Pseudomonas Exotoxin A
MEMBRANE BINDING, INSERTION, AND TRAVERSAL*

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Using vesicle targets composed of phosphatidylycholine and cholesterol (1:1 molar ratio), we found that Pseudomonas aeruginosa exotoxin A (PTx) binding and insertion are not only dependent on pH (Zalman, L. S., and Wisnieski, B. J. (1985) Infect. Immun. 50, 630–635) but also on ionic strength, reaching a maximum in pH 4 buffer that contains 150–200 mM NaCl. Insertion was monitored by photolabeling with an intramembranous probe. Higher levels of binding and insertion were attained with vesicles that contained 2.5 mol % dicetylphosphate than with neutral vesicles. Positively charged vesicles (2.7 mol % stearylamine) were the least effective targets. At pH 7.4, all binding levels were depressed. While PTx binding increased with increasing temperature, the relative proportion of the vesicle-associated toxin that was photolabeled decreased. The most likely explanation for the decrease is that the bilayer translocation rates increased with increasing temperature, and hence fewer PTx molecules were accessible at the time of photolabeling. At 37 °C, binding and insertion both plateaued within 10 min of lowering the pH to 4. After 10 min, the amount of bound toxin decreased slightly with time but there was a dramatic decrease in photolabeling, indicating that inserted PTx had begun to cross the bilayer. This was verified by the finding that when PTx was incubated with vesicles that contained trypsin, cleavage occurred only in those samples in which the pH was shifted down to pH 4. Entry is triggered by an acid-induced conformational change that promotes productive binding and insertion. After insertion, the kinetics of membrane traversal appear to be regulated by the physical properties of the bilayer.

PTx is produced by most clinical isolates of Pseudomonas aeruginosa (1). It has been implicated as the major virulence factor in burn victims and other compromised hosts (2, 3). PTx inhibits protein synthesis in sensitive cells by catalyzing the transfer of the ADP-ribosyl moiety of NAD+ onto a diphthamide residue of elongation factor 2 (4), a transferase activity it shares with DTx. Although PTx resembles DTx in several ways (5–7), there are no regions of homology in their sequences (6). Moreover, the ADP-ribosyltransferase activity resides in the carboxyl end of PTx and in the amino end of DTx (6, 8). The cell surface binding domain of PTx has not been identified, nor have any processing events been unequivocally associated with the acquisition of ADP-ribosyltransferase activity in vivo. Several treatments including cleavage (8, 10) and partial reduction of denatured PTx (11) have been shown to induce a sharp increase in enzymic activity in vitro and a concomitant decrease in toxicity with intact cells; hence, it is assumed that the intact PTx molecule undergoes some sort of processing after binding to cells.

Very little is known about the mechanism whereby PTx gains access to the cytosolic compartment of target cells (12). A better understanding of the protein-lipid interactions involved is of critical importance to resolving the problem. Studies by Alving et al. (13) and others (14) have shown that the binding of DTx to phospholipid vesicles at acidic pH is affected by the membrane composition and surface charge. Because of the similarity we have noted in the pH dependency of the PTx and DTx binding profiles (7, 15), we felt it was important to examine how pH, temperature, ionic strength, target membrane composition, and surface charge affect the binding, insertion, and translocation of PTx. Studies were performed with model membrane targets so that we could easily vary the assay conditions used to analyze the kinetics of these processes. Binding was measured after vesicle flotation through Ficoll 400 step gradients buffered at pH 7.4 to prevent further binding. Insertion was monitored by photolabeling with the membrane-restricted photoreactive probe, 12APS-GlcN (for a review, see Refs. 16 and 17). Approximately 95% of this probe partitions into the membrane bilayer within 1 min of mixing. Irradiation at 366 nm for 15 s induces covalent attachments to neighboring molecules, including newly inserted proteins (18, 19). Translocation across the bilayer was verified by adding PTx to vesicle targets that contained trypsin in their lumens and soybean trypsin inhibitor outside. Data obtained in the course of these investigations established the optimal conditions for PTx entry as an intact unit structure.

MATERIALS AND METHODS

Reagents—Egg PC was purchased from Avanti Biochemicals Inc. Other lipids, soybean trypsin inhibitor (Type I-S), and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Type XIII) were obtained from Sigma. PTx was purchased from the Swiss Serum and Vaccine Institute (Bern, Switzerland) and used as provided. The photoreactive probe 12APS-GlcN, specific activity 57 μCi/μmol, was synthesized as described (7) and stored at 3 °C in ethanol.

Vesicle Preparation—Vesicles were prepared by the reverse phase evaporation method (20). In a typical preparation, 11 μmol of egg PC and 11 μmol of CS were added to a 50-ml round-bottom flask. Some vesicles also contained stearylamine (2.7 mol %) or dicetylphosphate (2.5 mol %), conditions shown to give PC vesicles a surface charge of +2 and −2, respectively, at pH 4 (21). The solvent was removed by

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‡ The abbreviations used are: PTx, Pseudomonas exotoxin A; DTx, diphtheria toxin; PC, phosphatidylycholine; CS, cholesterol; 12APS-GlcN, 12-(4-azido-2-nitrophenoxy)stearoyl-1-14C-glucosamine.
rotary evaporation. The lipids were redissolved in 1 ml of diethyl ether and then 0.35 ml of buffer (20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 7.4) was added. For the samples shown in Fig. 1, the NaCl concentration of the buffer ranged from 50 to 350 mM, as noted in the text. The mixture was bath sonicated at 4 °C for ~5 min. The ether was slowly removed by rotary evaporation at 20–25 °C for 15–20 min; another 0.23 ml of buffer was added and the suspension was evaporated for an additional 15–20 min. Trypsin-containing vesicles were made by the same procedure using 0.33 ml of buffer that contained trypsin (1.65 mg/ml) and either passed through a Sepharose 4B column or floated through a density gradient of Ficoll 400 to remove unencapsulated material. Phospholipid concentrations were determined by the method of Raheja et al. (22).

Photolabeling and Binding—The probe 12APS-GlcN was added at 60,000 cpm/tube, dried, and re-wet with 1.5 μl of ethanol (ethanol was always less than 1% of the total sample volume). Vesicles (100 μg of PC in 200 μl of buffer) were added and incubated at 37 °C for 10 min. Under these conditions, >95% of the probe inserts into the lipid bilayer. After addition of PTx (typically 20 pg in 10 μl of buffer to each vesicle sample), the pH was adjusted with 0.25 N HCl or NaOH and each sample was incubated according to specified conditions. The samples were then irradiated for 30 s at 366 nm with a high intensity mercury lamp (75–100 watts). Irradiated samples were immediately mixed with 200 μl of 40% (v/v) Ficoll 400 prepared in buffer at pH 7.4, overlayed with 180 μl of 10% (w/v) Ficoll 400, and then with buffer (~70 μl). The samples were centrifuged for 1 h at 180,000 × g in a Beckman SW30.1 rotor equipped with 0.7-ml tube adaptors. Vesicles banding at the 0%/10% Ficoll interface were collected, solubilized in sodium dodecyl sulfate sample buffer under reducing conditions (23) and run on 10% polyacrylamide gels (24). After staining with Coomassie Blue (G-250 plus R-250), the gels were treated with sodium salicylate (25), dried, and exposed to Kodak X-Omat AR film for 5 weeks at ~70 °C. Photograph prints of gels and fluorograms were scanned with a Soft Laser Scanning Densitometer (BioMed Instruments Inc., Fullerton, CA). Peak integration was done on an Apple IIe computer with the “Videophoresis II: Electrophoresis Reporting Integrator Program” (BioMed Instruments Inc.). Each gel contained lanes of toxin standards for calibration purposes. Results shown are the average of two or more experiments. The calculated standard deviations of the data points ranged from 4 to 9%. The photolabeling efficiency at pH 4 was typically 1–8 nol %, depending on the specific sample conditions and the incubation time before irradiation.

RESULTS

Dependence of PTx Binding and Insertion on Ionic Strength—In a recent study, Zalman and Wisnieski (7) found that the levels of membrane binding and insertion of PTx increase with decreasing pH. The experiments described in this report were designed to quantify this dependency and to elucidate other factors that might regulate toxin entry. Since the previous experiments had been done in Tris buffer without NaCl, we felt it was important to establish how PTx binding and insertion vary as a function of ionic strength. We used vesicle targets composed of egg PC plus CS. The amount of PTx associated with floated vesicles was quantified by densitometric scans of Coomassie Blue-stained gels and the relative level of photolabeling was monitored by densitometric scans of the respective fluorograms (Fig. 1). The membrane-restricted photoprobe 12APS-GlcN covalently labels components inside the hydrocarbon core of the outer leaflet of the membrane bilayer (26, 27). Fig. 1 shows that at pH 4 the levels of binding and photolabeling of PTx increase dramatically with increasing salt concentration up to ~200 mM NaCl. At pH 7.4, binding was negligible throughout (data not shown). Since electrostatic interactions typically decrease with increasing ionic strength, these data indicate that toxin-membrane interactions are at least partially controlled by hydrophobic forces.

The goal of our next set of experiments was to establish the optimum conditions for PTx binding in physiological saline solution (buffer containing 150 mM NaCl). As seen in Fig. 2, the binding of PTx increased sharply with decreasing pH. There was a parallel increase in the level of photolabeling (Fig. 2B). Binding to these targets (egg PC/CS) increased 10-fold with every 1.55 pH unit drop, whereas the level of photolabeling increased 10-fold with every 2.57 pH unit drop. Binding and insertion were monitored after a 30-min incubation period at pH 4. A subsequent analysis of the kinetics of these processes showed that at 37 °C, binding and photolabeling of PTx both reached a maximum within 10 min at pH 4 (Fig. 3). While binding remained relatively constant for the next 20 min, photolabeling began to decrease steadily,
contained soybean trypsin inhibitor (5 pg/ml). Probe and toxin were lowered to added as described under "Materials and Methods." The samples were incubated an additional 30 min at pH 8 for trypsin cleavage. The vesicles were harvested by flotation. The Coomassie Blue-stained gel is on the left and the fluorogram is on the right. Lane 1, molecular mass standards in kilodaltons; lane 2, 6 μg of PTx; lane 3, the PTx-vesicle sample irradiated after 10 min at pH 4; lane 4, the PTx-vesicle sample irradiated after 30 min at pH 4. Lanes 3' and 4' show the corresponding fluorographic results. Arrowheads on right denote positions of the 61-, 36-, and 30-kDa fragments of PTx.

indicating that the toxin molecules had begun to cross the bilayer and were no longer accessible to the photoreactive probe (Fig. 3).

Effect of Surface Charge in PTx Binding and Insertion—To determine if the significant increase in PTx binding at low pH was due to charge titration at the membrane surface, we compared the PTx binding and insertion profiles obtained with vesicle targets composed of egg PC/CS, egg PC/CS/stearylamine, and egg PC/CS/dicetylphosphate. Fig. 3A shows the effect of vesicle surface charge on the time course of PTx interaction with membranes. A significant difference was observed between the kinetics of binding to positively and negatively charged vesicles. The amount of PTx bound to negatively charged or neutral (PC/CS) vesicles reached a maximum within 10 min and then showed a slight, slow decrease after 30 min. The level of PTx bound to positively charged vesicles increased rapidly between 0 and 10 min, and then continued to increase very slowly for up to 1 h. Overall, binding to negative and neutral vesicles was significantly more efficient than binding to positive vesicles. To determine if the lower level of binding to positive vesicles was due to a less efficient recovery of these vesicles from the Ficoll gradient, all three types of vesicles were prepared from radiolabeled PC. After flotation through Ficoll step gradients, vesicle recovery was analyzed and found to be independent of composition. The relative levels of PTx photolabeling achieved with these targets are shown in Fig. 3B. With neutral and negative vesicles, the amount of PTx inside the membrane bilayer reached a maximum within 10 min of lowering the pH to 4 and then dropped dramatically. In contrast, the amount of PTx inside the bilayer of positive vesicles increased gradually throughout the entire 60-min time period. The binding and insertion profiles of PTx followed the same general trend for a given vesicle type until the point in time at which they reached a maximum. Thereafter, any drop in photolabeling
relative to binding is indicative of toxin translocation across the bilayer. Thus, we conclude that positively charged vesicles are not very effective targets for PTx binding, insertion, and translocation.

Temperature Dependence of PTx Binding and Insertion—The temperature dependence of PTx binding to vesicles is shown in Fig. 4A. We noted a dramatic increase in the level of PTx binding to all of the vesicles tested as the temperature was increased from 0 to 41 °C. The efficiency of PTx binding to positive vesicles was always the lowest (Fig. 4A). Photolabeling of the vesicle-associated toxin showed the same trend (Fig. 4B). When the photolabeling data was converted to photolabeling efficiency per µg of bound toxin (Fig. 4C), we found that more of the vesicle-bound PTx was inside the bilayer (for each type of vesicle) at 0 °C than at any other temperature. Since the proportion of the vesicle-associated toxin that was photolabeled after 30 min at pH 4 actually decreased steadily with increasing temperature (Fig. 4C), we conclude that toxin translocation rates also increase significantly with increasing temperature.

Translocation of PTx Across the Bilayer—To determine the fate of PTx after binding and insertion, trypsin was trapped inside PC/CS vesicles prepared at pH 8.0. These vesicles were incubated with PTx in the presence of soybean trypsin inhibitor (present in an amount sufficient to prevent PTx cleavage when the vesicles were lysed with Triton X-100 at 0.1%). Binding of the PTx was induced by lowering the pH to 4. After 10 or 30 min at 37 °C, the pH of the reaction mixture was increased to pH 8.0 and the incubation was continued for an additional 30 min. We have shown that raising the pH back to 7 or 8, inhibits further PTx binding. Samples were irradiated after the first 10 or 30 min incubation (at pH 4, or pH 8 for the controls). As shown in Fig. 5, after dropping the pH to 4 the exogenously added PTx (M, 66,583) could be cleaved by vesicle-entrapped trypsin. Three fragments, 61, 36, and 30 kDa, were produced. The photolabeling data (lanes 3' and 4') shows that these fragments were or had previously been in contact with the lipid milieu of the membrane. No cleaved toxin was observed in the unbound toxin fractions of the pH 4 or 8 samples (Fig. 6, lanes 1 and 2); only intact PTx and soybean trypsin inhibitor were detected. This indicates that cleavage did not occur from outside the vesicles, nor was toxin released from the vesicles after cleavage had occurred. Lane 4 of Fig. 6 shows the type of cleavage pattern obtained when empty vesicles with bound toxin were exposed to trypsin externally, and lane 5 shows the results obtained with soluble PTx (no vesicles). The two patterns can be distinguished from each other as well as from the pattern obtained when PTx is cleaved from inside the vesicles (shown in Fig. 5). Moreover, the solution cleavage pattern did not change significantly with time (lanes 6, 7, and 8; 60, 30, and 15 min of digestion); it retained its characteristic features.

DISCUSSION

Expression of PTx toxicity involves at least five steps: 1) binding to cell surface receptors, 2) bilayer insertion, 3) translocation to the cytosol, 4) processing to activate the ADP-ribosyltransferase domain, and 5) transfer of the ADPribosyl portion of NAD+ onto elongation factor 2. Step 5 is the only process that has been characterized in detail (11, 12). Although recent studies indicate that PTx entry into cells involves interaction with cell surface receptors and uptake via coated vesicles (28-30), we have found that toxin exposure to low pH leads to high levels of membrane binding and insertion in the absence of specific receptors (Fig. 2; Ref. 7). This result indicates that a pH-dependent change in toxin conformation between steps 1 and 2 might facilitate avid membrane contact and insertion. Supporting evidence with cellular targets comes from the work of Moehring and co-workers on Chinese hamster ovary cell lines selected for resistance to PTx. Such cells were found to be cross-resistant to Dtx and several viruses (31, 32). Studies by Merion et al. (29) revealed that these mutant cell lines were defective in an ATP-dependent acidification of endosomes. Moreover, the resistance of these cells was lost in low pH environment (32).

The data presented here indicate that exposure of PTx to an acid pH environment causes a dramatic increase in the levels of membrane binding and insertion, and suggest that low pH induces a conformational change in the toxin molecule. Although acid conditions might promote lipid-protein interactions by altering the surface charge of the vesicles (and/or the toxin itself), the increase in toxin binding that occurred when the ionic strength of the medium was increased (Fig. 1A) indicates that the major attractive forces are hydrophobic in nature. The finding that much higher levels of binding were attained with negatively charged and neutral vesicles than with positively charged vesicles rules out the possibility that the role of low pH is to make the vesicle surface more positive. Indeed, PTx binding to positively charged vesicles was significantly lower at all temperatures tested (Figs. 3A and 4A) and time course studies showed that with negatively charged vesicles, close to maximum binding levels were achieved within 10 min of lowering the pH (Fig. 3A). The slight preference of PTx for negatively charged vesicles implies that electrostatic interactions may play some role in toxin-membrane interactions; however, the nature of the vesicle charge alone is not the sole determinant of toxin binding levels because at neutral pH, PTx did not bind appreciably to any of the vesicle targets.

The kinetic profiles of PTx photolabeling were not identical to the binding profiles. While PTx binding and insertion levels both peaked within 10 min of incubation with neutral or negatively charged vesicles at pH 4, there was a notable drop in the levels of photolabeling relative to binding at subsequent time points. In contrast, the levels of photolabeling achieved with positively charged vesicles were more closely correlated with the binding levels and both slowly increased throughout the 1-h test period. A decrease in photolabeling relative to binding indicates that the inserted toxin had begun to cross the lipid bilayer toward the vesicle lumen (see Ref. 21). Hence these studies provide the first evidence that PTx can rapidly insert into the hydrophobic domain of a membrane and then begin to migrate across the bilayer. The migration of PTx molecules is a relatively slow process compared to the migration rate of the cholera toxin A1 subunit (27). The temperature dependence of PTx binding and insertion (Fig. 4C) revealed that the probability of a given molecule being photolabeled after 30 min at pH 4 increased with decreasing temperature with all three types of target vesicles. This is consistent with data obtained in a previous study (7) with dimyristoylphosphatidylcholine targets and provides strong evidence that toxin translocation rates are linked to the physical state of the target bilayer. Thus, while binding and insertion appear to be exquisitely coordinated or even part and parcel of the same process initially, at some point in time the photolabeling efficiencies begin to reflect translocation rates. It should be pointed out that with dimyristoylphosphatidylcholine targets (Tm ~ 23 °C), the overall levels of binding and photolabeling of Dtx increased with decreasing temperature (15); therefore, we expect that different protein-lipid interactions are involved in the Dtx entry pathway.

Direct proof of translocation was that toxin allowed to bind
and insert at pH 4.0 could be cleaved with trypsin trapped inside the vesicles. Data from this experiment revealed that intact toxin (M, 66,583) was cleaved into three major fragments (M, 61,000, 36,000, and 30,000). This cleavage pattern could be distinguished from patterns obtained by external digestion and by digestion of soluble PTx in the absence of vesicles. In solution, no difference was noted between the pH 8-cleavage patterns of pH 4- and pH 8-treated toxin; however, PTx was slightly more susceptible to cleavage at pH 4. Our preliminary investigations of toxin structure demonstrate a reversible acid-induced conformational change, and we propose that this change facilitates avid membrane binding which significantly affects the overall levels of translocation.

The results presented here demonstrate that at low pH, PTx acquires the capability to bind and insert into target membranes. It is not yet clear whether these two events are truly distinct, but membrane insertion usually leads to toxin translocation. Binding appears to be primarily hydrophobic in nature although electrostatic interactions between positively charged toxin and negatively charged membranes may enhance the binding efficiency. The observation that trypsin-cleavage of photolabeled PTx generates three major photolabeled fragments (e.g. 30 kDa) shows that both ends of the toxin molecule are exposed to lipid side chains during insertion and consequently that the toxin undergoes translocation as a unit structure. The finding (7) that chymotrypsin-cleaved PTx creates large pores (2.8 nm diameter) at pH 4 needs to be examined in a physiological context. Since the toxin’s susceptibility to chymotrypsin increased upon pre-exposure to pH 4, it is possible that lysosomes may be involved both in toxin entry and processing (7). While our results are consistent with an endosomal or lysosomal route of entry, several steps in the entry pathway remain obscure: the place where processing occurs as well as the nature of that process (e.g. disulfide bridge reduction and/or enzyme cleavage), the domain responsible for cell receptor binding at neutral pH, and the destination of any cleavage products that might be formed during processing. Studies made with membrane targets in conjunction with target cell lines should rapidly augment our understanding of the physicochemical events involved in the PTx entry pathway.

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