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The Acid-Triggered Entry Pathway of *Pseudomonas* Exotoxin A†

Zohreh Toossi Farahbakhsh and Bernadine J. Wisnieski*

Department of Microbiology and The Molecular Biology Institute, University of California, Los Angeles, California 90024

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ABSTRACT: In this study we examined the pH requirements and reversibility of early events in the *Pseudomonas* toxin entry pathway, namely, membrane binding, insertion, and translocation. At pH 7.4, toxin binding to vesicles and insertion into the bilayer are very inefficient. Decreasing the pH greatly increases the efficiencies of these processes. Acid-treated toxin exhibits pH 7.4 binding and insertion levels. This indicates that hydrophobic regions that become exposed upon toxin acidification become buried again when the pH is raised to 7.4. In contrast, the change in toxin conformation that occurs upon membrane binding is irreversible. Returning samples to pH 7.4, incubation with excess toxin, or dilution with buffer up to 1000-fold leads to very little loss of bound toxin. Bound toxin exhibits an extremely high susceptibility to trypsin compared to free toxin (at both pH 4 and pH 7.4). At pH 4, membrane-associated toxin slowly proceeds to a trypsin-protected state; neutralization halts this process. At low pH, toxin was found to bind and insert into DMPC vesicles very efficiently at temperatures both above and below 23 °C, the lipid melting point. With fluid targets, the proportion of bound toxin that was photolabeled from within the bilayer peaked rapidly and then decreased with time. With frozen targets, the efficiency of photolabeling peaked but then remained fairly constant. The results suggest that after insertion PTx can cross a fluid bilayer much more efficiently than it can a frozen one. We conclude that the reversible pH-triggered changes in toxin conformation [Farahbakhsh, Z. T., Baldwin, R. L., & Wisnieski, B. J. (1987) *J. Biol. Chem. 262*, 2256–2261] have a functional role in promoting membrane binding, insertion, and translocation. The kinetics of translocation is governed by the pH and the physical state of the target membrane.

PTx[1] is one of the extracellular products secreted by toxicogenic strains of *Pseudomonas aeruginosa* (Callahan, 1974; Liu, 1974). Its activity as an inhibitor of protein synthesis makes it a critical virulence factor during infection (Cryz, 1985). PTx is secreted as a single polypeptide chain with a molecular weight of 66 583 (Gray et al., 1984). Like diphtheria toxin (DTx), it catalyzes the ADP-ribosylation of elongation factor 2 (Iglewski & Kabat, 1975; Lory & Collier, 1980). Although the entry mechanisms of these two toxins exhibit some similarities, they do not appear to be identical (Gray et al., 1984; Middlebrook & Dorland, 1984; Zalman & Wisnieski, 1985; Sundan et al., 1984; Olsnes & Sandvig, 1988). The three-dimensional structure of PTx has recently been determined by X-ray crystallography (Allured et al., 1986) and shown to contain three structural domains. On the basis of this information, plasmids that code for specific portions of the toxin molecule have been used to generate peptides. Preliminary studies with these peptides indicate that the three domains of the toxin are required for cell recognition, translocation, and ADP-ribosylation, respectively (Hwang et al., 1987).

Despite the structural and functional characterization of the toxin, the mechanism by which the ADPr-transferase domain gains access to the target cell cytoplasm is still under intense investigation. The process seems to involve receptor-mediated endocytosis (Moehring & Moehring, 1983; Morris & Saelinger, 1986; Robbins et al., 1984). The importance of exposure to a low-pH environment derives from observations that compounds that increase the pH of normally acidic organelles protect the cells against PTx (FitzGerald et al., 1980; Sundan et al., 1984) and that this block can be overcome by lowering the external pH (Moehring & Moehring, 1983; Morris & Saelinger, 1986). We have noted that incubation of toxin below pH 6 leads to exposure of hydrophobic regions (Farahbakhsh et al., 1987). Most of the acid-induced conformational changes observed were reversible ($t_{1/2}$ <30 s), and they occurred within a narrow pH range (Farahbakhsh et al., 1987).

The goal of this investigation was to explore the functional role of such changes and any further changes that might occur upon membrane binding. Although we have shown that toxin inserts into the membrane bilayer upon acidification (Zalman & Wisnieski, 1985; Farahbakhsh et al., 1986), factors that govern its translocation have not yet been established. Photolabeling experiments were conducted to establish the effects

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* Address correspondence to this author at the Department of Microbiology, University of California, Los Angeles, CA 90024.

[1] Abbreviations: PTx, *Pseudomonas* exotoxin A; DTx, diphtheria toxin; PC, egg phosphatidylcholine; CS, cholesterol; 12APS-GlcN, *N*-[12-(4-azido-2-nitrophenoxy)stearoyl][1-^{14}C]glucosamine; TSE, 20 mM Tris-HCl, 150 mM NaCl, and 1 mM ethylenediaminetetraacetic acid, pH 7.4; DMPC, dimyristoylphosphatidylcholine; ANS, 1-anilino-8-naphthalenesulfonic acid; T_m, membrane melting point; CHO, Chinese hamster ovary cells; SDS, sodium dodecyl sulfate.

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of lipid physical state on the kinetics of toxin insertion and translocation; other studies were designed to characterize relevant conformational changes in toxin structure and the reversibility and pH dependence of each step in the entry pathway. Evidence is presented that after insertion of toxin into fluid membranes translocation proceeds unidirectionally as long as the toxin is exposed to an acidic milieu. Our results lend strong direct support to the notion that PTx entry involves passage through the boundary membrane of an acidic compartment. No processing, other than the conformational changes provoked by acidification, appears to be required.

Experimental Procedures

Materials. Egg PC was purchased from Avanti Biochemicals, Inc. Other lipids, soybean trypsin inhibitor (type I-S), L-1-(tosylamino)-2-phenylethyl chloromethyl ketone treated trypsin (type XIII), and TPCK-treated trypsin (from bovine pancreas, 50 units/1.25 mL of suspension) attached to agarose beads were obtained from Sigma. PTx was purchased from the Swiss Serum and Vaccine Institute (Berne, Switzerland) and used as provided. The photoreactive probe 12APS-GlcN, specific activity 57 μCi/μmol, was synthesized as described previously (Zalman & Wisnieski, 1985) and stored at 4 °C in ethanol. Na^{125}I, 15 mCi/μg, was from Amersham.

Preparation of Target Membranes. PC/CS vesicles were prepared by the reverse-phase evaporation method (Szoka & Papahadjopoulos, 1978). Briefly, 11 μmol of egg PC and 11 μmol of CS were added to a 50-mL round-bottom flask. The solvent was removed by rotary evaporation. The lipids were redissolved in 1 mL of diethyl ether, and then 0.3 mL of TSE buffer was added. This mixture was bath sonicated at 4 °C until a homogeneous phase was formed. The ether was rotary evaporated for ~15 min, then 0.3 mL of TSE was added, and evaporation was continued for another 15 min. The diameter of these unilamellar vesicles is ~500–1000 Å. DMPC vesicles were prepared as described by Hu et al. (1981) and determined by electron microscopy to be ~500 Å in diameter. Briefly, 10 mg of DMPC was suspended in 1 mL of TSE buffer by interval sonication under a steady stream of N_2 (ten 30-s pulses at 40 W with 15-s intervals). A water bath was used to maintain the temperature at 30 °C during sonication. The vesicle suspension was then incubated at 37 °C for 0.5 h and spun in a Beckman microfuge for 5 min. The supernatant served as the source of DMPC vesicles used in our assays. Phospholipid concentrations were determined by the method of Raheja et al. (1973).

^{125}I-Labeled PTx. PTx was labeled with Na^{125}I by the lactoperoxidase method (Morrison et al., 1971). For a 32-μL reaction volume, 10 μg of toxin, 2 mCi of Na^{125}I, 0.05 μg of lactoperoxidase, and 0.0002% H_2O_2 were employed. After 15 min on ice, the reaction was stopped by adding 5 μL of NaI (53 mM). The mixture was applied to a Sephadex G-25 column equilibrated with TSE buffer. Fractions corresponding to radiolabeled toxin were pooled and used in binding studies (conditions described in Table I and Figure 2). Toxin bound to vesicles was separated from unbound toxin by flotation (see below). Iodinated toxin retained full biological activity with human U937 cells; also no breakdown products were noted on SDS–polyacrylamide gels.

Photolabeling Procedure. Probe 12APS-GlcN (60000 cpm) in ethanol was added to the bottom of a test tube, dried, and rewetted with 1.5 μL of ethanol. DMPC vesicles (100 μg) in 200 μL of buffer were added and incubated at 37 °C for 10 min, followed by addition of 20 μg of PTx. The pH was adjusted by addition of a small amount of 0.25 N HCl, and samples were incubated as described in Figure 3. The samples were then irradiated for 30 s at 366 nm with a high-intensity mercury lamp (75–100 W). The samples were brought to pH 7.4 and mixed with Ficoll 400 (50% w/v in TSE) to a final Ficoll concentration of 30%, then overlaid with 25% Ficoll and 60 μL of buffer. Samples were centrifuged for 1 h at 40000 rpm (180000*g*) in a Beckman SW50.1 rotor equipped with 0.7-mL tube adaptors. Vesicles collecting at the 25%/0% Ficoll interface were removed, solubilized in SDS sample buffer under reducing conditions (O'Farrell, 1975), and subjected to electrophoresis on 11% polyacrylamide gels (Laemmli, 1970). After being stained with Coomassie blue (G250 plus R250), the gels were treated with sodium salicylate (Chamberlain, 1979), dried, and exposed to Kodak X-Omat AR film for 8 weeks at −70 °C. The levels of binding and photolabeling were quantified as described previously (Farahbakhsh et al., 1986).

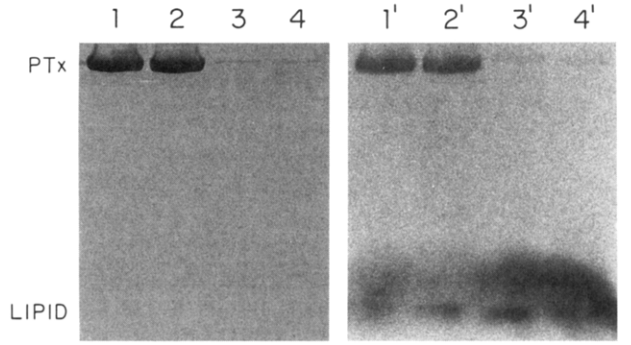

FIGURE 1: Binding and insertion of acid-treated toxin. PTx (20 μg) was incubated in 175 μL of TSE buffer, pH 7.4, at 37 °C for 10 min; the pH was then dropped to 4.0 followed by a 1- or 10-min incubation. The samples were either left at pH 4.0 or returned to pH 7.4. Each toxin sample was then mixed with probe-containing PC/CS vesicles (100 μg as phospholipid) that were suspended in 25 μL of buffer of corresponding pH. After 30 min at 37 °C, the vesicle samples were irradiated, harvested by flotation, and subjected to SDS gel electrophoresis. Lane 1, the amount of PTx bound to vesicles (pH 4, 30 min) after toxin acidification for 1 min; lane 2, PTx bound to vesicles (pH 4, 30 min) after toxin acidification for 10 min; lane 3, PTx bound to vesicles (pH 7.4, 30 min) after toxin acidification for 1 min; lane 4, PTx bound to vesicles (pH 7.4, 30 min) after toxin acidification for 10 min. Gel lanes 1–4 show the amounts of toxin bound to the vesicles. Lanes designated 1′–4′ show the corresponding lanes of the fluorogram.

Results

Binding and Insertion of Acid-Treated Toxin. In a previous study, we examined the effects of acidification on the conformation of PTx (Farahbakhsh et al., 1987). For the most part the changes were fully reversible, including the acquisition of a hydrophobic binding site that is likely to play a role in membrane binding and insertion under acidic conditions. To test the prediction that the reversible conformational changes occur in regions of the toxin that are responsible for membrane binding, we examined the efficiency of PTx binding to vesicle targets after acidification for 1 or 10 min before incubation with vesicles at pH 7.4. As shown in Figure 1, acid-treated toxin did not bind to vesicles at pH 7.4 (Figure 1, lanes 3 and 4). However, when the acid-treated toxin was incubated with vesicles at low pH, very high levels of binding and insertion were observed after 10 min; these results are entirely consistent with our hypothesis. Acidification of toxin has no negative effect on basal or activated levels of ADPr-transferase activity when assayed at pH 7.4. Typically acid-treated toxin exhibited higher than normal basal activity (undenatured, unreduced PTx), indicating that the one irreversible change we did observe in conformation might be in the region of the toxin responsible for the ADP-ribosylation of elongation factor 2.

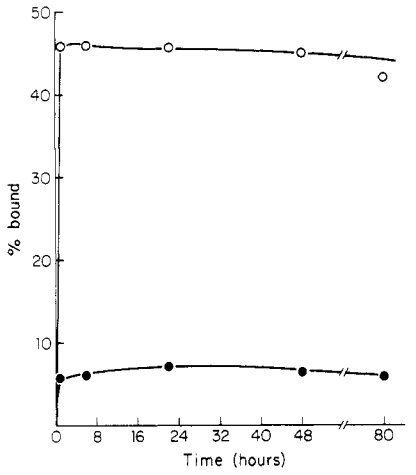

FIGURE 2: PTx binding to vesicles as a function of pH. PTx (10 μg of unlabeled plus 0.02 μg of ^{125}I-labeled toxin) was incubated with PC/CS vesicles (50 μg as phospholipid) at 37 °C in pH 4 or pH 5 buffer (100-μL total volume). At the specified times, vesicle-bound toxin was separated from unbound toxin by flotation. The radioactivity of each fraction was determined; then the fractions were subjected to electrophoresis on SDS gels. Stained gels were subjected to densitometric scanning to quantify the protein content of each fraction. Both methods of protein quantitation gave similar results. Data shown are the average of two independent studies. Symbols: (○) pH 4, 37 °C; (●) pH 5.0, 37 °C.

The Extent of Toxin Binding to Membranes Is a Function of pH. Next, we asked whether the extent of toxin binding at pH values greater than 4 ever reaches the levels observed at pH 4. At 37 °C, we found that the binding of ^{125}I-labeled PTx to vesicles peaked within minutes of lowering the pH. At pH 5, the amount of binding observed at 10 min was 73% of that obtained at 1 h, when the binding level plateaued. Furthermore, as shown in Figure 2, the amount of toxin bound to vesicles at pH 4 was always significantly greater than the amount bound at pH 5, even after incubation for 80 h. The efficiency of binding showed a strong dependence on temperature; i.e., a significant decrease occurred when the samples were incubated at 0 °C. The dependence of binding on temperature was more pronounced at pH 4 than at pH 5. The initial low level of PTx binding to vesicles at pH 7.4 did not change appreciably over a 4-day incubation period (data not shown). Optimal binding, in terms of both rates and absolute levels achieved, requires that toxin be exposed to vesicles at low pH and high temperature.

Evidence for Irreversible Binding of Toxin to Target Membranes. An important goal of this study was to ascertain whether binding to target membranes was reversible. Table I shows the effect of raising the pH to 7.4 on toxin that was prebound to vesicles at pH 4.0. These data indicate that most of the vesicle-bound toxin is nondissociable in the absence or presence of unlabeled PTx. This conclusion was confirmed by a second study in which triplicate samples of vesicles were incubated with ^{125}I-labeled toxin for 30 min at pH 4, returned to pH 7.4, and then mixed with from 0 to 20 μg of cold toxin before harvesting; on average, 88% of the labeled toxin remained bound. Finally, an attempt was made to dissociate bound toxin by sample dilution with pH 7.4 buffer; regardless of the extent of dilution (up to 1000-fold), ~85% of the toxin remained bound.

Effect of Bilayer Fluidity on Toxin Binding, Insertion, and Translocation. Addition of PTx to DMPC vesicles resulted in very efficient binding and insertion at low pH. Use of DMPC vesicles (T_m = 23 °C) enabled us to study the effect of lipid physical state on the kinetics of these processes. In these studies, a photoactivable glycolipid probe (12APS-GlcN) was used to monitor bilayer insertion. Binding to DMPC vesicles plateaued at 1 min and then remained relatively constant at both 13 and 33 °C. At 13 °C, 35% of the PTx was bound; at 33 °C, 57% of the PTx was bound. The level of photolabeling at 13 °C always mirrored the level of binding. By expression of the data as the amount of bound protein that is in the membrane at any given time after sample acidification (Figure 3), it appears that inserted toxin molecules slowly begin to cross a fluid bilayer. Although the toxin is essentially irreversibly associated with vesicles, with time it becomes increasingly inaccessible to the photoreactive membrane probe. Unlike insertion, translocation requires temperatures above the lipid-phase transition. Below the phase transition temperature (i.e., at 13 °C) bound toxin remained anchored in the vesicle bilayer throughout the 60-min assay period (Figure 3). Here, no time-dependent change in photolabeling efficiency could be detected after the insertion plateaued.

PTx Sensitivity to Trypsin Cleavage in Soluble and Membrane-Bound States. Studies of toxin binding to target membranes revealed that exposure to low pH induced a strong association between toxin molecules and membranes. Experiments were subsequently carried out to determine whether

Table I: Dissociation of Membrane-Bound Toxin[a]

incubation condition[b]	PTx recovered (cpm) after first float	PTx recovered (cpm) after second float	recovery efficiency (%)
1 h, 37 °C	992	810	81.7
1 h, 37 °C, 10 μg of PTx	670	557	83.1
1 h, 4 °C	995	843	88.3
1 h, 4 °C, 10 μg of PTx	755	698	92.5
30 min, 4 °C, and 30 min, 37 °C	940	784	83.4

[a] PC/CS vesicles were prepared by reverse-phase evaporation. ^{125}I-Labeled PTx (3200 cpm) mixed with 0.5 μg of unlabeled PTx was incubated with PC/CS vesicles (100 μg of phospholipid) in 200 μL of buffer at 37 °C, pH 4.0, for 10 min. The samples were returned to pH 7.4, floated through Ficoll, and harvested. They were then incubated at pH 7.4 under the specified conditions and refloated to remove any toxin that may have dissociated. The average recovery efficiency of vesicles (no toxin) monitored by employment of [^{14}C]DMPC was 85 ± 0.9% under these conditions. Data shown are not corrected for vesicle-recovery efficiency. [b] Conditions of incubation before second flotation.

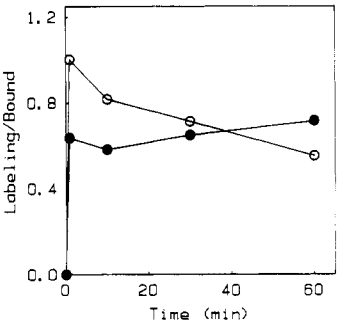

FIGURE 3: Effect of temperature on PTx–membrane interactions. PTx (20 μg per sample) was incubated with DMPC vesicles (100 μg) that contained 12APS-GlcN in 200 μL of pH 7.4 buffer for 10 min at 13 or 33 °C. The pH was dropped to 4 and incubation continued for the specified time before irradiation at 366 nm. After quantification of the level of PTx binding to vesicles and the level of photolabeling, the photolabeling efficiency (i.e., the level of photolabeling achieved per microgram of vesicle-associated PTx) was calculated. Symbols: (●) photolabeling efficiency at 13 °C; (○) photolabeling efficiency at 33 °C.

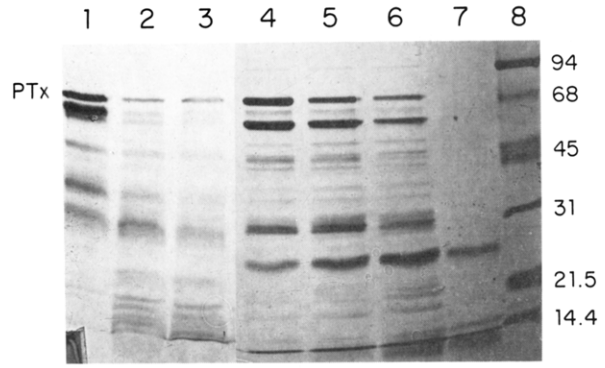

FIGURE 4: PTx susceptibility to trypsin increases after membrane binding. PTx (20 μg per sample) was incubated with PC/CS vesicles (100 μg as phospholipid) in 200 μL of buffer, pH 7.4, for 10 min at 37 °C. The pH was adjusted to 4, and samples were incubated for 30 min at 37 °C. Vesicles were harvested by flotation and then treated with trypsin in 50 μL of pH 7.4 buffer for 1 h at 23 °C. Lanes 1–3, membrane-bound toxin plus 0.007, 0.035, and 0.070 μg of trypsin, respectively. Lanes 4–6, soluble toxin plus 2.5, 5, and 10 μg of trypsin (1 h at 23 °C), respectively. The amount of soluble toxin used was the same as the amount bound to the floated vesicles. Lanes 7 and 8, trypsin (5 μg) and molecular weight standard ($\times 10^{-3}$), respectively.

PTx binding to vesicles triggered additional changes in protein conformation. These experiments focused on an analysis of toxin susceptibility to trypsin cleavage in soluble and membrane-bound forms. If membrane binding at pH 4 induced an irreversible conformational change in the protein, we would expect that any difference in sensitivity to trypsin would remain after a return to pH 7.4. As shown in Figure 4, the toxin was much more susceptible (~300–500-fold) to trypsin after binding to vesicles. Susceptibility was based on densitometric scanning data. Calculation of fold change took into account the percent reduction in intact toxin (68–kDa band) and the enzyme concentration. The conformational state induced by acidification differs in a second significant way from that induced upon membrane binding: the low-pH conformation of PTx in solution is fully reversible as assessed by trypsin cleavage (Farahbakhsh et al., 1987) while that of bound toxin is not.

Accessibility of PTx to Cleavage after Binding to Membranes. Surface accessibility to trypsin was used to determine whether toxin molecules were translocated across the membrane bilayer during exposure to low pH. Toxin was incubated with vesicles for either 15 min or 4.5 h at pH 4.0, and then unbound toxin was separated from vesicles by flotation. Vesicle samples were subsequently subjected to trypsin cleavage, followed by SDS–polyacrylamide gel electrophoresis. Figure 5 shows that after 15 min at pH 4 ~95% of the toxin bound to membranes remained accessible to trypsin, whereas toxin bound for 4.5 h was significantly protected (~25% remained intact). These results indicate that PTx traverses the membrane bilayer relatively slowly and in a pH-dependent manner, since toxin that was bound to membranes at pH 4 for 15 min and returned to pH 7.4 did not become cryptic with time (up to 5 h; data not shown). Moreover, toxin incubated at pH 4 in solution (no vesicles) for 4.5 h remained fully susceptible to trypsin.

Discussion

Recent studies have demonstrated that PTx entry into cells involves receptor-mediated endocytosis of bound toxin, followed by penetration through the membranes of acidic organelles (Morris & Saelinger, 1986; FitzGerald et al., 1980). CHO cells that are deficient in endosomal acidification are resistant to both DTx and PTx (Moehring & Moehring, 1983; Didsbury

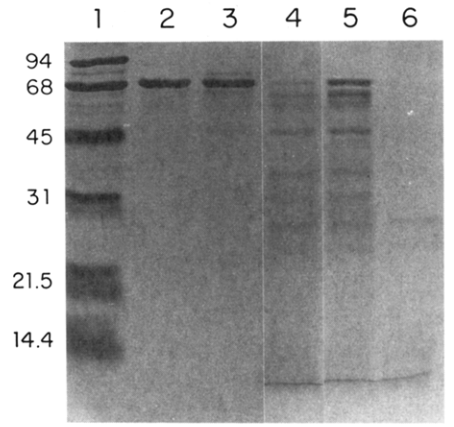

FIGURE 5: Vesicle-bound PTx becomes inaccessible to trypsin. PTx (20 μg per sample) was incubated with PC/CS vesicles (100 μg as phospholipid) in 200 μL of pH 7.4 buffer for 10 min at 37 °C. The pH was dropped to 4.0. Incubation was continued for either 15 min or 4.5 h. Vesicles were harvested by flotation and incubated with 0.43 unit of bead-bound trypsin in 50 μL of pH 7.4 buffer for 30 min at 37 °C. Samples (including the trypsin beads) were immediately boiled for 2 min in reducing sample buffer and subjected to electrophoresis on 11% SDS–polyacrylamide gels. Lane 1, molecular weight standards. Lanes 2 and 3, PTx bound to membranes at pH 4.0 after incubation for 15 min and 4.5 h, respectively (no treatment). Lanes 4 and 5, PTx bound to membranes at pH 4.0 after incubation for 15 min and 4.5 h, respectively, and subsequent treatment with trypsin. Lane 6, 0.43 unit of bead-bound trypsin that was incubated in the absence of vesicles.

et al., 1983; Merion et al., 1983). Agents that raise the pH of acidic organelles, such as monensin, methylamine, and ammonium chloride, protect cells from both DTx and PTx (Middlebrook & Dorland, 1984; Saelinger et al., 1985). Furthermore, the protective effects of these so-called lysosomotropic agents can be overcome by exposing toxin-bound cells to low pH (Moehring & Moehring, 1983; Morris & Saelinger, 1986); the step requiring low pH has been shown to occur before a stage that is inhibited by incubation at 19 °C (Morris & Saelinger, 1986). Previous work with model membrane targets has indicated that low pH triggers a hydrophilic to hydrophobic change in toxin structure (Zalman & Wisnieski, 1985; Farahbakhsh et al., 1986, 1987). A model for PTx entry, which summarizes our current findings, is depicted in Figure 6.

We have shown that the pH-dependent changes observed in the conformation of PTx are for the most part rapidly reversible (Farahbakhsh et al., 1987). The first role for the acid conformation of PTx was discovered from our vesicle binding studies; as depicted in Figure 1, acidified toxin lost its ability to bind to vesicles if the pH was raised to pH 7.4 before vesicles were added. This is in contrast to data obtained with DTx in which preexposure to low pH was found to potentiate toxin binding and insertion into membranes at neutral pH (Hu & Holmes, 1985), a finding we confirmed with the same vesicle targets and flotation harvesting techniques used here for examining PTx behavior (unpublished data).

Data presented in this paper establish that binding and insertion of intact toxin are dependent on temperature and pH. Incubation at pH 5 never led to binding levels as high as those observed at pH 4; this suggests that the pH 5 conformation of PTx is distinct. It did not convert to a pH 4 conformation, in terms of membrane-binding efficiency, even after an 80-h incubation. Thus, it appears that the discrete changes that we observed in toxin conformation upon lowering the pH (Farahbakhsh et al., 1987) are correlated with discrete changes in binding activity. Temperatures in the physiological range are required for optimal binding, regardless of membrane

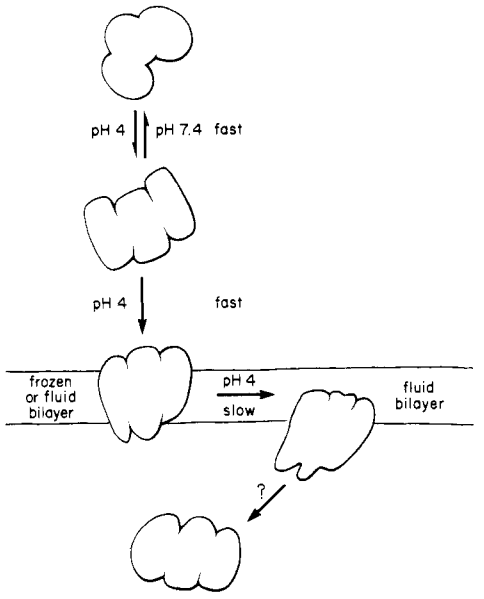

FIGURE 6: Acid-triggered entry pathway of PTx. The acid-triggered change in toxin conformation that facilitates membrane binding is reversible (step 1). If a membrane target is present, the toxin will bind and insert regardless of the physical state of the bilayer (step 2); however, a low-pH environment is required. Within 10 min, the inserted toxin acquires a transmembrane orientation as demonstrated by accessibility to vesicle-entrapped trypsin (Farahbakhsh et al., 1986). It also becomes exquisitely sensitive to external trypsin. If the target bilayer is fluid and the pH remains acidic, toxin will migrate across the bilayer (step 3). Accessibility to external trypsin and to the photoreactive glycolipid probe diminishes. The question of whether toxin is translocated completely (step 4) is currently under investigation.

composition or physical state. This suggests that an additional temperature-dependent conformational change is required for maximum toxin binding to the membrane. Similar results have been observed with DTx; high temperature increased the sensitivity of the native conformation to changes induced by low pH (Zhao & London, 1986).

An analysis of the membrane-inserted conformation of PTx and factors that regulate membrane penetration revealed several interesting features of the toxin–membrane complex. After binding at low pH, very little toxin could be dissociated from the membrane (e.g., after neutralization), indicating that bound PTx undergoes a second change in conformation that is irreversible (with respect to pH and other environmental changes). Binding and insertion are essentially irreversible processes. This is in contrast to findings with colicin E1, which appears to extrude partially out of the membrane when samples are neutralized (Davidson et al., 1985). The uniqueness of the membrane-bound conformation of PTx was demonstrated by its exquisite sensitivity to trypsin. The irreversibility of this change was established by the observation that the sensitivity of membrane-bound PTx to trypsin is the same regardless of whether the assay is performed at pH 4 or pH 7.4. A dramatic increase in sensitivity to chymotrypsin and Pronase was also observed after toxin bound to membranes (data not shown). Such increases in susceptibility to cleavage might facilitate enzymatic processing (the enzymically active conformation of PTx in vivo is unknown).

The final stage in entry of the toxin is translocation of the ADPr-transferase domain into the cytosol. In contrast to DTx, which must be cleaved and reduced to express enzymatic activity (Collier, 1975), intact PTx can serve as an active ADPr-transferase upon denaturation and reduction (Lory & Collier, 1980; Leppla, 1976). So far there is no evidence to suggest that PTx must be cleaved in vivo; however, cleaved toxin has been shown to express enhanced activity in vitro (Lory & Collier, 1980; Sanai et al., 1980; Vasil et al., 1977). Expression of toxicity in vivo requires that PTx be translocated across a membrane barrier. Again, a low-pH milieu appears to be required. Early translocation of at least part of the toxin molecule was established by the observation that vesicle-bound PTx becomes available to cleavage by vesicle-entrapped trypsin (Farahbakhsh et al., 1986), giving rise to a unique profile of cleavage products. The evidence for translocation is strengthened by studies described here in which insertion was followed by photolabeling with a glycolipid probe. The proportion of vesicle-associated toxin that was photolabeled decreased with incubation time at pH 4. The time-dependent decrease in the level of photolabeling compared to that of the level of binding indicates that toxin molecules begin to cross the membrane. Efficient translocation requires that the target membrane be in a fluid state (Figure 3). The crucial role of low pH in toxin translocation was demonstrated by the discovery that during incubation at pH 4, but not at pH 7.4, prebound toxin (vesicle bound at pH 4) becomes increasingly cryptic to subsequent trypsin treatment (Figure 5). This cryptic characteristic holds regardless of the pH at which the trypsin assay is conducted.

The photolabeling data (Figure 3) demonstrate that about 25% of the PTx mass inserted in the membrane at 1 min is no longer accessible to the probe 30 min later. The trypsin accessibility data (Figure 5; also see Figure 4) tell us that ~5% of the vesicle-bound PTx molecules are completely protected from trypsin after 15–30 min at pH 4. There is no reason to assume that these two percentages should be identical; the fact that both percentages increase with time strongly supports the pH-dependent translocation model presented in Figure 6. The loss of PTx accessibility to exogenous trypsin cannot at this time be taken as proof that every part of the toxin molecule has become translocated to the lumen. However, the observed loss of trypsin-susceptible sites at the vesicle surface coupled with the fact that new trypsin-sensitive sites begin to appear lumenally very early after acidification (Farahbakhsh et al., 1986) is proof that at least some portion of the inserted toxin molecule does migrate toward the lumen. The time-dependent decrease in photolabeling provides strong support for this model. It is interesting to note that the data of Moskaug et al. (1987) indicated that the acid-induced entry of 5–10% of the cell-bound DTx was sufficient to account for the decrease in protein synthesis observed (i.e., internalization of 200–400 toxin molecules per cell).

Data presented in this paper are entirely consistent with an endosomal entry pathway. At the first stage, toxin binds to receptor and enters cells by receptor-mediated endocytosis. When the pH of the endosome is reduced, hydrophobic regions of the toxin are exposed to facilitate insertion into the boundary membrane (see Figure 6). Intoxication of the cells occurs when the ADPr-transferase domain of the toxin is translocated to the cytoplasm, an event regulated by pH and membrane fluidity. Further studies are needed to address the question of how much of the toxin molecule reaches the cytoplasm and the nature of the processes that lead to activation of enzyme function in vivo. Such studies should continue to provide invaluable insight into the translocation mechanisms of large biologically active proteins, as well as the ways in which membrane structure and function modulate their transport. In view of the fact that a PTx-containing immunotoxin (FitzGerald et al., 1987) and oncotoxin (Lorberboum-Galski et al., 1988) have now been constructed and shown to be highly specific cytotoxic agents, the importance of elucidating the

entry mechanism of PTx has become a problem of very widespread interest. PTx has already been shown to be a toxic factor that complicates infections of individuals suffering from burns, cystic fibrosis, and immunodeficiency syndromes; perhaps in the future it will be exploited as a valuable magic bullet for use in cancer therapy and other medically related interventions.

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