Purification of Anthrax Edema Factor from *Escherichia coli* and Identification of Residues Required for Binding to Anthrax Protective Antigen

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The structural gene for anthrax edema factor (EF) was expressed in *Escherichia coli* under the control of a powerful T5 promoter to yield the 89-kDa recombinant protein that reacted with anti-EF antibodies. Recombinant EF was purified to homogeneity by a two-step procedure involving metal chelate affinity chromatography and cation-exchange chromatography. From 1 liter of culture, 2.5 mg of biologically active EF was easily purified. This is the first report of purification of anthrax EF from *E. coli*. EF purified from *E. coli* was biologically and functionally active as its *Bacillus anthracis* counterpart. The recombinant protein could compete with lethal factor for binding to protective antigen. Sequence analysis revealed a stretch of seven amino acids, Val Tyr Tyr Glu Ile Gly Lys, present both in EF (residues 136 to 142) and lethal factor (residues 147 to 153). To investigate the role of these seven residues in binding to protective antigen, the residues were individually mutated to alanine in EF. Mutations in residues Tyr137, Tyr138, Ile140, and Lys142 of EF specifically blocked its interaction with anthrax protective antigen. The adenylyl cyclase activity of the mutants remained unaffected. The results suggested that residues Tyr137, Tyr138, Ile140, and Lys142 are required for binding of EF to anthrax protective antigen, which facilitates its entry into susceptible cells.

*Bacillus anthracis*, the causative agent of anthrax, is a highly pathogenic bacterium. It primarily affects animals. Humans acquire the disease via infected animals or contaminated animal products. Two major virulence factors of this bacterial pathogen are antiphagocytic poly-d-glutamic acid capsule (8) and the three-component protein exotoxin called the anthrax toxin complex (17). The three proteins that form the complex are protective antigen (PA; 83 kDa; 735 amino acids), edema factor (EF; 89 kDa; 767 amino acids), and lethal factor (LF; 90 kDa; 776 amino acids). Individually, all three proteins are nontoxic. However, a combination of PA and LF, called the lethal toxin, causes death in experimental animals (24) and lysis of mouse peritoneal macrophages and macrophage-like cell lines (1, 3, 6, 7), whereas a combination of PA and EF, known as the edema toxin, induces an increase in intracellular cyclic AMP (cAMP) levels in eukaryotic cells (16) and elicits skin edema after subcutaneous injection (25).

Anthrax toxin fits the A-B model of classification of toxins, according to which the A (activity) moiety and the B (binding) moiety reside on different proteins that interact during intoxication of cells. Anthrax toxin utilizes a single B moiety (PA) to deliver one of the alternative A moieties (EF or LF) into the cell cytosol (17). PA binds to cell surface receptors and is cleaved by a cell surface protease such as furin (15). Proteolysis releases an N-terminal 20-kDa fragment, PA$_{20}$, from the cell surface and exposes a high-affinity binding site on the 63-kDa fragment, PA$_{63}$, still bound to the receptor. PA$_{63}$ then binds to EF or LF, and the entire complex undergoes receptor-mediated endocytosis (6). Acidification of the endosome results in insertion of PA$_{63}$ into the endosomal membrane and translocation of EF and LF into the cytosol, where they exert their toxic effects. It appears that ion-conductive channels, formed upon oligomerization of PA$_{63}$ fragments, facilitate the translocation process (19).

EF is a bacterial adenylyl cyclase which, upon activation by its eukaryotic cofactor, calmodulin, causes a rapid increase in the intracellular cAMP levels of host cells (16). Anthrax edema toxin can differentially regulate lipopolysaccharide-induced production of tumor necrosis factor alpha and interleukin-6 by increasing intracellular cAMP in monocytes (12). The disruption of the cytokine network may contribute to clinical symptoms of anthrax, such as edema formation.

The genes for PA, LF, and EF (called *pagA*, *lef*, and *cya*, respectively) reside on a 185-kb plasmid, pXO1, of *B. anthracis*. Strains lacking pXO1 do not produce toxin and are essentially avirulent (13). All three genes have been cloned and sequenced (4, 5, 21, 26). Efforts have been made to express and purify PA and LF from other expression hosts, such as *Bacillus subtilis* (14) and *Escherichia coli*. Large amounts of biologically active PA and LF can now be obtained from *E. coli* with relative ease (10, 11). Unfortunately, attempts have not been made to overexpress and purify PA and LF from other expression hosts. Therefore, culture supernatant of *B. anthracis* remains by and large the major source for the purification of EF (18, 20). Purification of EF from *B. anthracis* cultures requires P-3 containment facilities. Moreover, *B. anthracis* cultures give poor yields of EF that are often contaminated with other proteins. This has discouraged researchers from undertaking studies of EF (23, 27).

The aim of the present study was to develop a rapid and efficient system for purification of EF. EF was expressed in *E. coli* as a six-histidine-tagged protein and was purified to homogeneity using a two-step procedure involving metal chelate affinity chromatography and cation-exchange chromatography. Further, we have used this system for cloning, expres-
sion, and purification of mutant proteins of anthrax EF to investigate the role of a stretch of seven amino acids (9), Val Tyr Gln Ile Gly Lys, that is present both in EF (residues 136 to 142) and LF (residues 147 to 153).

**Plasmid construction.** The full-length structural gene for EF was amplified by PCR using pXO1 as a template and primers that added BamHI and KpnI sites to the 5’ and 3’ ends of the PCR product, respectively. The sequences of the forward and reverse primers were 5’ GAT GCC GCG CAT TGA ATG AAC ATT ACA CTG AGA G 3 and 5’ GAT GCC GGG GTA CCT TAT TTT TCA TCA ATA ATT TTT TGG 3’, respectively. The amplified PCR product was digested with the restriction enzymes BamHI and KpnI and then ligated to the BamHI- and KpnI-digested plasmid pQE30 (Qiagen) to generate the construct pPN-EF. Cloning of the gene was verified by restriction analysis and sequencing (22). The construct pPN-EF has a six-histidine coding sequence at the 5’ end of the gene. The expression of the EF gene is under the control of a powerful T5 promoter. There are two lac operator sequences, which in combination with the lac repressor protein ensure tight regulation of gene expression.

**Expression and purification of EF.** For high-level expression of the gene, pPN-EF was transformed into *E. coli* SG13009 (pREP4) competent cells. Cells bearing pPN-EF were grown at 37°C and 250 rpm in Luria broth containing 100 μg of ampicillin and 25 μg of kanamycin per ml. When the *A_600* reached 0.8, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After 4 h of induction, the cells were harvested by centrifugation. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of these cells showed EF migrating at 89 kDa. The protein reacted with rabbit polyclonal anti-EF antibodies on immunoblots. The periplasm, cytosol, and inclusion bodies were checked for the presence of EF, which was found to be mainly localized in the cytosol.

For the purification of EF from the cytosol of SG13009 cells, all procedures were performed at 4°C. The pellet obtained from 1 liter of culture was resuspended in 30 ml of sonication buffer (50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). Lysozyme (1 mg/ml) was added, and the cell suspension was incubated on ice for 30 min. The cells were disrupted by sonication at 4°C. Cell debris was removed by centrifugation, and the supernatant was mixed with 5 ml of 50% Ni-nitritolriacetic acid resin previously equilibrated with sonication buffer. The matrix was washed with the sonication buffer containing 20 mM imidazole until the *A_{280} of the flowthrough was less than 0.01. The protein was then eluted with 300 mM imidazole chloride in elution buffer (20 mM potassium phosphate, pH 7.0, 50 mM NaCl, 1% glycerol, 2 mM β-mercaptoethanol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). Affinity chromatography resulted in 456-fold purification of EF (calculated by dividing the specific activity of the protein eluting from the column by that obtained for the total cell lysate).

For further purification of EF, the fractions containing EF were pooled and dialyzed against binding buffer (20 mM potassium phosphate, pH 7.0, 1% glycerol, 2 mM β-mercaptoethanol, and 1 mM EDTA) to remove imidazole and NaCl. The dialyzed sample was loaded onto an SP-Sepharose (Sig-
However, beyond a saturating dose of EF (1 μg/ml), no further increase in cAMP was observed. PA or EF alone can neither increase cAMP nor induce morphological changes in CHO cells. EF purified from E. coli and B. anthracis was found to be equally potent in generating cAMP response in cultured cells (data not shown).

**Competition with LF.** EF or LF binds with high affinity to the site exposed on domain 1 of PA upon cleavage with proteases, such as trypsin and furin. It is expected that the binding of the alternate activity moiety to this site would make the site unavailable for the other moiety. This competition between LF and E. coli-purified EF for the binding site of PA was demonstrated on both J774A.1 and CHO cells.

CHO cells were incubated with edema toxin in the presence and absence of LF. The cAMP response of CHO cells to edema toxin was found to decrease when the dose of LF was increased (Table 1). A 10-fold excess of LF over recombinant EF effectively reduces intracellular cAMP to basal levels.

Intoxication of J774A.1 cells by lethal toxin was similarly affected by the presence of recombinant EF. Complete cell death occurs within 3 h when J774A.1 cells are incubated with lethal toxin (1 μg [each] of PA and LF/ml). However, in the presence of EF, J774A.1 cells are protected against the cytotoxicity of the lethal toxin (Table 2). Complete protection is achieved when the concentration of EF is 10 times that of LF.

**Role of residues 136-Val Tyr Tyr Glu Ile Gly Lys-142.** To explain the competition between EF and LF for the binding site on PA, the sequences of the two proteins were compared. The N-terminal 300 amino acids of EF and LF have significant homology and are considered to be the domains that bind to PAαβ. The most notable feature of this region of homology is a stretch of seven amino acids, Val Tyr Tyr Glu Ile Gly Lys, which is present in EF (residues 136 to 142) as well as LF (residues 147 to 153). To investigate the roles of these amino acids in binding to PAαβ, each of the seven amino acids was individually replaced with Ala. Stragatena’s Quikchange site-directed mutagenesis kit was used for introducing mutations. pPN-EF was used as the template for PCR with primers that contained the desired mutation. Seven mutant constructs were made. Sequencing of the mutant constructs confirmed that only specific mutations were introduced (V136A, Y137A, Y138A, E139A, I140A, G141A, and K142A). The constructs were then transformed into E. coli SG13009(pREP4) competent cells for expression of the mutant protein. The EF mutants were purified to homogeneity as detailed before for the wild-type EF.

To evaluate the biological activities of the mutant proteins, the cAMP responses generated by the mutants in CHO cells were studied. Mutants Y137A, Y138A, I140A, and K142A (when added along with PA) failed to elicit an elongation response in CHO cells, unlike wild-type EF or the other three mutants, V136A, E139A, and G141A. Quantitative estimation of the intracellular cAMP of the treated cells revealed that the abilities of the mutant proteins Y137A, Y138A, I140A, and K142A to increase intracellular cAMP levels were impaired (Fig. 1). The cAMP response elicited by the mutant E139A in CHO cells was also slightly reduced in comparison to that generated by the wild-type protein.

Next, the enzymatic activities of the mutant proteins were determined. It was observed that the adenylate cyclase activities of all of the mutant proteins were comparable to that of the wild-type EF. This suggested that the impaired cAMP response generated in CHO cells by the mutants Y137A, Y138A, I140A, and K142A was not due to reduced adenylate cyclase activity but was due to a defect in their ability to enter the cells.

To gain entry into susceptible cells, EF binds to receptor-bound PA. To study the interaction of EF mutant proteins with PA, the mutant proteins were allowed to compete with radiolabeled wild-type EF for binding to receptor-bound PA. The results from the competition assay showed that cold wild-type EF, as well as the mutants V136A, E139A, and G141A, was able to compete with radiolabeled wild-type EF and inhibit its binding to receptor-bound PA. However, the mutants Y137A, Y138A, I140A, and K142A could not compete with the radiolabeled wild-type protein in binding to receptor-bound PA (Fig. 2). This suggested that the mutants Y137A, Y138A, I140A, and K142A were defective in the ability to bind PA.

Similar results were obtained in vitro when the mutant proteins were allowed to bind to PA in solution. Mutants V136A, E139A, and G141A were able to bind to trypsin-digested PA.

### Table 1. cAMP response in CHO cells treated with anthrax toxin proteins

<table>
<thead>
<tr>
<th>Amt of toxin protein added (μg/ml)</th>
<th>Intracellular cAMP in CHO cells (pmol/mg of CHO protein)</th>
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<tbody>
<tr>
<td>PA</td>
<td>LF</td>
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<tr>
<td>0</td>
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<td>1</td>
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<td>1.5</td>
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* CHO cells plated in a 96-well tissue culture plate were treated with the indicated amounts of toxin components. After 2 h, intracellular cAMP was measured in the cultured cells. The average protein content of CHO cells per well was 7 μg.

* The values are representative of three experiments done in triplicate, with standard errors of less than 5%.

### Table 2. Cytotoxicity produced in J774A.1 cells in response to anthrax toxin proteins

<table>
<thead>
<tr>
<th>Amt of toxin protein added (μg/ml)</th>
<th>% Viability of J774A.1 cells</th>
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<tbody>
<tr>
<td>PA</td>
<td>LF</td>
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<tr>
<td>1</td>
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* J774A.1 cells plated in 96-well plates were treated with the indicated amounts of toxin proteins. After 3 h of incubation, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake, as described previously (2).

* The values are representative of three experiments done in triplicate, with standard errors of less than 5%.
and retard its mobility on native polyacrylamide gel electrophoresis. However, mutants Y137A, Y138A, I140A, and K142A did not bind to trypsin-digested PA in solution (Fig. 3). Thus, it was concluded that the residues Tyr137, Tyr138, Ile140, and Lys142 are required by EF for binding to anthrax PA, which facilitates its entry into susceptible cells.

To summarize, a system for expression and purification of \textit{B. anthracis} EF has been presented above. The procedure does not involve handling of the pathogenic bacteria, since the expression host is \textit{E. coli}. From 1 liter of culture, 2.5 mg of purified EF can easily be obtained, which is a significant improvement over the yields (0.4 to 0.8 mg/liter) obtained from \textit{B. anthracis} cultures. The recombinant protein is purified to homogeneity. Furthermore, we have demonstrated that EF purified from \textit{E. coli} is biologically and functionally comparable to its \textit{B. anthracis} counterpart. The rapidity of the procedure (just two chromatographic steps) ensures minimal loss of biological activity. The high reproducibility and simplicity of the method make it a convenient system to adopt routinely.

EF merits study not just because it is an important virulence factor of \textit{B. anthracis} but also because it is the only toxin known so far which enters susceptible cells through receptor-mediated endocytosis, where it displays intrinsic adenylate cyclase activity. \textit{Bordetella pertussis} adenylate cyclase is another toxin with inherent adenylate cyclase activity, but unlike its \textit{B. anthracis} counterpart, it enters cells through direct penetration of the plasma membrane. Most other toxins increase intracellular cAMP levels by modulating the adenylate cyclase activity of the host cell.

Edema toxin provides an interesting model to study how cAMP regulates basic cellular and metabolic processes. It is through its ability to influence intracellular cAMP levels that edema toxin modulates cytokine synthesis in the infected host (11). Disruption of cytokine networks impairs the host’s ability to defend against the invading bacteria and contributes to clinical symptoms of anthrax, such as edema formation.

Hitherto, biochemical investigations of the structure-function relationship of anthrax EF were hampered by the difficulty of purifying protein free from the other two toxin components. The availability of homogeneous protein preparations will trig-
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FIG. 3. Binding of EF mutants to PA in solution. Wild-type EF (EFwt) or its mutants (1 μg) were allowed to incubate with trypsin-nicked PA (1 μg) for 15 min, and the samples were analyzed on a nondenaturing 5 to 10% gradient gel. The gel was stained with silver stain.