolyl isomerase: enzymatic catalysis of slow protein-folding reactions. *Annu. Rev. Biophys. Biomol. Struct.* **22:**123–142

Shen, W.H., Choe, S., Eisenberg, D., Collier, R.J. 1994. Participation of lysine 516 and phenylalanine 530 of diphtheria toxin in receptor recognition. *J. Biol. Chem.* **269:**29077–29084

Silverman, J.A., Mindell, J.A., Zhan, H., Finkelstein, A., and Collier, R.J. 1994. Structure-function relationships in diphtheria toxin channels: I. Determining a minimal channel-forming domain. *J. Membrane Biol.* **137:**17–28

Steinberg, I.Z., Harrington, W.F., Berger, A., Sela, M., Katchalski, E. 1960. The configurational changes of poly-L-proline in solution. *J. Amer. Chem. Soc.* **82:**5263–5279

Studier, F.W., Mofat, B.A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189:**113–130

Wang, Y., Malenbaum, S.E., Kachel, K., Zhan, H., Collier, R.J., London, E. 1997. Identification of shallow and deep membrane-penetrating forms of diphtheria toxin T domain that are regulated by protein concentration and bilayer width. *J. Biol. Chem.* **272:**25091–25098

Yaron, A., Naider, F. 1993. Proline-dependent structural and biological properties of peptides and proteins. *Critical Rev. Biochem. Mol. Biol.* **28:**31–81

Zhan, H., Choe, S., Huynh, P.D., Finkelstein, A., Eisenberg, D., Collier, R.J. 1994. Dynamic transitions of the transmembrane domain of diphtheria toxin: disulfide trapping and fluorescence proximity studies. *Biochemistry* **33:**11254–11263

Zhan, H., Oh, K.J., Shin, Y.-K., Hubbell, W.L., Collier, R.J. 1995. Interaction of the isolated transmembrane domain of diphtheria toxin with membranes. *Biochemistry* **34:**4856–4863