Effects of Eliminating a Disulfide Bridge within Domain II of *Pseudomonas aeruginosa* Exotoxin A

INGER HELENE MADSHUS† and R. JOHN COLLIER*

Department of Microbiology and Molecular Genetics, Harvard Medical School, and Shipley Institute of Medicine, Boston, Massachusetts 02115

Received 22 December 1988/Accepted 18 March 1989

Cysteines 265 and 287 of *Pseudomonas aeruginosa* exotoxin A (ETA) were substituted by serine, thereby eliminating a disulfide bridge within domain II, the putative membrane insertion-translocation domain. Purified mutant toxin was 80-fold less toxic for mouse L cells than was wild-type ETA while retaining the same specific activity in the ADP-ribosyltransferase reaction as did wild-type toxin. Binding of the nonionic detergent Triton X-114 by mutant ETA occurred at a slightly higher pH than did binding by wild-type ETA, suggesting that the mutant protein more readily undergoes a conformational change exposing hydrophobic regions. Data are presented supporting the notion that the mutant and wild-type toxins enter from the same intracellular compartment. The lower cytotoxicity of the mutant protein could be due to accelerated intracellular degradation or abortive, premature membrane insertion.

*Pseudomonas aeruginosa* exotoxin A (ETA) kills eucaryotic cells by catalyzing the ADP ribosylation of elongation factor 2 within the cytosol (14). The mechanism whereby ETA enters the cytosol is poorly understood. Indeed, the mechanism by which any protein translocates through highly hydrophobic lipid bilayer membranes is not understood in detail. Toxins such as *Pseudomonas* ETA and diphtheria toxin are useful model proteins in studies of translocation mechanisms because their entry can be measured by their effects on protein synthesis once they are inside the cytosol.

ETA is known to act by a multistep mechanism, beginning with binding to receptors on the cell surface (18). The toxin is endocytosed from coated pits and possibly transferred to the Golgi apparatus (20). It has been documented that low intravesicular pH is required for efficient entry of the toxin (9, 24). Sandvig and Moskau (22) reported that *Pseudomonas* toxin binds Triton at low pH in a similar manner to that of diphtheria toxin. Farahbakhsh et al. (8) described an increase in intrinsic fluorescence of ETA as well as increased binding of the fluorophore 1-anilino-8-naphthalene sulfonic acid with decreasing pH. Low pH is therefore believed to affect the translocation of ETA by triggering a conformational change in the toxin whereby normally hidden hydrophobic regions are exposed. This acquisition of hydrophobic properties is probably crucial for the insertion-translocation process. The translocation results in transfer of at least the catalytic domain to the cytosol and thereby promotes ADP ribosylation of elongation factor 2.

The X-ray crystallographic structure of the toxin shows three domains (I). Domain I has been identified as the receptor-binding moiety (12, 13) and domain III as that responsible for enzymatic activity (11, 18). Data indicating that domain II is involved in membrane insertion and translocation have been presented (13).

To probe the process of membrane insertion and translocation, we have begun to apply directed mutagenesis to the cloned gene. As a first step, we chose to probe the function of the disulfide bridge within domain II (Cys-265–Cys-287). This disulfide, one of the four within ETA, connects two of the six alpha helices comprising domain II. We hypothesized that the disulfide provides a conformational constraint on the molecule and that its removal might influence the conformational change at low pH.

**MATERIALS AND METHODS**

**Strains and plasmids.** The following *Escherichia coli* K-12 strains were used: JM103 ([lac pro] thi rpsL supE endA xbcB15 hsdR4 F' traD36 proAB [lacF' lacZ M15] hsdR hsdM*') and TG1 ([lac pro] supE thi hsdD5 F' traD36 proAB [lacF' lacZ M15]). M13mp18 was purchased from New England BioLabs, Inc. (Beverly, Mass.). pCDPT2, a pBR322 derivative bearing the ETA gene behind the tac promoter, was constructed in this laboratory (6). All restriction enzyme digestions and ligations were performed as described by Maniatis et al. (19), using restriction enzymes and T4 DNA ligase from New England BioLabs.

**Site-directed mutagenesis.** A *Kpn1-Xho1* fragment from pCDPT2 was cloned into the polylinker region of *M13mp18*. The construction was confirmed by restriction digest analysis. Oligonucleotide-directed mutagenesis was used to change the codons for cysteine 265 and cysteine 287 to codons for serine. The mutagenic oligonucleotides 5′-CG GCAAGTGGCTGGCCCTGG-3′ and 5′-GGATAGCCTGGAC TGCCCTCC-3′ were synthesized on an Applied Biosystems model 381A synthesizer and passed through Sep-Pak C18 cartridges (Waters Associates, Inc., Milford, Mass.). Site-directed mutagenesis was performed with an oligonucleotide mutagenesis kit as described by the manufacturer (Amer- sham Corp., Arlington Heights, Ill.). Replicative-form molecules synthesized in vitro were transfected into *E. coli* TGI. Bacteriophage M13 particles in supernatant fractions from putative mutants were bound to nitrocellulose using a slot blot apparatus (Schleicher & Schuell, Inc., Keene, N.H.). The phage particles were lysed with 0.1 M NaOH and neutralized with 1 M Tris hydrochloride (pH 7), and the filter was exposed to UV light for 60 s. Filters were probed with end-labeled mutagenic oligonucleotide [32P]ATP; 5,000 Ci/mmol). Positive clones were plaque purified, reblotted, and...
sequenced to verify the mutation. Dideoxy sequencing was performed with reagents in the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) as recommended by the manufacturer. [32]SdATP (500 Ci/mmol) was from Amersham. A BgII-ApaI fragment from a mutant clone was ligated back into the expression vector, pCDPT2, to create the vector pCDPTC265SC287S. From this vector, the Klpn-XhoI restriction fragment was cloned back into the polylinker region of M13mp18, and the whole Apal-BgII fragment was sequenced to verify that no secondary mutations had occurred.

Extracts of mutant and wild-type clones. Extracts were prepared in the following way: E. coli JM103, transformed with pCDPTC265SC287S, was grown overnight in L broth containing 100 μg of ampicillin per ml. A 40-ml culture was inoculated at an initial A600 of 0.1 and grown to an A600 of 0.3. Isopropyl-p-D-thiogalactopyranoside (IPTG) (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 1 mM (4), and the cells were incubated to a final A600 of 3.0. Cells were harvested by centrifugation at 4,000 × g, washed with 50 mM Tris hydrochloride (pH 8.2)-1 mM EDTA, suspended in 0.01 the initial culture volume, and sonicated. Insoluble material was removed by centrifugation at 100,000 × g for 30 min, and the supernatant fraction was applied to a 10-ml Sephadex G-50 column equilibrated with the same buffer. Peak fractions (as determined from A280) were pooled; 5 μg of soybean trypsin inhibitor per ml, 1 mM phenylmethylsulfonyl fluoride, and 5% glycerol were added; and equal portions were stored frozen at −70°C.

ADP ribosylation assays. Samples were assayed for ADP ribosylation activity essentially as described by Lory and Collier (16). The samples were activated for 30 min at 25°C in the presence of 4 M urea and 10 mM dithiothreitol. After activation, the samples were incubated for 30 min with 0.01 μCi of [14]Cladenylate-NAD (600 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) and wheat germ elongation factor 2 in 50 mM Tris hydrochloride (pH 8.2)-1 mM EDTA-1 mM dithiothreitol in a final volume of 100 μl. The assay mixture was precipitated on filter paper saturated with 10% trichloroacetic acid, and the paper was washed twice for 30 min with 5% trichloroacetic acid. Finally, the filtered paper was soaked in methanol for 5 min, dried, and counted in a scintillation counter.

Antiserum preparation. Serum from immunized rabbits or sheep was purified as described previously (6).

Polyacrylamide gel electrophoresis and Western blots (immunoblots). Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS) by the procedure described by Laemmli (15). Samples were analyzed for total protein by Coomassie blue staining (7) or for ETA-related material by immunoblot analysis (25). Western blots were probed with 125I-labeled rabbit anti-ETA and exposed to X-ray film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.) for 1 to 24 h. When bands were to be quantified, the film was preflashed and an LKB laser densitometer was used.

Cytotoxicity assay. Mouse L cells (L-M929) were used in cytotoxicity assays in 24-well Falcon plates at a density of 10⁵ cells per ml. Cells were grown in minimal essential medium with 5% fetal bovine serum. At the start of the experiment, the medium in the well was aspirated, and 0.5 ml of the same medium (unless otherwise indicated in the figure legend) was added. Toxin was added, and the cells were incubated at 37°C for 6 h in the presence of 5% CO₂ unless otherwise indicated. At the end of the experiment, residual protein synthesis was measured by incubating the cells in leucine-free minimal essential medium containing 1 μCi of [3H]leucine per ml (Amersham). The cells were washed twice with 10% trichloroacetic acid, and the precipitated protein was dissolved in 200 μl of 0.2 M KOH. The content of each well was transferred to scintillation vials and counted.

Purification of the mutant toxin. An overnight culture of JM103 (pCDPTC265SC287S) in L broth with 100 μg of ampicillin per ml was diluted to an initial A600 of 0.1 in 10 liters of the same medium with 1 ml of Antifoam B (J. T. Baker Chemical Co., Phillipsburg, N.J.). Cultures were grown in a Microferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) with oxygen at a flow rate of 10 liters/min. IPTG was added at an A600 of 0.2 to a final concentration of 1 mM. At an A600 of 3.5, the cells were harvested by a Pellicon cassette system (HVLP 000 C5; 465 cm²; Millipore Corp., Bedford, Mass.). The cells were washed with 10 mM Tris hydrochloride (8.0) by centrifugation in a GS-3 rotor (Ivan Sorvall, Inc., Norwalk, Conn.) at 5,000 rpm for 30 min and suspended in 100 ml of 100 mM Tris hydrochloride (pH 8.2)-0.1 mM EDTA. The cell suspension was sonicated in a sonic oscillator (Branson Sonic Power Co., Danbury, Conn.), and insoluble material was removed by centrifugation at 50,000 × g at 4°C for 30 min.

Immunoadsorption purification of the mutant was performed as described by C. M. Douglas and R. J. Collier (manuscript in preparation). Immunoglobulin G fractions from ETA-immunized rabbit and sheep sera were prepared as described elsewhere (6). Antibodies were coupled to Affigel 10 (Bio-rad Laboratories, Richmond, Calif.) as specified by the manufacturer. Immunoglobulin G-reacted resin (100 ml) was packed, and the supernatant fraction from the sonicated cell suspension was circulated through the affinity column for 2 to 3 h. Unbound protein was washed from the column with 5 column volumes of 5 mM Tris hydrochloride (pH 8.2)-150 mM NaCl-0.5 mM EDTA. Bound protein was eluted with 3 M potassium thiocyanate (KSCN) in the same buffer. The eluted protein was concentrated with Centriprep 30 concentrators (Amicon Corp., Lexington, Mass.) to a volume of 20 ml and then dialyzed against 10 mM Tris hydrochloride (pH 8.2)-0.5 mM EDTA.

The concentrated and dialyzed fractions were applied to a fast protein liquid chromatography (FPLC) anion-exchange column (Mono Q; Pharmacia Inc., Piscataway, N.J.) in 10 mM Tris hydrochloride (pH 8.2)-0.5 mM EDTA at a flow rate of 1 ml/min. The protein was eluted in the same buffer with a linear NaCl gradient. Protein peaks were run on 11.25% reducing SDS-polyacrylamide gels and stained by Coomassie blue as well as probed by 125I-labeled anti-ETA on Western blots. Most pure ETA eluted at about 0.3 M NaCl. Peak fractions were pooled and concentrated in a Centricron 30 concentrator (Amicon). The concentrated sample was applied to an FPLC sizing column (Superose 12). The peak fractions eluted with 100 mM Tris hydrochloride (pH 8.0) were analyzed by SDS-polyacrylamide gel electrophoresis.

Measurements of protein concentrations. Concentrations of protein were determined by using laser densitometry of Coomassie blue-stained polyacrylamide gels (7), with known amounts of proteins as standards run on the same gels. For quantifying proteins on Western blots, the film was preflashed and the autoradiogram was read by laser densitometry (7).

Triton X-114 preparation. To prepare a stock solution of detergent, 40 g of Triton X-114 (Sigma) was dissolved in 1
liter of 20 mM sodium phosphate (pH 7.5) containing 0.14 M NaCl by stirring overnight at 4°C and then incubated at 37°C until two phases appeared. The upper phase, which consisted of buffer, was discarded, and new buffer was added. This procedure was repeated three times. Finally, the pretreated Triton X-114 was made up to 200 ml by addition of 20 mM sodium phosphate (pH 7.5) containing 0.14 M NaCl, giving a final detergent concentration of 20%. The pH in different detergent solutions was adjusted by addition of citric acid.

Detergent partition experiments. Toxin (10 µl) was added at 4°C to 0.5 ml of Triton X-114 prepared as described above. The samples with different pHs were incubated at 37°C for 30 min and then centrifuged for 4 min in an Eppendorf centrifuge. A 200-µl sample of the water phase was collected and neutralized, and dilutions were made in phosphate-buffered saline (pH 7.5) containing 0.1 mg of bovine serum albumin per ml. The amount of toxin in each sample was compared with the amount present in the aqueous phase from the pH 7.0 sample by assaying for cytotoxicity as described above.

\[ ^{125}I \] labeled wild-type and mutant toxins. Toxins were prepared by the iodogen method (10) and by oxidation with chloramine T. Specific activities were \( 1 \times 10^5 \) to \( 2 \times 10^5 \) cpm/µg of protein.

RESULTS AND DISCUSSION

Site-directed mutagenesis. Cys-265 and Cys-287 were substituted by Ser by oligonucleotide-directed mutagenesis in the M13 system (27). The mutagenesis was performed in two steps, and DNA sequence analysis of plaque-purified M13 mutants confirmed the base changes.

A BglII-Apal restriction fragment from mutant M13 replicative form was exchanged for the corresponding wild-type fragment in pCDPT2. The resulting plasmid, pCDPTC265SC287S, encoded the Ser-265/Ser-287 form of ETA. A clone that produced full-length cross-reacting material, as detected by Western blot analysis, was chosen for further study.

Purification of the mutant toxin. Ser-265/Ser-287 ETA was purified from E. coli JM103 bearing the plasmid pCDPTC265SC287S. Douglas and Collier (in preparation) found that wild-type ETA produced in E. coli comigrated with ETA produced in Pseudomonas aeruginosa on a denaturing polyacrylamide gel and that the toxin was stable and had the same cytotoxicity and specific ADP-ribosyltransferase activity as did ETA produced in P. aeruginosa.

The cellular extract from a fermentor batch of 10 liters was sonicated and centrifuged. The supernatant fraction was chromatographed on an immunoglobulin G immunoaffinity column; the toxin eluted with a high concentration of KSCN, as described by Douglas and Collier (in preparation). Douglas and Collier showed that relative to immunoreactivity, the enzymatic and cytotoxic activities of wild-type ETA remained constant throughout this purification, indicating that the toxin was not inactivated by the purification.

The semipurified toxin preparation (containing most of the degradation fragments as well as some immunoglobulin G from the immunoaffinity column; Fig. 1) was then further purified on an FPLC anion-exchange column. The product at this stage was almost 50% pure (Fig. 1). A degradation fragment of approximately 40,000 daltons, which we were unable to remove by anion-exchange chromatography, was separated from full-length mutant ETA on an FPLC sizing column. This step yielded material of \( >95\% \) purity, as judged by Coomassie staining of the preparation run on an 11.25% SDS-polyacrylamide gel (Fig. 1).

The yield of the purified protein was low because of the susceptibility of the protein to proteolytic enzymes. Immediately upon harvesting and lysing of the cells, several degradation fragments were seen on a Western blot. This was not observed with wild-type ETA produced in an identical fashion (data not shown). From approximately 1 mg of immunoreactive material in the crude extract, we recovered approximately 15 µg of pure protein. The largest losses were due to ongoing proteolysis and to loss of material in the immunoaffinity step and in the concentration of the sample before application to the FPLC sizing column.

Characterization of the purified mutant toxin. (i) ADP-ribosyltransferase activity. Mutant toxin treated with dithiothreitol and urea had the same ADP-ribosyltransferase activity as did wild-type toxin, and neither protein had detectable basal (unactivated) enzymatic activity (data not shown). The 40,000-dalton fragment present in the preparation before purification on the FPLC sizing column was shown to be highly active in ADP-ribosylating elongation factor 2 (data not shown).

Cytotoxicity. The cytotoxic activities of wild-type and mutant toxins were compared by incubating mouse L cells with equal amounts of the pure toxins at 37°C for 6 h and

![Figure 1](http://iai.asm.org/)

**FIG. 1.** (A) SDS-polyacrylamide gel electrophoresis of fractions from purification of ETA Ser-265/Ser-287. Proteins were separated on an 11.25% gel and stained with Coomassie blue. Amounts of protein loaded per lane, as estimated by the method of Bradford (3), are given below in parentheses. Lanes: 1. molecular weight markers (indicated in kilodaltons); 2. high-speed supernatant of sonicated cells (40 µg); 3. immunonaffinity pool (15 µg); 4. Mono Q pool (5 µg); 5. Superose pool (0.3 µg); 6. ETA (0.7 µg). (B) Western blot of gel loaded with same fractions and amounts of protein as in panel A.

![Figure 2](http://iai.asm.org/)

**FIG. 2.** Cytotoxic activities of wild-type ETA and ETA Ser-265/Ser-287. Increasing amounts of either protein in 10 µl were added to cells in 24-well plates. The residual protein synthesis was measured as described in Materials and Methods.
then assaying for cellular protein synthesis. Ser-265/Ser-287 ETA was less cytotoxic than wild-type toxin by a factor of ca. 80 (Fig. 2). The difference in cytotoxicity between wild-type and mutant toxins was the same when crude extracts, partially purified forms of the toxins, or fully purified toxin preparations were compared. This finding shows that the extracts contained no N-terminal fragments active in blocking binding of the mutant toxin to the cellular receptors and that the cytotoxic activity of the mutant protein was not altered during the purification process. Toxin in extracts or partially purified toxin was quantified as described in Materials and Methods.

When the cytotoxicity of the mutant toxin was assayed in the absence of serum, the ID<sub>50</sub> (toxin concentration reducing protein synthesis 50%) was decreased approximately threefold (data not shown). This was not observed with wild-type toxin. The addition of leupeptin, phenylmethylsulfonyl fluoride, and trypsin inhibitor also decreased slightly the ID<sub>50</sub> of the mutant protein in the presence of serum (data not shown). This finding suggests that there may be partial inactivation of the mutant toxin during the incubation by proteases present in the serum.

The decreased cytotoxicity of the mutant toxin can possibly be explained by decreased stability. Before and during purification, the protein was rapidly cleaved into several fragments even in the presence of protease inhibitors. It was recently reported that degradation of proteins takes place within endosomes because of the presence of proteolytic enzymes such as cathepsin D (5), cathepsin B (21, 26), and carboxypeptidase (23).

**Effect of low pH on the conformation of Ser-265/Ser-287 ETA.** Sandvig and Moskaug (22) have shown that ETA binds Triton X-114 at pH values below 5.0. Triton X-114 is a nonionic detergent that is miscible with water at 0°C, whereas two phases are formed at temperatures above 20°C. When proteins are added to the detergent at low temperature and the mixture is incubated at 37°C, hydrophobic proteins are enriched in the detergent-poor phase, whereas amphiphilic proteins are partitioned into the detergent phase (2). The finding of Sandvig and Moskaug (22) implies that cryptic hydrophobic regions of ETA become exposed at low pH. This, in turn, suggests that the demonstrated requirement for low pH during the entry process is to facilitate the transport of the toxin across the vesicular membrane by rendering the molecule hydrophobic. It is highly unlikely that ETA in its native hydrophilic form would be able to penetrate a lipid bilayer membrane.

We examined the detergent binding of mutant and wild-type ETAs as a function of pH. Wild-type and mutant ETAs were added to Triton X-114 at 4°C. The two phases were separated after incubation at 37°C, and the amount of toxin in the water phase was determined by assaying cytotoxicity. The percentage of toxin in the Triton phase at the different pH values was calculated as percentage of total toxin added. For both toxins, the titration curve was sharp (Fig. 3). The mutant toxin entered the Triton phase at a higher pH (ca. 0.25 pH units) than did wild-type toxin. Control experiments showed that both the wild-type and mutant toxins retained cytotoxic activity after being exposed to the low pH required for detergent binding.

Partition of label into the detergent-rich and -poor phases was also determined by using 125I-labeled toxins. Iodinated wild-type ETA entered the detergent phase essentially as shown for noniodinated toxin (Fig. 3). In contrast to wild-type ETA, the mutant toxin showed a gradual increase in the partition into the detergent phase as a function of decreasing pH (data not shown). The fact that the titration curve for the iodinated mutant protein was more gradual suggested some conformational alteration of the protein due to the iodination. This finding shows that a functional assay in this case is preferable, since no modification of the toxin is necessary. The results of this experiment support the notion that a conformational change exposing cryptic hydrophobic surfaces is more readily induced by low pH for the mutant than for the wild-type ETA.

With the altered detergent-binding profile of the mutant protein, we would expect that this protein more readily inserts into a lipid bilayer membrane at low pH. Premature insertion-translocation would result in decreased cytotoxicity if proteolytic processing by enzymes present only in the Golgi apparatus is a prerequisite step in the intoxication process. The actual pH required to trigger a conformational change in vivo may be different from that determined by a detergent-binding assay in vitro because receptor binding and unknown factors may contribute in this process. This was shown to be the case for poliovirus; in these studies, the pH dependence for detergent binding was shifted depending on whether the virus had been exposed to its natural receptor (17).

**Protective effect of NH<sub>4</sub>Cl on cytotoxicity induced by mutant and wild-type ETAs.** Entry of ETA into the cytoplasm of eucaryotic cells occurs by an acid-dependent mechanism, as illustrated by inhibition of the process by lysosomotropic drugs such as NH<sub>4</sub>Cl. These compounds act by raising the pH of intracellular acidic compartments. Several authors have found that abolishing the pH gradient across vesicular membranes does not completely block cellular intoxication. Some nonspecific toxin entry occurs via fluid-phase endocytosis or perhaps some other uncharacterized acid-independent mechanism. As a percentage of the total, however, the amount of toxin entering via acid-independent pathways is minimal (9, 24).

NH<sub>4</sub>Cl at a final concentration of 15 mM protected L cells more than 100-fold against intoxication by wild-type ETA (Fig. 4A) but only about 10-fold against intoxication by the mutant ETA (Fig. 4B). In the presence of NH<sub>4</sub>Cl, the ID<sub>50</sub> of the mutant toxin was approximately the same as that of the wild type.

One explanation for the reduction in cytotoxicity of the mutant toxin, and for the minimal protection observed by NH<sub>4</sub>Cl, would be that the mutant toxin enters the cells by a different type of vesicle than is used by wild-type toxin. To explore this possibility, we made use of the observation by
ELIMINATION OF A DISULFIDE BRIDGE IN PSEUDOMONAS TOXIN

FIG. 4. Effect of NH₄Cl on the cytotoxic activities of wild-type ETA (A) and ETA Ser-265/Ser-287 (B). The ordinary growth medium (aspirated cells) was replaced with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered minimal essential medium containing 5% fetal bovine serum with and without 15 mM NH₄Cl. The cells were incubated with this medium for 30 min at 37°C before toxin was added. Incubation was for 6 h before the residual protein synthesis was measured as described in Materials and Methods.

Morris and Saelinger (20) that there is a temperature block in the entry of ETA into the cytoplasm of L cells at 19°C. The entry of the toxin was shown to be less efficient at 19°C than at 26 and 37°C, suggesting that a fusion event is involved and that ETA therefore probably enters the cytosol from the Golgi complex (20). We therefore tested the effect of low temperature on the entry of the two toxins. Cells were incubated at 26 or 19°C, wild-type or mutant toxin was added, and reduction in protein synthesis at the two temperatures was measured after 6 h. For both proteins, the ID₅₀ was approximately 10-fold higher at 19°C than at 26°C. Only a minimal difference in ID₅₀ was observed between cells incubated at 26 and 37°C (data not shown). This finding shows that for both proteins there is a temperature block, inhibiting entry to the same extent, and implies that both toxins enter the cytosol after a fusion event. This suggests that entry of both toxins occurs from the same vesicular compartment. However, we cannot completely rule out the possibility that the two forms of ETA enter from different secondary compartments and that a greater proportion of mutant than wild-type toxin enters by an acid-independent pathway.

To show that incubation of cells with NH₄Cl did not interfere with the binding of either form of the toxin to cellular receptors, the following experiment was performed. NH₄Cl was added to cells at 37°C, and the cells were incubated for 15 min before being chilled on ice. Mutant and wild-type toxins were added at 4°C and incubated with the cells for 60 min. The cells were then washed and finally incubated further in ordinary medium without NH₄Cl for 6 h at 37°C. In this case there was no effect of NH₄Cl (data not shown).

The observation that NH₄Cl has less effect on cytotoxicity of the mutant ETA than the wild-type ETA could be explained by the possibility that an increase in intravesicular pH due to the presence of NH₄Cl stabilizes mutant ETA that would otherwise be susceptible to degradation or to unfolding, causing premature, abortive membrane insertion.

In conclusion, substitution of cysteines 265 and 287 in domain II of ETA by serines, thereby eliminating the disulfide bridge between helix A and helix B of this domain, yielded a mutant protein that was about 80-fold less toxic than wild-type toxin for mouse L cells. The ability of the protein to ADP ribosylate elongation factor 2, a function of domain III, was the same as for the wild-type protein; in addition, we doubt that the domain I function, binding of the toxin to cells, was significantly altered, although we were never able to obtain enough pure protein to do competition binding experiments under saturating conditions. The data presented are consistent with the notion that removal of the disulfide bridge alters the functional properties of domain II, causing either altered membrane permeation properties, a change in intracellular routing, or destabilization of the protein.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-22021 and AI-22848 from the National Institutes of Health. Inger Helene Madshus was supported by a long-term fellowship from the European Molecular Biology Organization.

LITERATURE CITED


