Mechanism of insertion of diphtheria toxin: Peptide entry and pore size determinations

(photoreactive probes/membrane penetration/permeability/liposomes/Sendai virus)

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ABSTRACT Diphtheria toxin (DTx) is an extremely potent inhibitor of protein synthesis. It is secreted as a linear polypeptide, which is cleaved to produce disulfide-linked A and B fragments. Fragment A, the inhibitor of protein synthesis, requires fragment B, the recognition subunit, for entry into intact cells. Fragment B has been proposed to form a transmembrane channel through which A gains access to the cytosol. If it were demonstrated that the B subunit had an exclusive association with membrane lipid acyl chains, this might indicate that A is secluded in a proteinaceous B channel. However, our results from intramembranous photolabeling studies show that both subunits of DTx enter the hydrocarbon domain of the bilayer. Toxin cleavage is not required for penetration. Decreasing pH leads to increased binding and hence indirectly to increased penetration. Parallel permeability studies indicate that cleaved DTx does indeed form pores (24 Å in diameter) and they are larger than those previously reported (5 Å) with native toxin. The data suggest that these are dimeric structures. Cleaved DTx is much more effective than intact DTx at pore formation. Thus, we conclude that, while pore formation is a feature of toxin-membrane interaction, the pore structure does not protect A from contact with lipid side chains and may in fact consist of both the A and B domains in a dimeric configuration, (AB)₂.

Diphtheria toxin (DTx) is produced by *Corynebacterium diphtheriae* that contain the bacteriophage β (for review of DTx see ref. 1). DTx is secreted as a linear polypeptide of $M_r \approx 63,000$, which is cleaved by cosecreted proteases to produce disulfide-linked A and B fragments, $M_r \approx 21,000$ and 40,000, respectively. Fragment A catalyzes the ADP-ribosylation of elongation factor 2, an essential protein component of ribosomal protein synthesis. The modified form of elongation factor 2 is inactive; protein synthesis stops and the cell dies.

While fragment A is an active inhibitor of cell-free protein synthesis, it requires fragment B for entry into intact cells. Fragment B is the binding-recognition subunit. The receptor for B has been identified as a glycoprotein in several cell types (2). However, toxin has been shown to bind to protein-free membranes under low pH conditions (3) and the functional insertion of A has been detected in liposome targets (J. J. Donovan, personal communication). Conductance studies with a segment of DTx called cross-reacting material 45 (CRM45) led to the proposal that B forms a membrane channel through which A travels to the cytosol (4, 5). CRM45 is produced by *C. diphtheriae* cells lysogenic for the mutant phage $\beta 45$ and unlike DTx has an exposed hydrophobic domain on a shortened B segment (5). CRM45 is relatively nontoxic to intact cells, apparently lacking the cell surface recognition domain (6). Kagan and co-workers observed that at low pH the CRM45 B segment as well as CRM45 itself

formed cation-selective channels in planar lipid membranes and 18-Å pores in liposomes (4, 7). In contrast, Donovan *et al.* (8) found that native DTx forms anion-selective channels under acidic conditions and concluded these pores were too small (5 Å) to allow A to cross the membrane. While the calculated channel size has been questioned, to our knowledge no other pore size determinations have been performed with native DTx.

Because direct information about the nature of toxin-membrane interactions is critical to solving the entry pathway, we used a photoreactive glycolipid probe (9, 10) to ascertain which of the two domains of the toxin penetrates the membrane bilayer. These experiments were carried out with simple biological and artificial targets and both cleaved and uncleaved forms of DTx. The effect of low pH on toxin entry was also monitored in light of previous models of lysosomal involvement in the toxin entry process (4, 11–13). Sendai virus served as the biological target. Our preliminary studies showed that DTx binds to paramyxoviruses (Sendai and Newcastle disease virus) and the photolabeling of viral envelope proteins is independent of temperature and pH in the ranges employed. Parallel studies of the rates of diffusion of different-sized solutes through liposomes containing toxin were performed to assess the relative effectiveness of cleaved and uncleaved toxin at channel formation. Use of a well-established liposomal swelling assay (14, 15) enabled us to delineate the size and structure of the resultant channels.

MATERIALS AND METHODS

Materials. Native DTx was generously provided by D. B. Cawley; α -toxin, by A. Bernheimer. Sendai virus was grown in 10-day-old embryonated chicken eggs and isolated 48 hr later by procedures developed for rapid harvesting of Newcastle disease virus (16). Sendai virus is not as dense as Newcastle disease virus strain HP₁₆ and hence bands at a lighter density in each of the gradients (16) employed for purification. The photoreactive probe *N*-[12-(4-azido-2-nitrophenoxy)stearoyl][1-¹⁴C]glucosamine (12APS-[¹⁴C]GlcN), specific activity 60 μ Ci/ μ mol (1 Ci = 37 GBq), was synthesized essentially as described (16), using dicyclohexyl carbodiimide instead of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline.

Photolabeling of Native DTx. Probe 12APS-[¹⁴C]GlcN (60,000 cpm) in ethanol was added to the bottom of a test tube and partially dried. Sendai virus (50 μ g of protein per sample) was added in 200 μ l of phosphate-buffered saline/ethylenediaminetetraacetic acid (P_i/NaCl/EDTA; 15 mM sodium phosphate buffer/150 mM NaCl/1 mM EDTA, pH 7.0). After 15 min at 37°C, native DTx (40 μ g of protein) in

Abbreviations: DTx, diphtheria toxin; CRM45, cross-reacting material 45, form of diphtheria toxin lacking part of the B domain; 12APS-[¹⁴C]GlcN, *N*-[12-(4-azido-2-nitrophenoxy)stearoyl][1-¹⁴C]glucosamine.

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P_i /NaCl/EDTA (150 μ l) was then added, the pH was adjusted, and the 37°C incubation was continued for 15 min. The samples were then irradiated at 366 nm for 30 s with a high-intensity mercury lamp (75–100 W), layered onto 300- μ l pads of 15% (wt/vol) Ficoll 400 in P_i /NaCl/EDTA, and spun for 1 hr at 40,000 rpm (180,000 $\times g$) in a Beckman SW 50.1 rotor equipped with 0.7-ml tube adaptors. The pellets were then solubilized in sodium dodecyl sulfate reducing sample buffer (17) and electrophoresed on 10% polyacrylamide gels (18).

Photolabeling of Cleaved DTx. The cleaved form of DTx was prepared by incubating whole toxin (usually 0.3–0.6 mg/ml) with trypsin (Sigma) at 1 μ g/ml for 1 hr at room temperature. Naturally cleaved DTx behaves identically to trypsin-cleaved DTx, and the addition of trypsin inactivators after cleavage had no effect on the results. Vesicles were prepared from dimyristoyl phosphatidylcholine by reverse phase evaporation (19). Each sample contained 90–100 μ g of phospholipid in 200 μ l of P_i /NaCl/EDTA and was incubated with probe (60,000 cpm) for 15 min at 37°C. DTx (50 μ g in 60 μ l of P_i /NaCl/EDTA) was then added. After 5 min at 37°C, the pH of the samples was adjusted, and the incubation was continued for 1 hr. Samples were then irradiated at 366 nm for 30 s with a high-intensity mercury lamp (75–100 W). The samples were mixed with 2 vol of 35% (wt/vol) Ficoll in P_i /NaCl/EDTA, overlaid with 16% Ficoll in P_i /NaCl/EDTA and then with P_i /NaCl/EDTA. Samples were centrifuged for 1.5 hr at 40,000 rpm (180,000 $\times g$) in an SW 50.1 rotor equipped with 0.7-ml tube adaptors. Vesicles banding at the 16%/0% Ficoll interface were removed, solubilized in sodium dodecyl sulfate reducing sample buffer (18), and electrophoresed on 10% polyacrylamide gels (19). The Sendai virus samples shown in Fig. 2 were treated as described for Fig. 1; however, they contained DTx that was partially cleaved with trypsin. Viral samples were incubated with toxin for 1 hr prior to irradiation.

Pore Size Determinations. These experiments were carried out as described (14, 15) with minor modifications. For comparison, the permeability curves from two other pore-forming proteins are shown (Fig. 3). The porin of *Pseudomonas aeruginosa* has a channel diameter of 20 Å (20) and the porin of *Escherichia coli* has a channel diameter of 12 Å (15). Acetone-extracted egg phosphatidylcholine (Sigma) (3 μ mol) and dicetylphosphate (0.075 μ mol) were dried onto the bottom of a large test tube (2 cm diameter) under a stream of N_2 and evacuated for 1 hr. Cleaved DTx was then added (40 μ g in 200 μ l for the experiment shown in Fig. 3; 25–50 μ g/200 μ l in other experiments). The mixture was sonicated for 2 min (Bransonic 12 ultrasonic cleaner), dried onto the test tube under reduced pressure, and desiccated overnight under reduced pressure. At this point 15% (wt/vol) dextran (Sigma; average M_r 17,000) was added in 0.6 ml of 10 mM Tris-HCl, pH 4. Liposomes were formed by manually shaking the tube and allowing it to incubate at 30°C for 1–2 hr. The assay itself involved quickly mixing 30 μ l of the liposome solution with 0.7 ml of an isotonic sugar solution in 10 mM Tris-HCl, pH 7.4, in a cuvette. The isotonic concentration was determined by using a large sugar, such as stachyose, that penetrates the protein pore slowly if at all. At isotonic concentrations ($\approx$ 50–60 mM), the optical density at 500 nm remains constant. When the pore was permeable to a specific sugar solute, the rate of influx of sugar and water was recorded as a decrease in the optical density at 500 nm (15, 21). The rate of sugar influx obtained in the liposome swelling assay was used to determine the size (15) of the toxin pore by using the Renkin equation (22). The number of protein molecules forming the pore was measured by monitoring the proportional increase in the rate of swelling with increase in protein concentration (25–50 μ g per liposome preparation). Results obtained with other pH values and with native toxin are described in the text.

RESULTS

The photoreactive probe used to monitor membrane insertion of DTx was the glycolipid 12APS-[14 C]GlcN. Approximately 95% of this probe spontaneously partitions into the membrane bilayer within 1 min at 37°C. When used with membrane systems, it will photolabel only integral membrane components (10), as demonstrated here (Fig. 1, lane 2') by the photolabeling of some Sendai virus proteins (HN, F₁, and M) but not others (P, NP, F₂).

As shown in Fig. 1, the binding of DTx to Sendai virus was strongly dependent on the pH. Binding is defined as an association that is maintained after target flotation (vesicles) or sedimentation (viruses) through Ficoll 400 step gradients. At neutral pH, the native toxin (lane 1; partially cleaved) bound very weakly to the viral membrane (lane 3). As the pH was decreased, more toxin bound and at pH 4 binding was at least 10-fold higher than at pH 7 (lane 4). Photolabeling from within the viral envelope revealed that the toxin not only bound to but inserted into the membrane bilayer at pH 4 (lane 4'). Surprisingly, fragments A and B both inserted into the membrane lipid domain as determined by accessibility to

FIG. 1. Effect of low pH on the binding and penetration of DTx. Stained gel lanes are 1–4; corresponding fluorogram lanes are 2'–4'. Lane 1 contains 30 μ g of untreated DTx as a standard. Lanes 3 and 4 show native DTx (40 μ g) incubated with Sendai virus (containing 12APS-[14 C]GlcN) for 15 min at 37°C before irradiation. The sample in lane 3 was held at pH 7; the sample in lane 4 was brought to pH 4 after toxin was added. Lane 2 shows a sample that contained virus and α -toxin (α -Tx) from *Staphylococcus aureus*. The α -toxin has no bands that overlap with viral proteins and hence this sample can be viewed as a virus control. The positions of the A and B fragments are indicated, as are those of six viral proteins (VPs).

probe (see refs. 10 and 23–25 for a discussion of probe reactivity and location). This would mean that A is certainly not completely sequestered in a transmembrane channel formed by B. At pH 7, intact DTx was labeled to a lesser extent (lane 3'), reflecting the poor efficiency of binding. Minor labeling of DTx fragments A and B was also observed at pH 7. Fragment A migrates very close to a fragment of viral protein HN, which does not stain effectively but is photolabeled (lanes 2', 3', and 4'). When Sendai virus was exposed to a preparation of DTx that was partially cleaved, the same pH dependence was observed on binding (Fig. 2, lanes 7, 8, and 9) and subsequent penetration (Fig. 2, lanes 7', 8', and 9'). Again intact and cleaved DTx were most efficiently labeled at pH 4 (lane 7').

Binding and insertion of native toxin was shown to be pH dependent with vesicle targets as well as with viruses. As shown in Fig. 2, much more DTx bound to vesicles at pH 4 (lane 5) than at pH 7 (lane 6). At pH 7 and 8, the amount of toxin bound was barely detectable (gel data not shown). Using ^{125}I -labeled toxin, we observed that, at pH 7, about 1.2% of the added toxin was bound to vesicles after 1 hr at 37°C. At pH 4, about 30% of the added toxin was bound. The photolabeling data (lanes 5' and 4') indicates that insertion is limited by the amount bound to the membrane. When vesicle targets were prepared from egg yolk phosphatidylcholine and cholesterol (50 mol%), we observed an increase in total binding and subsequent penetration over that obtained with

dimyristoyl phosphatidylcholine vesicles but the same pH dependence was noted (data not shown).

With trypsin-cleaved toxin, in which the A and B fragments are linked solely by cystine, DTx binding to vesicles was also strongly dependent on pH (Fig. 2). At pH 4, almost all of the cleaved toxin added bound to the vesicles (lane 1). At pH 6, the level of the membrane-bound toxin decreased (lane 2). At pH 7 and 8, toxin binding was significantly reduced (lanes 3 and 4). The photolabeling results with vesicle targets showed that the toxin insertion profile paralleled the pH dependence of binding. Most of the increase in photolabeling with decrease in pH can be accounted for by increased binding. Hence, the role of low pH on toxin appears to be to facilitate more efficient binding and not just better insertion of prebound toxin. The function of toxin specific receptors on cells may be merely to promote membrane associations at neutral pH. In this regard, it should be mentioned that the existence of toxin receptors is controversial (1).

Membrane conductance studies suggest that DTx forms anion-selective pores 5 Å in diameter (8). To determine channel dimensions on the basis of passage of neutral compounds, we used the liposome swelling assay developed by Luckey and Nikaido (14) to measure nonelectrolyte penetration rates. The kinetics of vesicle solute permeation (Fig. 3) demonstrated that trypsin-cleaved DTx does indeed form pores with high efficiency whereas native toxin does not

FIG. 2. Entry of DTx fragments A and B. Coomassie blue-stained gel lanes are 1–9; corresponding fluorogram lanes are 1'–9'. Lanes 1–4 show vesicle samples containing cleaved toxin at pH 4, 6, 7, and 8, respectively. Lanes 5 and 6 show vesicle samples containing native toxin at pH 4 and pH 7, respectively. Lanes 7, 8, and 9 show Sendai virus samples containing partially cleaved toxin at pH 4, 6, and 7, respectively. Partially cleaved toxin was obtained by mild trypsin treatment of native toxin.

(data not shown). The question in terms of toxin entry is whether the pore is large enough to accommodate fragment A. From the Renkin equation (22), the pore was calculated to have a channel diameter of about 24 Å. While fragment A could penetrate through a pore of this dimension, the photolabeling results suggest that, at some point during membrane traversal, fragment A is in contact with the acyl chains of membrane lipids.

The pore-forming activity of cleaved DTx was found to be greatest when the DTx-containing liposomes were made at pH 4 and tested at pH 7 (rather than the reverse), a finding consistent with other pH data. When the DTx-containing liposomes were formed and tested at pH 4, the toxin was still active, but the observed swelling rates decreased by about 50%. When liposomes were made and tested at neutral pH, the swelling rates were very low, less than 20% of those observed at pH 4. When 2-mercaptoethanol (0.6%) was added to the assay system, all of the swelling rates increased by 20–30%, indicating that pore formation may be facilitated by reduction of the cystine bridge between the A and B domains. In one study in which the concentration of DTx was varied, we noted a 4-fold increase in swelling rate with a 2-fold increase in DTx, suggesting that the actual pore may be a dimeric structure.

The permeability profiles of liposomes containing *E. coli* porin and *P. aeruginosa* porin are provided for comparison (Fig. 3). These two proteins form channels in bacterial outer membranes. The *E. coli* porin forms a 12-Å channel with a 600-dalton exclusion limit (15); the *P. aeruginosa* porin forms a 20-Å channel with a 4000- to 6000-dalton exclusion limit (20). The DTx channel is clearly larger, with a calculated diameter of 24 Å. The DTx curve shown was obtained

FIG. 3. Swelling rates of liposomes composed of cleaved DTx and phospholipids. Liposomes containing 15% dextran in 10 mM Tris-HCl, pH 4, were prepared with nicked DTx (40 µg). Permeability was determined from the initial rate of change in optical density at 500 nm over a 2-min period. The results were normalized to the rate obtained with arabinose. The sugars used (from left to right) were arabinose (M_r , 150), glucose and fructose (M_r , 180), sucrose (M_r , 342), and raffinose (M_r , 504).

with 40 µg of cleaved toxin. A similar curve was obtained with native toxin; however, the efficiency of pore formation was decreased to only 13% of that seen with native toxin (i.e., 7.5 times more protein was needed to achieve the same rates). Much of the pore-forming activity of native toxin can be accounted for by endogenous levels of cleaved toxin ($\approx 10\%$). With native DTx, only minimal pore-forming activity was detected regardless of the pH at which the vesicles were made and tested. In a duplicate study, the relative rates of permeation by arabinose varied as follows with pH and cleavage (50 µg of DTx): native toxin at pH 4, 0.05; cleaved toxin at pH 7, 0.03; and cleaved toxin at pH 4, 0.24. Although the rates were lower for all sugars tested, with native toxin the standardized data fit the same curve as that shown in Fig. 3, indicating a similar pore size but much lower efficiency of pore formation. The photolabeling studies show that intact toxin is very similar to cleaved toxin in terms of membrane binding and penetration. Consequently, membrane penetration does not always lead to pore formation.

DISCUSSION

The photolabeling results obtained with DTx are similar to those obtained with ricin toxin (26) in that the A and B domains of both of these potent inhibitors of protein synthesis penetrate the membrane bilayer. Thus the B domains of ricin and DTx do not merely bind and remain at the membrane surface, as was found with cholera toxin (9, 10, 27, 28); instead they proceed to enter the membrane bilayer. One clear difference between DTx and ricin toxin is that the former penetrates the membrane better at low pH (pH 4) and the latter at high pH (pH 8) (unpublished observations), data consistent with toxicity studies (12, 29). This indicates that pH is not affecting the target membrane *per se*, but the toxin. The difference in pH dependence indicates that the two toxins may employ distinct routes for entering cells: DTx via endocytosis and escape from a lysosomal compartment and ricin via a more direct pathway. While cleavage of DTx dramatically enhances its ability to form pores and is a requirement for toxicity, data obtained with DTx as well as with cholera toxin show that toxin cleavage is not necessary for penetration. The intact A subunit of cholera toxin as well as the enzymatically active A_1 portion of the disulfide-linked A_1A_2 cleavage product are equally capable of penetrating the bilayer and show a similar kinetic profile. The evidence indicated, however, that only the A_1 domain of the cholera toxin A subunit is capable of membrane penetration (9, 10). Results with ricin and cholera toxin are mentioned because, like DTx, the former inhibits protein synthesis while the latter has ADP-ribosylating activity.

Since our photolabeling results with cholera toxin have been verified by two independent groups (27, 28), one using an entirely different approach (28), we are confident of the ability of our protocol to define protein-lipid interactions under a variety of experimental conditions. With respect to DTx, the data establish directly that the A and B domains both participate in the entry process. Permeability data further show that DTx forms 24-Å-diameter pores, possibly by aggregation into dimeric structures. Prior toxin exposure to low pH promotes increases in toxin binding and concomitantly in penetration. Preliminary observations suggest that incubation at low temperature has the same effect; moreover, there appears to be little lag time between binding and penetration at low pH (unpublished data). Low pH also enhances pore formation, possibly involving A as well as B domains. Intact toxin is extremely inefficient at pore formation. The data are compatible with a cytosolic entry pathway involving escape from a lysosomal compartment, where acid conditions may enhance functional binding. They are incompatible with A traversal entirely through a B channel. Know-

ing the location of the photolabeled residues in the sequence and structure of DTx should reveal other important details of the entry pathway.

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