

Roles of Glu 349 and Asp 352 in membrane insertion and translocation by diphtheria toxin

POONAM KAUL,¹ JARED SILVERMAN,^{1,3} WEI HAI SHEN,^{1,4} STEVEN R. BLANKE,¹
PAUL D. HUYNH,² ALAN FINKELSTEIN,² AND R. JOHN COLLIER¹

¹ Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

² Departments of Physiology & Biophysics and Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

(RECEIVED October 31, 1995; ACCEPTED January 5, 1996)

Abstract

Acidic conditions within the endosomal lumen induce the T domain of receptor-bound diphtheria toxin (DT) to insert into the endosomal membrane and mediate translocation of the toxin's catalytic domain to the cytosol. A conformational rearrangement in the toxin occurring near pH 5 allows a buried apolar helical hairpin of the native T domain (helices TH8 and TH9) to undergo membrane insertion. If the inserted hairpin spans the bilayer, as hypothesized, then the two acidic residues within the TL5 interhelical loop, Glu 349 and Asp 352, should become exposed at the neutral cytosolic face of the membrane and reionize. To investigate the roles of these residues in toxin action, we characterized mutant toxins in which one or both acidic residues had been replaced with nonionizable ones. Each of two double mutants examined showed a several-fold reduction in cytotoxicity in 24-h Vero cell assays (sixfold for E349A+D352A and fourfold for E349Q+D352N), whereas the individual E349Q and D352N mutations caused smaller reductions in toxicity. The single and double mutations also attenuated the toxin's ability to permeabilize Vero cells to Rb⁺ at low pH and decreased channel formation by the toxin in artificial planar bilayers. Neither of the double mutations affected the pH-dependence profile of the toxin's conformational rearrangement in solution, as measured by binding of the hydrophobic fluorophore, 2-*p*-toluidinyl-naphthalene 6-sulfonate. The results demonstrate that, although there is no absolute requirement for an acidic residue within the TL5 loop for toxicity, Glu 349 and Asp 352 do significantly enhance the biological activity of the protein. The data are consistent with a model in which ionization of these residues at the cytosolic face of the endosomal membrane stabilizes the TH8/TH9 hairpin in a transmembrane configuration, thereby facilitating channel formation and translocation of the toxin's catalytic chain.

Keywords: diphtheria toxin; membrane insertion; membrane translocation; pH dependence

Many protein toxins undergo direct interactions with the plasma membrane or membranes of internal compartments of their target cells. Some form channels in the plasma membrane and ultimately cause colloid osmotic lysis, whereas others undergo

more complex interactions, resulting in transfer of their catalytic moieties across membranes and into the cytosolic compartment. In recent years, crystallographic structures of toxins of both types have been elucidated (Allured et al., 1986; Sixma et al., 1991; Choe et al., 1992; Parker et al., 1992, 1994; Stein et al., 1994), providing a useful framework for understanding the interactions of such toxins with membranes.

A prominent structural feature in certain membrane-interactive toxins is an α -helical domain that inserts into the bilayer and converts the protein into an integral membrane form, as an essential step in the toxin's action. The most extensively studied of such domains are those of colicin A, which kills susceptible *Escherichia coli* strains by forming ion-conductive channels in the inner membrane (Parker et al., 1992), and diphtheria toxin, which kills susceptible mammalian cells by introducing its catalytic moiety into the cytosol and inhibiting protein synthesis (Choe et al., 1992). In both of these toxins, partial unfold-

Reprint requests to: R. John Collier, Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115; e-mail: collier@warren.med.harvard.edu.

³ Present address: Section of Infectious Diseases, Yale University School of Medicine, LCI 808, 333 Cedar Street, New Haven, Connecticut 06510-8056.

⁴ Present address: Genetics Institute, 85 Bolton Street, Cambridge, Massachusetts 02140.

Abbreviations: DT, diphtheria toxin; ψ wt DT, pseudo-wild-type diphtheria toxin; TNS, 2-*p*-toluidinyl-naphthalene 6-sulfonate; MEM, minimal essential media; PBS, phosphate-buffered saline; ANTS, 8-aminonaphthalene-1,3,6 trisulfonic acid disodium salt; DPX, *p*-xylylene-bis(pyridinium bromide).

ing of a 10-helix bundle mediates insertion of the toxin into model phospholipid bilayers and formation of ion-conductive channels.

In DT, the α -helical domain (termed the T, or transmembrane, domain) is located in the primary structure between the amino-terminal catalytic (C) and carboxyl-terminal receptor-binding (R) domains (Choe et al., 1992). After binding to its receptor via the R domain, the toxin undergoes receptor-mediated endocytosis and is conveyed to the endosomal compartment (Collier, 1994). The acidic conditions there promote conformational changes in the toxin that induce the T domain to insert into the endosomal membrane, a step required for efficient translocation of the C domain to the cytosol. It has been proposed that the channel formed by the T domain serves as a conduit through which the unfolded C domain traverses the membrane (Kagan et al., 1981), but direct evidence for this is lacking.

A feature common to the α -helical domains of DT, colicin A, and certain other colicins is a buried pair of long apolar helices. There is evidence that these "hydrophobic hairpins" become unshielded through conformational rearrangements triggered by environmental conditions and/or intermolecular contacts, allowing the hairpins to insert into their respective target bilayers. In the case of DT, evidence from several approaches indicates that acidic conditions within the endosomal compartment (\sim pH 5.3) trigger a conformational alteration of the T domain that allows the overlying helices to separate from the buried hairpin (helices TH8 and TH9) and permits the hairpin to insert into the endosomal membrane (Cabiaux et al., 1993; Mindell et al., 1994a, 1994b; Silverman et al., 1994a, 1994b; Zhan et al., 1994, 1995).

The hydrophobic hairpin of DT's T domain is distinguished from colicin A's by, among other things, two acidic residues (Glu 349 and Asp 352) within the interhelical loop (TL5). Choe et al. (1992) and O'Keefe et al. (1992) proposed that the acidic pH of the endosomal lumen partially protonates these residues, facilitating insertion of the TH8-TH9 hairpin. If the inserted helices remain in the hairpin configuration and span the bilayer, Glu 349 and Asp 352 presumably traverse the hydrocarbon core in protonated form, and their side-chain carboxyl groups penetrate to the cytosolic face of the membrane and reionize there at the neutral pH of the cytosol. To probe the roles of these acidic residues in toxin action, we have characterized mutant forms of DT in which the residues have been replaced by non-ionizable ones.

Results

By oligonucleotide-directed mutagenesis, we substituted non-ionizable residues for E349 and/or D352 within an enzymically attenuated form of DT (termed pseudo-wild-type, ψ wt, toxin). The E148S active-site mutation within this form reduces ADP-ribosylation activity by \sim 1,000-fold (Barbieri & Collier, 1987; Wilson et al., 1990), permitting the toxin to be expressed in *E. coli* in accordance with the Recombinant DNA Advisory Committee guidelines. Two double mutants in the T domain were constructed, one in which E349 and D352 were replaced with their amide congeners (Gln and Asn, respectively), and another in which both residues were replaced with Ala. From the increase in the concentration of toxin required to inhibit protein synthesis by 50% (IC_{50}) in 24-h Vero cell assays, we estimated cytotoxic activity to be \sim sixfold lower in the E349A+D352A mutant and \sim fourfold lower in the E349Q+D352N mutant (Fig. 1A).

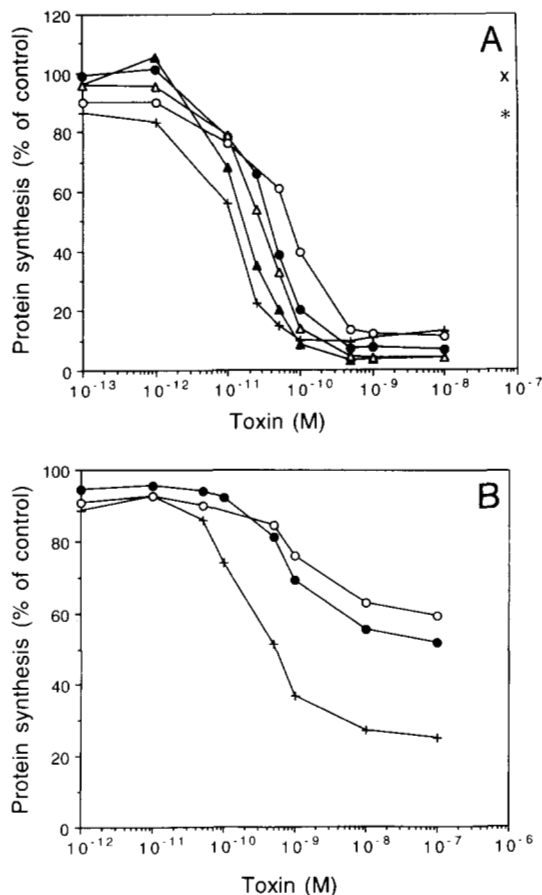


Fig. 1. Inhibition of protein synthesis in Vero cells by mutant forms of DT. Cells were incubated with nicked toxin at the concentration indicated for 24 h (A) or 4 h (B) at 37 °C. Protein synthesis was then measured during a 1-h incubation with radiolabeled leucine. Activity is expressed as percent of radioactivity incorporated in the absence of toxin. Results shown are representative of three similar experiments; each data point is the mean of duplicate samples, with variation from the mean being \pm 10% or less. Toxin: +, ψ wt; \blacktriangle , E349Q; \triangle , D352N; \bullet , E349Q+D352N; \circ , E349A+D352A; *, E349K (D352K gave identical results); X, E349R (D352R gave identical results).

Comparison with results of a 4-h assay, in which inhibition of protein synthesis was incomplete even at the highest concentrations of toxin tested (10^{-7} M), indicated that these double mutants slowed the rate of toxin action (Fig. 1B). The E349Q mutation alone caused only a slight reduction in cytotoxicity, and D352N alone caused a somewhat greater reduction (\sim threefold); the effects of these two mutations individually were approximately additive in the E349Q+D352N double mutant. The magnitude of these effects was small relative to those caused by substitution of Lys or Arg for E349 or D352, which almost completely blocked toxic activity (Fig. 1A).

To probe the basis of the change in cytotoxicity caused by neutral substitutions at positions 349 and 352, we first checked the ability of the mutants to permeabilize the plasma membrane of Vero cells. Receptor-bound DT is known to form cation-selective channels in the plasma membrane upon exposure to low pH (Sandvig & Olsnes, 1988), an activity that may be assayed by measuring release of $^{86}Rb^+$ from cells preloaded with this

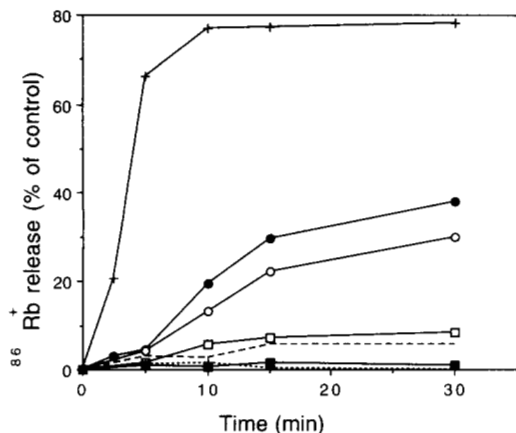


Fig. 2. Low pH-dependent permeabilization of Vero cells by mutant forms of DT. Cells preincubated with $^{86}\text{Rb}^+$ were incubated with varying concentrations of nicked toxin for 1 h, washed and exposed to pH 5.0 at 37 °C, and $^{86}\text{Rb}^+$ release was measured by sampling supernatant at intervals. Release is expressed as percent of total isotope released. The results shown are representative of three similar experiments; each data point is the mean of duplicate samples, which differed by no greater than $\pm 10\%$ from the mean. Toxin: +, ψwt ; ●, E349Q+D352N; ○, E349A+D352A; □, E349K; --, D352K; ---, E349R; ■, D352R.

K^+ analogue. Mutant toxins were allowed to bind to $^{86}\text{Rb}^+$ -containing Vero cells at 4 °C, a temperature that inhibited endocytosis of the toxin. After removal of unbound toxin, MES/Tris-buffered saline, pH 5.0, was added, and release of $^{86}\text{Rb}^+$ into the medium was assayed as a function of time after acidification of the medium. As shown in Figure 2, the E349Q+D352N and E349A+D352A double mutations caused major reductions in $^{86}\text{Rb}^+$ release, with E349A+D352A having a slightly greater effect than E349Q+D352N. Replacement of Glu 349 or Asp 352 with either Lys or Arg had much greater effects, virtually abolishing release. In other experiments, the D352N mutation alone was found to cause a reduction in ion release only slightly less than that of the E349Q+D352N double mutation, whereas the reduction caused by the E349Q mutation alone was only about half that of the D352N mutation (data not shown). These results are consistent with data obtained earlier showing differential influences of E349 and D352 on single-channel conductance (Mindell et al., 1994a). Concentration-dependence curves showed saturation at ~ 3 nM for both the mutant and control toxins (data not shown), which is close to the value reported for the apparent dissociation constant of the toxin with its receptor (Itelson & Gill, 1973).

DT has been found to form cation-selective channels in planar bilayer membranes (Donovan et al., 1981; Kagan et al., 1981). The rate of channel formation is sensitive to both the *cis* and *trans* pH, increasing dramatically when either the former is lowered or the latter is raised.⁵ As shown in Figure 3A, when the *trans* pH was raised from 5.3 to 7.1, the rate of channel formation increased by about 700-fold. In contrast, for the E349Q+

⁵ The single-channel conductance is also pH dependent, increasing when either the *cis* or *trans* pH is raised, but the magnitude of the pH effect on channel conductance is much smaller than that on the rate of channel formation. For example, in the experiments described in this section in which the *trans* pH is raised from 5.3 to 7.1, the single-channel conductance increased by only about 50%.

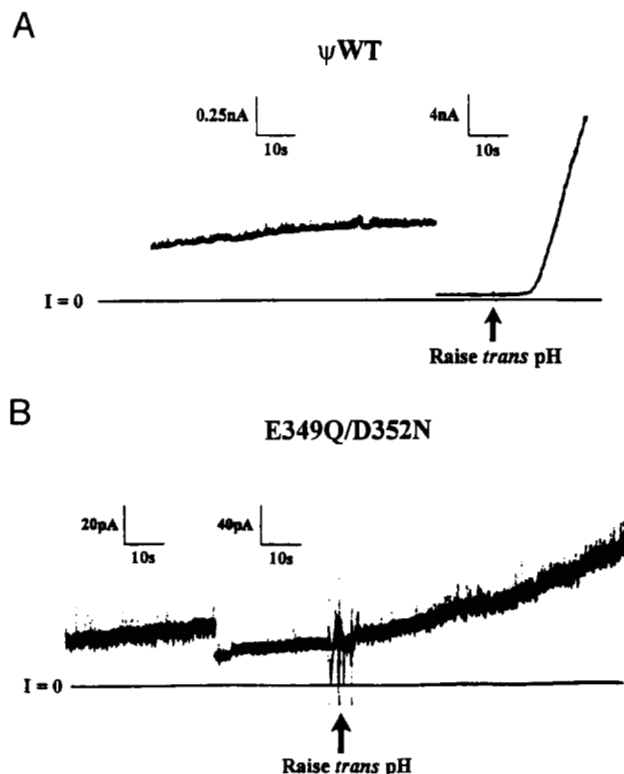


Fig. 3. Comparison of the effect of raising the *trans* pH on the rate of channel formation by ψwt and mutant toxin in planar lipid bilayers. **A:** Four minutes prior to the start of the record, 640 ng of nicked ψwt toxin were added to the *cis* compartment, pH 5.3. In the first segment of the record, the rate of current rise was 1.9 pA/s (the downward shift in the trace reflects the change in current scale). At the arrow, the *trans* pH was raised from 5.3 to 7.1 by the addition of concentrated Hepes buffer. Note the dramatic increase in the rate of current rise to 1,400 pA/s, which is more than 700-fold greater than before raising the *trans* pH. **B:** Seven minutes before the start of the record, 200 ng of E349Q+D352N were added to the *cis* compartment, pH 5.3. In the first segment of the record, the rate of current rise was 0.17 pA/s (the downward shift in the trace reflects the change in current scale). At the arrow, the *trans* pH was raised from 5.3 to 7.1 by the addition of concentrated Hepes buffer. The rate of current rise increased to 2.8 pA/s, which is only a factor of 16 greater than before raising the *trans* pH. Both the *cis* and *trans* compartments were stirred continuously throughout the experiments, and the membrane potential was held at +60 mV for the entire duration of the records shown.

D352N mutant, the same elevation of *trans* pH increased the rate of channel formation by only 16-fold (Fig. 3B), and for the E349A+D352A double mutant, a 12-fold increase was found. For the single E349Q and D352N mutants, raising the *trans* pH increased the rate of channel formation by 30–40-fold, a rate intermediate between that obtained with ψwt DT and that with its double-amide mutant.

The mutant toxins were also examined for changes in conformational state as a function of pH, using the fluorescent probe TNS, which exhibits a higher quantum yield in nonpolar than in polar media (Burns et al., 1986; Collins & Collier, 1987). Mutant or ψwt toxin (100 nM) was incubated with TNS (150 μM) for 15 min at room temperature in buffers at varying pH before readings were taken. TNS fluorescence increased sharply below pH 5.5 as accessibility of hydrophobic residues to the probe in-

creased. The mutant and control proteins showed essentially identical titration curves, indicating that the mutations did not cause major perturbation of conformation.

Discussion

Although there is considerable evidence that the TH8/TH9 hairpin buried within diphtheria toxin's T domain undergoes insertion into the endosomal membrane under the influence of low pH, the precise structure of this element in the membrane remains to be determined. The apolar nature of the helices, together with the presence of two acidic residues in the hairpin's interhelical loop, suggested that the inserted hairpin might adopt a transmembrane configuration in which part or all of the loop was exposed at the cytosolic face of the membrane (Choe et al., 1992; O'Keefe et al., 1992). This model is supported by the finding that substitution of Lys for Glu 349, Asp 352, or Ile 364 causes drastic reductions in toxicity and channel formation, a result expected if these activities require that the TL5 loop penetrate the bilayer (O'Keefe et al., 1992; Cabiaux et al., 1993; Silverman et al., 1994a). The E349R and D352R mutations, characterized in the current work, produced similar effects. Also, in electrophysiological studies of DT channels in planar lipid bilayers, substitution of Lys for Glu 349 or Asp 352 caused drastic reductions in channel activity (Silverman et al., 1994a), and Asp 352 mapped to the *trans* face of planar lipid bilayers (Mindell et al., 1994a), whereas ionizable residues at the opposite end of the TH8 helix mapped to the *cis* face (Mindell et al., 1994b). Additionally, electron paramagnetic measurements of site-directed spin-labeled T-domain have yielded results that are consistent with the proposed model (Zhan et al., 1995).

The current work shows that substitution of both Glu 349 and Asp 352, with either Ala or the corresponding amide-containing congeners, caused a reduction in cytotoxicity of four- to sixfold, with the double Ala substitution causing a slightly greater effect than the double-amide substitution.⁶ These results indicate, on the one hand, that there is no absolute requirement for an acidic residue within the TL5 loop for toxin action and, on the other, that such residues significantly enhance toxic activity of the protein. Either the E349Q or the D352N mutation alone caused an intermediate reduction of cytotoxicity, and the functions of Glu 349 and Asp 352 appear, to a first approximation, to be additive.

These effects are generally consistent with a model in which the translocation function of DT is dependent upon the inserted TH8/TH9 hairpin's spanning the membrane, and upon stabilization of this configuration by ionization of the acidic residues. From the fact that the mutations caused no change in the pH-dependence profile of TNS binding in solution, we infer that the effects on toxicity result from changes in the toxin's interaction with the bilayer, rather than its unfolding. Elimination of the charge of Glu 349 and Asp 352 could alter the partitioning of the toxin between inserted and noninserted states, or, perhaps more likely, permit the inserted hairpin to adopt other

configurations in the membrane that are inactive in promoting translocation. Studies with the channel-forming C-terminal fragment of colicin A have led to a proposal that the membrane-inserted hydrophobic helical hairpin of this protein lies parallel to the membrane surface (Lakey et al., 1993; Duché et al., 1994). The double-Ala mutant of DT should be more amenable than the double-amide mutant to configurations in which the TH8-TH9 hairpin is suspended in the membrane, without spanning it, and this may explain the greater loss of activity seen in this mutant.

Measurements of the changes in toxicity resulting from the double mutations were complemented by studies of their effects on low-pH induced ⁸⁶Rb⁺ release in Vero cells. The release, which is a function of both the rate of channel formation and the specific conductance of individual channels, was found to be significantly reduced by the double mutations, with the double-Ala substitution again showing a greater effect than the double-amide. The single D352N mutation caused a slightly smaller reduction, and the E349Q produced a still smaller effect. The greater effect of the D352N substitution is likely attributable to the fact that the charge state of the residue at this site strongly affects single-channel conductance, whereas that of Glu 349 has essentially no effect (Mindell et al., 1994a). For similar reasons, the effects of the double-Ala and double-amide mutations may reflect both a decreased single-channel conductance, due to the elimination of the negative charge at position 352, and an alteration in the insertion state or configuration of the toxin in the membrane. Substitution of either of the acidic residues with Lys or Arg virtually completely blocked ⁸⁶Rb⁺ release, consistent with our earlier report (Silverman et al., 1994a) and with the notion that a positively charged residue in the TL5 loop drastically reduces the probability of the hairpin's penetrating the membrane.

The effects of the mutations in Glu 349 and Asp 352 on the channel-forming activity of DT in artificial lipid bilayers are consistent with their effects on toxicity and permeabilizing activity on Vero cells. The strong dependence of channel formation on a pH gradient across the membrane implies a pH-sensing mechanism within the protein, which might be related to the ionization properties of Glu 349 and Asp 352. If channel formation depends on the TH8/TH9 hairpin's spanning the membrane, as proposed (O'Keefe et al., 1992; Silverman et al., 1994a), the elevation of the *trans* pH from acidic to neutral values would be expected to decrease the protonation of *trans*-exposed carboxyl groups of Glu 349 and Asp 352, thereby stabilizing the hairpin in a channel-competent transmembrane configuration. The finding that the *trans* pH dependence of channel formation is greatly altered by the E349Q+D352N double mutant, and less so by the single mutants, is consistent with this model. An effect of *cis* pH was also noted on channel formation by this double mutant, however (unpubl. obs.), indicating that the pH-sensing mechanism is complex and depends on other residues within the protein, as well. Although *in vivo* the toxin's interaction with membranes, including the nature of the channel formed under acidic conditions (Choe et al., 1992; O'Keefe et al., 1992), is affected by its interaction with its receptor, the correlation between the changes in membrane-permeabilizing properties seen in Vero cells with the mutants and those seen in planar bilayers implies a common basis for the effects.

The data presented here show that the ionization properties of Glu 349 and Asp 352 are functionally significant in the in-

⁶ In an earlier study, Silverman et al. (1994a) reported no effect of the E349Q or D352N mutations on either cytotoxicity or channel formation. Because the assays in this study were performed with impure mutant toxin preparations and the studies were designed primarily to screen for major (> fivefold) changes in activity, it is perhaps not surprising that the effects we now report were not detected.

toxication of mammalian cells by DT and support the model proposed earlier for membrane insertion by the TH8/TH9 hairpin. A more precise understanding of the structure of the hairpin in the membrane and of how insertion of the hairpin promotes translocation of the catalytic domain of DT awaits further studies.

Materials and methods

Materials, strains, and plasmids

Restriction endonucleases and T4 DNA ligase were from New England Biolabs; site-directed mutagenesis materials from Amersham Corp; DNA sequencing materials from United States Biochemical; and the pET15b vector and nickel chelate chromatography resin from Novagen. Vero cells were from American Type Culture Center (no. CCL 81). [³⁵S] Methionine, ⁸⁶Rb, and [³H] leucine were from NEN Dupont. *E. coli* XL1-Blue and BL21(DE3), used for expression of proteins, were from Stratagene. The plasmid pET15b was obtained from Novagen.

Site-directed mutagenesis

The DT coding sequence lacking the signal sequence was first cloned into the polylinker region of M13mp19 phage vector using the *Eco*R 1–*Hind* III sites. Site-directed mutagenesis was then performed with synthetic oligonucleotides synthesised on an applied Biosystems Model 381 DNA synthesizer. *E. coli* strain TG1 was used for all phage-related manipulations. Mutations were confirmed by dideoxy sequencing. The *Nsi* 1–*Sph* 1 fragment of the phage M13 containing the mutated site was then subcloned into the corresponding sites of pETWHS105, and the ligation product was transformed into *E. coli* XL1blue. For protein expression, this plasmid was finally electroporated into BL21(DE3).

Production and purification of mutant toxins

Overnight cultures from freshly streaked *E. coli* BL-21 transformed with the DT mutants were diluted 100-fold in L broth containing 100 µg/mL ampicillin and allowed to grow to an optical density of 1 at 600 nm. The cultures were then induced by the addition of isopropyl-D-thiogalactopyranoside to 1 mM and grown for another 2–3 h. Cells were then harvested by centrifugation at 3,000 × *g* for 10 min at 4 °C. The pellets were re-suspended in 20 mM Tris, 0.5 M NaCl, 5 mM imidazole, pH 7.9, and sonicated on ice in a Branson model 350 sonifier at power level 5, 50% duty cycle, pulse output for three 30-s treatments, separated by 5-min pauses. Cellular debris was removed by centrifugation at 5,000 × *g* for 30 min at 4 °C, and the supernatants were loaded on a Ni²⁺ chelate affinity column and separated according to the manufacturer's instructions. The final purification was achieved by anion exchange chromatography (Mono Q column from Pharmacia) using a gradient of NaCl to 1 M. The protein eluted at ca. 100 mM NaCl. The ψwt toxin and mutant toxins in 20 mM Tris-HCl, pH 7.5, were converted to the "nicked" form by incubation in the presence of 1 mM NAD and 1% (w/w) of TPCK-treated trypsin for 20 min at 25 °C. The reaction was stopped by the addition of soybean trypsin inhibitor (fivefold excess).

Cytotoxicity assay

Vero cells (African Green Monkey kidney) were grown in Eagle's minimal essential medium (MEM) supplemented with Earl's salts, calf serum (10%), penicillin G (50 U/mL), streptomycin (50 µg/mL) and glutamine (2 mM). The cells were maintained as monolayers and were grown in an atmosphere containing 5% CO₂ at 37 °C. For the assay, 2 × 10⁵ cells per well were seeded in a 24-well assay plate and allowed to grow to confluence at 37 °C for 24 h. After aspiration of the medium, trypsin-activated toxin in MEM was added, and the cells were incubated for an additional 24 h, unless otherwise indicated. The medium was then aspirated, and the cells were washed twice with PBS and incubated with 0.5 mL L-[3,4,5-³H] leucine (1 µCi/mL) in MEM lacking unlabeled L-leucine for 1 h at 37 °C. The medium was then aspirated, and the cells were washed twice with PBS and treated with 10% trichloroacetic acid. Protein synthesis was then estimated from the radioactivity in the acid-insoluble material.

⁸⁶Rb release assay

Vero cells were seeded in a 6-well plate (5 mL/well) or 24-well plate (1 mL/well) at a concentration of 2 × 10⁵ cells/mL. After incubation at 37 °C for 8 h, the medium was aspirated and replaced with MEM containing 1 µCi/mL of ⁸⁶Rb, and the cells were incubated for an additional 12 h at 37 °C. Mutant and control toxins at a concentration of 1 µg/mL, or as otherwise indicated, were added to the cells, and binding was allowed to occur for 1 h at 4 °C. The medium was aspirated to remove unbound toxin, and the cells were washed twice with cold PBS and twice with warm PBS, and finally were exposed to 20 mM MES/Tris, 145 mM NaCl, pH 5.0, at 37 °C. Aliquots of the medium were collected at intervals, and the release of ⁸⁶Rb was determined. At the end of the experiment, 5% TCA was added to each well, and an aliquot of the extract was used to measure radioactivity remaining associated with the cells. The amount of radioactivity released was determined according to the method of Thelestam and Molby (see Thelestam, 1988). %Rb release = (sample induced release – spontaneous release)/(maximal release – spontaneous release).

Planar lipid bilayer experiments

The experimental arrangement for forming planar lipid bilayers and recording from them was as described in Silverman et al. (1994b). Briefly, membranes were formed at room temperature by a modification of the method of Montal (1974) across a hole (~100 µm diameter) in a polystyrene cup. The membranes separated identical solutions containing 1 M KCl, 2 mM CaCl₂, 1 mM EDTA, 5 mM MES, pH 5.3; both solutions were stirred continuously with small magnetic stirring bars throughout the course of an experiment. After a membrane was formed, wild-type DT or one of its mutants was added to the solution in the cup (defined as the *cis* side). The voltage difference across the membrane was then clamped at 60 mV, with the *cis* side of the membrane positive with respect to the opposite *trans* side, and the resulting current flow across the membrane was monitored. The current across the membrane began to increase and, within a minute or two, achieved a constant rate: this rate of current increase is directly proportional to the rate of channel formation in the membrane. After this constant rate

was achieved, the pH of the *trans* solution was raised to between 7.0 and 7.2 by the addition to it of HEPES buffer. Within a few seconds (the mixing time of the solution) the current rapidly increased and reached a new, higher constant rate. The ratio of this new rate to that prior to the elevation of the *trans* pH measures the effect on channel formation of raising the *trans* pH (except for a small effect on single-channel conductance).

Determination of pH-dependent unfolding of DT

Mutant and control toxins were incubated with 150 μ M TNS for 20 min at room temperature in 20 mM buffer (Tris-HCl for pH 7.0; MES for pH 6.5 and pH 6.0; sodium acetate for pH 5.5, 5.0, and 4.5) containing 150 mM NaCl and 1 mM EDTA. The excitation wavelength was 366 nm and emission was measured at 440 nm.

Acknowledgments

This work was supported by NIH grants T32-GM-07288 (P.D.H.), GM-29210 (A.F.), and AI-22021 (R.J.C.).

References

- Allured VS, Collier RJ, Carroll SF, McKay DB. 1986. Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0-Ångstrom resolution. *Proc Natl Acad Sci USA* 83:1320-1324.
- Barbieri JT, Collier RJ. 1987. Expression of a mutant, full-length form of diphtheria toxin in *Escherichia coli*. *Infect Immun* 55:1647-1651.
- Burns G, Abraham AK, Vedeler A. 1986. Nucleotide binding to elongation factor 2 inactivated by diphtheria toxin. *FEBS Lett* 208:217-220.
- Cabiaux V, Mindell J, Collier RJ. 1993. Membrane translocation and channel-forming activities of diphtheria toxin are blocked by replacing isoleucine 364 with lysine. *Infect Immun* 61:2200-2202.
- Choe S, Bennett MJ, Fujii G, Curmi PM, Kantardjieff KA, Collier RJ, Eisenberg D. 1992. The crystal structure of diphtheria toxin. *Nature* 357:216-222.
- Collins CM, Collier RJ. 1987. Effects of acidic pH on the conformation and activity of diphtheria toxin. In: Bonavida B, Collier RJ, eds. *Membrane-mediated cytotoxicity*. New York: Alan R. Liss, Inc. pp 41-52.
- Collier RJ. 1994. Diphtheria toxin: Crystallographic structure and function. In: Freer J, Aitken R, Alouf JE, Boulnois G, Falmagne P, Fehrenbach F, Montecucco C, Piemont Y, Rappuoli R, Wadstrom T, Witholt B, eds. *Bacterial protein toxins, FEMS Symposium No. 73*. New York: Gustav Fischer. pp 217-230.
- Donovan JJ, Simon MI, Draper RK, Montal M. 1981. Diphtheria toxin forms transmembrane channels in planar lipid bilayers. *Proc Natl Acad Sci USA* 78:172-176.
- Duché D, Parker MW, González-Mañas JM, Pattus F, Baty D. 1994. Uncoupled steps of the colicin A pore formation demonstrated by disulfide bond engineering. *J Biol Chem* 269:6332-6339.
- Ittelson TR, Gill DM. 1973. Diphtheria toxin: Specific competition for cell receptors. *Nature* 242:330-332.
- Kagan BL, Finkelstein A, Colombini M. 1981. Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. *Proc Natl Acad Sci USA* 78:4950-4954.
- Lakey JH, Duché D, González-Mañas JM, Baty D, Pattus F. 1993. Fluorescence energy transfer distance measurements. The hydrophobic helical hairpin of colicin A in the membrane bound state. *J Mol Biol* 230:1055-1067.
- Mindell JA, Silverman JA, Collier RJ, Finkelstein A. 1994a. Structure-function relationships in diphtheria toxin channels: II. A residue responsible for the channel's dependence on *trans* pH. *J Membr Biol* 137:29-44.
- Mindell JA, Silverman JA, Collier RJ, Finkelstein A. 1994b. Structure-function relationships in diphtheria toxin channels: III. Residues which affect the *cis* pH dependence of channel conductance. *J Membr Biol* 137:45-57.
- Montal M. 1974. Formation of bimolecular membranes from lipid monolayers. *Methods Enzymol* 32:545-554.
- O'Keefe DO, Cabiaux V, Choe S, Eisenberg D, Collier RJ. 1992. pH-dependent insertion of proteins into membranes: B-chain mutation of diphtheria toxin that inhibits membrane translocation, Glu-349 \rightarrow Lys. *Proc Natl Acad Sci USA* 89:6202-6206.
- Parker MW, Buckley JT, Postma JP, Tucker AD, Leonard K, Pattus F, Tsernoglou D. 1994. Structure of the *Aeromonas* toxin proaerolysin in its water-soluble and membrane-channel states. *Nature* 367:292-295.
- Parker MW, Postma JP, Pattus F, Tucker AD, Tsernoglou D. 1992. Refined structure of the pore-forming domain of colicin A at 2.4 Å resolution. *J Membr Biol* 224:639-657.
- Sandvig K, Olsnes S. 1988. Diphtheria toxin-induced channels in Vero cells selective for monovalent cations. *J Biol Chem* 263:12352-12359.
- Silverman JA, Mindell JA, Finkelstein A, Shen WH, Collier RJ. 1994a. Mutational analysis of the helical hairpin region of diphtheria toxin transmembrane domain. *J Biol Chem* 269:22524-22532.
- Silverman JA, Mindell JA, Zhan H, Finkelstein A, Collier RJ. 1994b. Structure-function relationships in diphtheria toxin channels: I. Determining a minimal channel-forming domain. *J Membr Biol* 137:17-28.
- Sixma TK, Pronk SE, Kalk KH, Wartna ES, van Zanten BAM, Witholt B, Hol WGJ. 1991. Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli*. *Nature* 351:371-377.
- Stein PE, Boodhoo A, Armstrong GD, Cockle SA, Klein MH, Read RJ. 1994. The crystal structure of pertussis toxin. *Structure* 2:45-57.
- Thelestam M. 1988. Assay of pore-forming toxins in cultured cells using radioisotopes. *Methods Enzymol* 165:278-285.
- Wilson BA, Reich KA, Weinstein BR, Collier RJ. 1990. Active-site mutations of diphtheria toxin: Effects of replacing glutamic acid-148 with aspartic acid, glutamine, or serine. *Biochemistry* 29:8643-8651.
- Zhan H, Choe S, Huynh PD, Finkelstein A, Eisenberg D, Collier RJ. 1994. Dynamic transitions of the transmembrane domain of diphtheria toxin: Disulfide trapping and fluorescence proximity studies. *Biochemistry* 33:11254-11263.
- Zhan H, Oh KJ, Shin YK, Hubbell WL, Collier RJ. 1995. Interaction of the isolated transmembrane domain of diphtheria toxin with membranes. *Biochemistry* 34:4856-4863.