Cell-mediated Cleavage of *Pseudomonas* Exotoxin between Arg\textsuperscript{279} and Gly\textsuperscript{280} Generates the Enzymatically Active Fragment Which Translocates to the Cytosol*

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*Pseudomonas* exotoxin (PE) is a three-domain toxin which is cleaved by a cellular protease within cells and then reduced to generate two prominent fragments (Ogata, M., Chaudhary, V. K., Pastan, I., and FitzGerald, D. J. (1990) *J. Biol. Chem.* 265, 20678–20685). The N-terminal fragment is 28 kDa in size and contains the binding domain. The 37-kDa C-terminal fragment, which translocates to the cytosol, contains the translocation domain and the ADP-ribosylation domain. Cleavage followed by reduction is essential for toxicity since mutant forms of the toxin that cannot be cleaved by cells are nontoxic. Previous results with these mutants suggested that cleavage occurred in an arginine-rich (arginine residues are at positions 274, 276, and 279) disulfide loop near the beginning of the translocation domain, but the exact site of cleavage was not determined. Since very few molecules of the 37-kDa fragment are generated within cells it was not possible to determine the site of cleavage by performing a conventional N-terminal sequence analysis of the 37-kDa fragment. Two experimental approaches were used to overcome this limitation. First, existing amino acids near the cleavage sites were replaced with methionine residues; this was followed by the addition of [\textsuperscript{35}S]methionine-labeled versions of these toxins to cells. The pattern of radioactive toxin fragments recovered from the cells indicated that the toxin was cleaved either just before or just after Arg\textsuperscript{279}. Second, [\textsuperscript{3}H]leucine-labeled toxin was produced and added to the cells. Sequential Edman degradations were performed on the small amount of radioactive 37-kDa fragment that could be recovered from toxin-treated cells. A peak of radioactivity in the fifth fraction indicated that leucine was the 5th amino acid on the C-terminal side of the cleavage site. This result confirmed that cleavage was between Arg\textsuperscript{279} and Gly\textsuperscript{280}.

*Pseudomonas* exotoxin (PE)\textsuperscript{1} is a single chain bacterial toxin that kills mammalian cells by gaining entry to the cytosol and inactivating protein synthesis (1, 2). The pathway of toxin action includes binding to a surface receptor (3, 4), internalization via coated pits and endosomes (5, 6), proteolytic processing, reduction of disulfide bonds, and finally the translocation of an enzymatically active 37-kDa C-terminal fragment to the cytosol (7). Translocation to the cytosol requires a specific sequence (REDLK) at the C terminus of this fragment which appears to function as an endoplasmic reticulum retention sequence (8, 9). Once in the cytosol, the fragment inhibits protein synthesis by the ADP-ribosylation of elongation factor 2 (10). Structural studies have shown that PE is composed of three distinct domains (11). Domain I at the N terminus binds to surface receptors, domain II in the middle of the protein has the translocating activity and is cleaved by a cellular protease, and domain III at the C terminus has the ADP-riboseylating activity and the endoplasmic retention sequence (7, 8, 12).

Diphtheria toxin and ricin are also single chain protein toxins that kill cells by translocating an enzymatically active fragment to the cytosol and inhibiting protein synthesis (13–15). Although all three toxins have similar functional properties, it has been easier to identify the active fragments of DT and ricin since these fragments can be generated before the toxin is added to cells. After DT is synthesized and secreted, a large proportion of the toxin is proteolytically nicked in the growth medium (14). When nicked toxin is reduced, distinct A and B fragments are produced. The toxin is nicked within an arginine-rich region bounded by a disulfide loop formed between cysteines 186 and 201 (16). The A fragment has the ADP-riboseylating activity and is the fragment which translocates to the cytosol. Since there are 3 arginines in this loop, it is possible to generate A fragments with different C termini (16). Olsnes and colleagues (17) have suggested that the fragment produced by cleavage after Arg\textsuperscript{190} is the only one that translocates to the cytosol. Murphy and colleagues (18) have made recombinant derivatives of DT with mutations in this region. Their results suggest that the presence of an arginine at residue 193 is most important for the activity of DT-derived chimeric toxins. However, the amino acid composition of the A fragment generated from these constructs was not determined.

For ricin, a 12-amino acid linker peptide (linking the A and B domains) has to be removed before the toxin can exhibit any cytotoxic activity (19). This linker is proteolytically excised within germinating castor bean seeds leaving the A and B fragments held together by the disulfide bond formed from cysteines 259 in the A fragment and 4 in the B fragment (20). Again it is the A fragment of the toxin which is translocated to the cytosol. It is presumed that the entire A fragment is

\textsuperscript{1} The abbreviations used are: PE, *Pseudomonas* exotoxin; DT, diphtheria toxin; PAGE, polyacrylamide gel electrophoresis.

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translocated to the cytosol, although this has never been addressed directly.

Since PE is secreted as a single chain protein and is not cleaved until it enters target cells, determining the precise composition of its active fragment has been more difficult. Previously this was addressed by following the cellular entry and metabolism of various mutant forms of \(^{15} \text{H}\)-labeled PE (7). The results indicated that the fragment recovered from the cytosol was 37 kDa in size and that its N terminus was derived from the arginine-rich loop (arginines are at residues 274, 276, and 279) located near the beginning of domain II. The exact amino acid sequence at the C terminus was not determined, but because the fragment extended for 37 kDa from the arginine-rich loop, the C-terminal end of the fragment was thought to include most of the residues found at the C terminus of PE itself. In addition, deletion analysis of residues at the C terminus of PE has shown that if more than 13 residues are removed, there is complete loss of ADP-ribosylating activity (8). Thus the active fragment from PE begins at residues 275–280 and extends past residue 600 and probably extends all the way to the last amino acid at residue 619. Here we determine the sequence at the N terminus of this fragment.

Since we could not by immunoprecipitation or other biochemical means recover enough of the 37-kDa fragment from cells to determine its N-terminal sequence by conventional microsequencing techniques, alternative approaches had to be used. All of the naturally occurring methionine residues in PE are found near the N terminus of PE in domain Ia (21). Therefore, when PE, metabolically labeled with \(^{35} \text{S}\)-methionine, is added to cells, radioactivity can be detected in immunoprecipitates containing either unprocessed toxin or the 28-kDa fragment (or small fragments derived from the 28-kDa fragment). No radioactivity is associated with the C-terminal 37-kDa fragment. To localize the site of cleavage we replaced amino acids on either side of the site of cleavage with methionine and studied the nature of the labeled fragments produced when the toxins is added to cells. In addition, PE metabolically labeled with \(^{3} \text{H}\)-leucine was added to cells, the radiolabeled 37-kDa fragment recovered by immunoprecipitation, and the distance of leucine residues downstream from the site of cleavage determined by repeated cycles of Edman degradation.

Both types of experiments indicate that within cells PE is cleaved between arginines 279 and glycine 280.

**MATERIALS AND METHODS**

**Plasmids and Strains**—Plasmids were propagated in HB101 or DH5a (Bethesda Research Laboratories). For expression of proteins, BL21 (DE3) was transformed with the appropriate plasmid and isopropyl 1-thio-β-D-galactopyranoside added for 90 min to induce the production of T7 polymerase (22). The structural gene for PE was located immediately downstream for the T7 promoter. Isopropyl 1-thio-β-D-galactopyranoside was added when the cells had grown in broth culture to the desired absorbance at 650 nm (usually 0.3–0.5 b). Native PE with an OmpA leader sequence was produced from the pVC45 vector (see Fig. 1B). The intermediate vector, pMOA1A2K352, was constructed by site-directed mutagenesis (see Fig. 1B). This vector encoded the same amino acids as pVC45f+(T), but the unique XhoI site at position 1646 was eliminated and moved to position 591 and the BsmBI site at 1251 was replaced by an XhoI site. Thus the 13-residue leader was exchanged for an MluI/SacII site (pMOIA7) and a MluI site (pMOIA7). In addition the sequence of plasmids pMOA4, pMOA6, pMOAI7, and pMOAI5 was checked by double-strandedideoxy sequencing using Sequenase and the kit supplied by U.S. Biochemical Corp.

Oligonucleotides were produced on an Applied Biosystems DNA synthesizer. The trityl group was left on, and the oligonucleotides were purified on OPC cartridges.

**Biosynthesis of Racaloblated PE and PE Mutants**—PE and PE mutant proteins were metabolically labeled as described (7). Briefly, Escherichia coli BL21 (DE3) was transformed with plasmids encoding PE or PE mutants, and the synthesis of proteins was addressed directly. The addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside when absorbance of the culture at 650 nm was in the range of 0.3–0.4. The bacteria were grown in chemically defined media containing glucose and thiamine. L-[\(^{3} \text{H}\)]Leucine or L-[\(^{35} \text{S}\)]methionine was added to the culture at 30 min after induction with isopropyl 1-thio-β-D-galactopyranoside. For each time point, either 20 μCi of [\(^{3} \text{H}\)]leucine or 10 μCi of [\(^{35} \text{S}\)]methionine was added per ml of culture. After a 90-min induction period, cells were harvested by centrifugation, the periplasm recovered by osmotic lysis, and radiolabeled proteins were purified either by ion exchange chromatography (Mono Q) alone or on ion exchange chromatography plus gel filtration chromatography (TSK-250 BioSili). In some experiments, crude periplasm was added to L929 cells. Since PE comprised 30–40% of the periplasmic protein and PE-related proteins could be recovered from cells by immunoprecipitation, this approach did not give results substantially different from those using purified radioactive proteins. The specific activity of [\(^{3} \text{H}\)]leucine-labeled toxins ranged from 4,000 to 9,000 dpm/ng and of [\(^{35} \text{S}\)]methionine-labeled toxins from 2,000 to 4,500 dpm/ng.

**Toxicity of Mutant PE Molecules**—All mutant forms derived from PE were checked for cellular toxicity. This was done by adding various dilutions of each mutant to L929 cells for 20 h. At the end of this incubation, [\(^{3} \text{H}\)]leucine was added to cells, and the level of protein synthesis was determined. Results were expressed as percent of control protein synthesis compared with cells that received no toxin. Relative activity was determined by comparing the IC50 values of mutant toxins with those of native PE. The activity of mutant toxins was assessed using procedures described previously (24). All mutants had 100% of this activity (data not shown).

**The Addition of \(^{35} \text{S}\)-Methionine-labeled Toxin to Cells**—Radiolabeled PE was added to L929 cells for 2.5 h at 37°C. Cells were then washed and lysed with radiolabeled precipitation buffer. Labeled fragments were recovered by immunoprecipitation and analyzed by SDS-PAGE and autoradiography (7).

**Sequencing of the 37-kDa Radiolabeled Fragment**—Mouse L929 cells (~5 × 10^6, total) were grown to confluence in Dulbecco’s modified Eagles’s medium supplemented with 5% fetal bovine serum and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 units/ml; and thiamine. ~1 [\(^{35} \text{S}\)]Methionine-labeled toxin was added to each plate to lyse the cells. The lysed cells were removed, pooled, and 100 μl of packed protein-A Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) was added to each tube. The mixture was placed on rotator at 4°C for 45 min. The protein-A was removed by centrifugation and discarded. Protein A-Sepharose preloaded with rabbit anti-PE was then mixed with the precleared lysate and incubated at 4°C overnight. The protein-A antibody complex was centrifuged, the pellet was washed twice with 100 μl of Tween/phosphate-buffered saline. Sample buffer was added to the pellet and the sample boiled for 4 min. The sample, which
**RESULTS**

**Strategy to Replace Residues near the Site of Cleavage with Methionine**—Previously we determined that PE is cleaved in an arginine-rich loop near the beginning of domain II to promote a 28-kDa N-terminal fragment (and smaller fragments derived from it) and a 37-kDa C-terminal fragment (7). After proteolytic cleavage, the 37-kDa fragment which contains the ADP-ribosylating activity is separated by reduction and later translocates to the cytosol where it inhibits protein synthesis. Because all methionine residues are located in domain 1a of PE, the 37-kDa fragment does not contain this amino acid (21). To help determine the site of cleavage methionine was substituted for existing residues. Only if methionine residues are substituted on the C-terminal side of the cleavage site will the 37-kDa fragment contain methionine. To use this strategy to determine the site of cleavage, radiolabeled PE mutants were generated by growing *E. coli* in chemically defined media containing [35S]methionine (see below).

**Construction of Methionine Replacement Mutants**—With the exception of arginine residues at 276 and 279, previously shown to be essential for toxicity (25), all amino acids from histidine 275 to glutamic acid 282 were individually replaced with methionine. PEmet275, PEmet277, PEmet281, and PEmet282 were made by site-directed mutagenesis (Fig. 1, B and C). Plasmids were constructed as outlined under “Materials and Methods” and their composition confirmed by dideoxy sequencing.

**Bioactivity of Methionine Mutants**—Since proteolytic processing of PE is required for toxicity and these substitutions were near the cleavage site, it was first necessary to determine if any of these substitutions altered the cytotoxic activity and/or processing of the toxin. Plasmids encoding each methionine substitution were transformed into BL21(XDE3) and the mutant proteins were expressed. These toxins were added to L929 cells and their activity determined by measuring inhibition of protein synthesis (Table I). Two substitutions showed reduced toxicity, the others had apparent wild type activity. The substitution of methionine for either proline 278 or tryptophan 281 reduced toxicity by 100-fold. Possible reasons for loss of activity are discussed below.

To examine the cellular processing of the various mutant forms, toxins were radiolabeled metabolically with [3H]leucine and added to L929 cells for 2.5 h. Full sized toxin molecules or processing of the toxin. Plasmids encoding each methionine substitution were transformed into BL21(XDE3) and the mutant proteins were expressed. These toxins were added to L929 cells and their activity determined by measuring inhibition of protein synthesis (Table I). Two substitutions showed reduced toxicity, the others had apparent wild type activity. The substitution of methionine for either proline 278 or tryptophan 281 reduced toxicity by 100-fold. Possible reasons for loss of activity are discussed below.

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A representative experiment is shown in Fig. 2. Routinely, the substitution of methionine for proline 278 showed slightly reduced processing, whereas the substitution of methionine for tryptophan 281 showed slightly enhanced processing.

Recovery of [35S]-labeled PE fragments locates cleavage site between Pro279 and Gly280. To determine the site of cleavage, [35S]-methylmethionine-labeled PE mutant proteins were incubated with cells for 2.5 h. Full-length toxin and fragments were recovered by immunoprecipitation and analyzed by SDS-PAGE and autoradiography. Results indicated that when histidine 275, glutamine 277, or proline 278 was replaced the 28-kDa fragment and smaller fragments derived from it were recovered as a radiolabeled species, but the 37-kDa fragment was not (Fig. 3). However, when glycine 280, tryptophan 281, or glutamic acid 282 was replaced by methionine, both 37- and 28-kDa fragments contained the radiolabel. These results indicate that amino acids 275–282 are on the C-terminal side, whereas amino acids 280–282 are on the C-terminal side. Thus the toxin was cleaved between proline 278 and glycine 280. This analysis could not distinguish between a cleavage that occurred between proline 278 and glycine 280. Since arginine 279 was shown previously to be essential for cleavage at this site, this amino acid was not replaced with methionine.

Fig. 3. Recovery of 35S-labeled PE mutants and fragments from cells. [35S]-Methylmethionine-labeled PE was added to L929 cells for 2.5 h and then immunoprecipitated using a rabbit anti-PE antibody. Samples were analyzed by SDS-PAGE and fluorography.

The composition of the active fragment derived from PE, which translocates to the cytosol begins with the amino acids GWEQLGQVYQRL etc.

DISCUSSION

The composition of the active fragment derived from PE, which translocates to the cytosol and ADP-ribosylates elon-

![Table 1](image)

| Toxin          | Amino Acids | Processing | Toxicity |
|---------------|-------------|------------|----------|
| PE            | TRHOPRGEQL | +++        | 100      |
| PE-met275     | M           | +++        | 100      |
| PE-gly276     | G           | -          | <0.03    |
| PE-gly277     | M           | +++        | 100      |
| PE-gly279     | G           | -          | <0.03    |
| PE-met280     | M           | ++         | 100      |
| PE-met281     | M           | ++++       | 100      |
| PE-met282     | M           | +++        | 100      |

Toxicity, which was determined by measuring the level of inhibition of protein synthesis, is expressed as a percentage of that seen when native PE was added to L929 cells. 

It was shown previously that these two mutations reduced toxicity to very low levels (25).
and then transferred to Immobilon membranes and fluorographed. The location of the 37-kDa fragment which was cut out of the membrane was obtained after immunoprecipitation, transfer to polyvinylidene difluoride membranes, Edman degradation of eluted sample, and detection of radioactivity in each of the fractions. These sensitive techniques, although by themselves are not new, are important for the toxicity of PE. The reasons why these mutations reduced toxin activity are unknown at the present but include the possibilities that they interfered with a step after cleavage or they changed the structure of the loop to cause cleavage at another amino acid. Since the arginine-rich loop resides between two prominent α-helices (11), proline at 278 may play an important role in future toxin research since only a few toxin molecules are usually needed to produce a biological effect.

In addition to revealing the site of cleavage, the methionine substitutions indicated that proline 278 and tryptophan 281 are important for the toxicity of PE. The reasons why these mutations reduced toxin activity are unknown at the present but include the possibilities that they interfered with a step after cleavage or they changed the structure of the loop to cause cleavage at another amino acid. Since the arginine-rich loop resides between two prominent α-helices (11), proline at 278 may play an important role in future toxin research since only a few toxin molecules are usually needed to produce a biological effect.

The results obtained in this study would not have been possible without the production of metabolically labeled toxin to high specific activity. The addition of 35S-labeled PEmet280–282 to cells generated radiolabeled 37-kDa fragments with strong enough signals to detect by radioautography despite the fact that there was only 1 methionine/fragment available for labeling. The addition of [3H]PE to approximately 5 × 10^6 cells for 6 h at a concentration of 1 µg/ml (120 µg, total) generated a radiolabeled 37-kDa fragment. However, only 10–20 ng of the 37-kDa fragment was recovered from 5 × 10^6 cells. Sequence information could only be obtained after immunoprecipitation, transfer to polyvinylidene difluoride membranes, Edman degradation of eluted sample, and detection of radioactivity in each of the fractions. These sensitive techniques, although by themselves are not new, may play an important role in future toxin research since only a few toxin molecules are usually needed to produce a biological effect.
and either reduce the amount of proteolysis or change the site of cleavage to one of the other arginines. This possibility is currently under investigation. The replacement of cryptophan with methionine did not interfere with the cleavage of the toxin. However, the resulting 37-kDa fragment is apparently not able to translocate to the cytosol. Preliminary data from recent studies indicate that cryptophan is important for a step in toxin action that occurs after proteolysis and before ADP-riboseylation in the cytosol. However, not every amino acid at the N terminus of the 37-kDa fragment is important for toxicity since PEmet280 and PEmet282 had apparent wild type activity.

As mentioned, the replacement of glycine 280 with methionine produced a mutant form of PE with full biological activity. Fortuitously, this substitution allows for the production of a recombinant form of 37-kDa fragment that begins with methionine (in place of glycine 280) and then continues with cryptophan and the other amino acids that make up the 37-kDa fragment (see 28). The production of large quantities of this active fragment will allow for the development of in vitro assays for toxin translocation that would not be possible if the only source of the fragment was the minute amount of protein that can be recovered from toxin-treated cells.

Defining the composition of toxin fragments that translocate to the cytosol has not been widely reported. In part this is because of the lack of sensitive techniques that can recover sufficient material for analysis. Also, other toxins tend to have their enzymatic domains at the N terminus of the protein. Thus, the critical amino acids for analysis are at the C terminus of the fragment, and C-terminal sequencing is difficult and inefficient. One report that tries to identify directly the composition of an active fragment is by Olsnes et al. (17) in which they show that only one of three possible cleavage products in the arginine-rich loop of DT can insert into membranes. Murphy and colleagues (18) have identified 1 of 3 arginine residues that is important for the activity of a chimeric toxin between DT and interleukin-2. Their analysis showed that proteolytic processing is needed for toxicity and that arginine at 193 is essential for the action of the intact fusion toxin.

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