Cleavage of a C-terminal Peptide Is Essential for Heptamerization of Clostridium perfringens ε-Toxin in the Synaptosomal Membrane*

Received for publication, December 20, 2000, and in revised form, January 23, 2001
Published, JBC Papers in Press, February 1, 2001, DOI 10.1074/jbc.M011527200

Shigeru Miyata‡, Osamu Matsushita‡, Junzaburo Minami§, Seichi Katayama‡,
Seiko Shimamoto‡, and Akinobu Okabe‡§

From the ‡Department of Microbiology, Faculty of Medicine, Kagawa Medical University, 1750-1 Miki-cho, Kita-gun, Kagawa 761-0793 and the §Department of Medical Technology, Kagawa Prefectural College of Health Sciences, 281-1 Mure-cho, Kita-gun, Kagawa 761-0123, Japan

Activation of Clostridium perfringens ε-protoxin by tryptic digestion is accompanied by removal of the 13 N-terminal and 22 C-terminal amino acid residues. In this study, we examined the toxicity of four constructs: an ε-protoxin derivative (PD), in which a factor Xa cleavage site was generated at the C-terminal trypsin-sensitive site; PD without the 13 N-terminal residues (ΔN-PD); PD without the 23 C-terminal residues (ΔC-PD); and PD without either the N- or C-terminal residues (ΔNΔC-PD). A mouse lethality test showed that ΔN-PD was inactive, as is PD, whereas ΔC-PD and ΔNΔC-PD were equally active. ΔC-PD and ΔNΔC-PD, but not the other constructs formed a large SDS-resistant complex in rat synaptosomal membranes as demonstrated by SDS-polyacrylamide gel electrophoresis. When ΔNΔC-PD and ΔC-PD, both labeled with 32P and mixed in various ratios, were incubated with membranes, eight distinct high molecular weight bands corresponding to six hetropolymers and two homopolymers were detected on a SDS-polyacrylamide gel, indicating the active toxin forms a heptameric complex. These results indicate that C-terminal processing is responsible for activation of the toxin and that it is essential for its heptamerization within the membrane.

ε-Toxin produced by Clostridium perfringens types B and D is the third most potent clostridial toxin after botulinum and tetanus toxins, and is responsible for the pathogenesis of fatal enterotoxemia in domestic animals caused by the organisms (1). This toxin exhibits toxicity toward neuronal cells via the glutamatergic system (2, 3) or extravesication in the brain (4) after infection of experimental animals. It has been suggested to be a pore-forming toxin based on the following observations. (i) ε-Toxin can form a large complex in the membrane of MDCK cells, and it permeabilizes them (5, 6); (ii) the large complex formed by ε-toxin is not dissociated by SDS treatment (6), which is a common feature of pore-forming toxins such as α-toxins (7), Clostridium septicum ε-toxin (8), and Pseudomonas aeruginosa cytotoxin (9); and (iii) the CD spectrum of ε-toxin shows it mainly consists of β-sheets (10), as is characteristic of pore-forming β-barrel toxins.

The structures of many bacterial pore-forming toxins or toxin components such as perfringolysin O (11), Bacillus thuringiensis δ-toxin (12), aerolysin (13), staphylococcal α-toxin (14), and protective antigen of anthrax toxin (15) have been determined. These pore-forming toxins are believed to undergo a drastic conformational change upon interaction with a membrane. Since these toxins are inserted into the cytoplasmic membrane without the aid of other proteins such as chaperones or the translocation machinery, characterization of their metabolism has been regarded as a novel means for studying membrane-protein interactions (16). A characteristic feature of ε-toxin is its potent neurotoxicity, which is not seen for the structurally well defined pore-forming toxins. Thus, it could serve as a useful tool for extending our knowledge of how proteins gain entry into a membrane. Another characteristic feature of ε-toxin is that activation of the inactive precursor form (ε-protoxin) by proteases such as trypsin, chymotrypsin (17), and α-protease (18, 19) is accompanied by removal of both N- and C-terminal peptides. In a previous study, we determined the N/C termini of ε-toxin activated by trypsin, trypsin plus chymotrypsin, and α-protease to be Lys-14/Lys-274, Lys-14/Tyr-267, and Met-11/Tyr-267, respectively (19).

This study aimed to answer the following questions. (i) Which peptide(s), i.e. the N-, or C-, or both terminal peptides, regulates the activity of ε-toxin; (ii) can the activated toxin form a large complex in the rat synaptosomal membrane; and (iii) how many toxin monomers are present in the membrane complex?

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—Escherichia coli NovaBlue (Novagen, Madison, WI) was used for the construction of all recombinant plasmids. DNA fragments encoding ε-protoxin and an N-terminally truncated form of it were obtained by PCR amplification using total DNA from C. perfringens type B NCIB 10691 (19) as the template and the following pairs of synthetic primers: 5'-GGCCAAGGAAATATCTA-TACAGCTATCTTAATGAA-3' (etx 1F primer) and 5'-GAATTCTTATTATTTTATTCCGTGTCCTATGATAAAG-3' (etx 1R primer) for ε-toxin (Lys-1 to Lys-296); and 5'-GGCCAAAGGTCTTATGATAAATGATA-GTACATT-3' (etx 2F primer) and 5'-GAAATCTTATTATTTTATTCCGTGTCCTATGATAAAG-3' (etx 2R primer) for ΔN-ε-protoxin (Lys-14 to Lys-296). These DNA fragments were cloned into the pT7Blue T-vector (Novagen), and then inserted into the MscI and EcoRI sites of pET22b (+) (Novagen) so that recombinant toxins could be directed to the periplasmic space of E. coli by a signal peptide encoded in the vector. The resultant plasmids, which expressed ε-protoxin and ΔN-ε-protoxin, were designated as pEP1 and pEN1, respectively.

To construct an ε-protoxin derivative (PD) and an N-terminally truncated form of it (ΔN-PD), in which a factor Xa recognition sequence,
IEGR, was inserted between Lys-273 and Lys-274 of ε-protoxin, sequential PCR amplification was carried out using pEP1 as the template and the following pairs of primers: 5’-TAATCGACTCTACTAATTGGG-3’ (T7 primer) and 5’-TTATCGACCTCGAGTTATTTACCTAGCTAT- ATATTGCTG-3’ (pBluescript II KS+). The resultant plasmids, sequence corresponding to IEGR and upstream of the signal sequence were digested with 5’-GATATGCTGACGTCGCTAGAGC-3’ and 5’-GATTAATCTGAGGCAGCTGAAGAATG-3’ (sense, sequence corresponding to IEGR is underlined) and then the cultures were grown and induced as described above. The fusion proteins were expressed PD and N-PD, respectively, by cleavage at the factor Xa-sensitive site. Digestion was confirmed by DNA sequencing. 

Expression and Purification of ε-Protoxin Derivatives—Transformants of E. coli BL21(DE3)pLysS (Novagen) carrying plasmids pEP1, pEN1, pEP2, and pEN2 were used to prepare ε-protoxin, N-ε-protoxin, PD, and N-PD, respectively. Each expressed protein was purified to homogeneity using a pentaepitope recognized by the cyclic-AMP-dependent protein kinase (20). The accuracy of all the final DNA constructions was confirmed by DNA sequencing.

Preparation of Rat Brain Synaptosomal Membranes—Fifteen nanograms of each 125I-labeled toxin derivative were added to 2.2 μl of synaptosomal membranes in 12 μl of minimum essential medium and then the cultures were grown for another 3.5 h. The cells were centrifuged at 15,000 × g for 1 h. The supernatant was centrifuged at 100,000 × g for 1 h. The fraction was re-suspended in buffer A containing 0.1 M sucrose, 10 mM Tris-HCl (pH 7.4). The specific activities of [125I]-labeled PD, AN-ε-PD, and ΔN-ε-PD were 430, 630, 540, and 390 kcpm/μg of protein, respectively.

Expression and Purification of ε-Protoxin Derivatives—Transformants of E. coli BL21(DE3)pLysS (Novagen) carrying plasmids pEP1, pEN1, pEP2, and pEN2 were used to prepare ε-protoxin, N-ε-protoxin, PD, and N-PD, respectively. Each expressed protein was purified to homogeneity using a pentaepitope recognized by the cyclic-AMP-dependent protein kinase (20). The accuracy of all the final DNA constructions was confirmed by DNA sequencing.

Preparation of Rat Brain Synaptosomal Membranes—Fifteen nanograms of each 125I-labeled toxin derivative were added to 2.2 μl of synaptosomal membranes in 12 μl of minimum essential medium and then the cultures were grown for another 3.5 h. The cells were centrifuged at 15,000 × g for 1 h. The supernatant was centrifuged at 100,000 × g for 1 h. The fraction was re-suspended in buffer A containing 0.1 M sucrose, 10 mM Tris-HCl (pH 7.4). The specific activities of [125I]-labeled PD, AN-ε-PD, and ΔN-ε-PD were 430, 630, 540, and 390 kcpm/μg of protein, respectively.

Phosphorylation of ε-protoxin derivatives with 32P was performed as follows. Approximately 300 μg of GST-PD or GST-ΔN-PD were loaded onto a glutathione-Sepharose 4B column (bed volume, 100 μl; Amersham Pharmacia Biotech), equilibrated with Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl (pH 7.4)). The specific activities of [32P]-labeled PD, AN-ε-PD, and ΔN-ε-PD were 430, 630, 540, and 390 kcpm/μg of protein, respectively.

Heteromeric polymerization of ε-protoxin derivatives was carried out using [32P]-labeled PD and [32P]-labeled GST-PD and GST-ΔN-PD in various ratios. A total of 20–40 nM of one of the mixed templates, and T7 and T7ter primers for a second PCR amplification. The specific PCR products were digested with AvrII and EcoRI, and then cloned into pET28a and pET28c. The fusion proteins were expressed PD and N-PD, respectively, by cleavage at the factor Xa-sensitive site within the C-terminal portion and also at a nonspecific but sensitive site in a GST linker sequence (see “Results”). Formation of an SDST-resistant Complex in Synaptosomal Membranes—Fifteen nanograms of each [125I]-labeled toxin derivative were added to 2.2 μl of synaptosomal membranes in 12 μl of TBS containing 0.1% BSA. After incubation at 37°C for 90 min, the reaction mixture was centrifuged at 17,000 × g for 5 min. The membrane pellet was washed three times with 200 μl of TBS at 4°C, and then dissolved by SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% β-mercaptoethanol, 1% SDS, 0.01% bromphenol blue) at 95°C for 5 min. Samples were electrophoresed on a SDS-PAGE gel, followed by exposure to an imaging plate (Fuji Photo Film, Kanagawa, Japan) for autoradiography.

Mouse Lethality Test and Cytotoxicity Assay—The lethality of ε-protoxin and its derivatives toward mice were determined as described previously (19). A group of 15 male ddY mice weighing 37 g each were injected intravenously with 0.25 ml of the toxin solution, and deaths occurring within 24 h were recorded.

MDCK cells were cultured in Eagle’s minimum essential medium containing Earle’s salts, penicillin (100 units/ml), and streptomycin (100 μg/ml), supplemented with 10% heat-inactivated fetal bovine ser- rum, in a cell culture incubator (under 5% CO2 at 37°C). Freshly trypsinized cells were cultured in 96-well microculture plates for 48 h to give monolayers. The medium was exchanged for 200 μl of minimum essential medium, followed by the addition of 50 μl of PBS or PBS containing one of the purified ε-protoxin derivatives. After a 6-h incubation, the cells in each well were washed with PBS containing Mg2+ and Ca2+. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-3-carboxyl tetrazolium (Promega, Madison, WI) conversion assay. The absorbance, which is proportional to the number of viable cells, was read at 490 nm using an enzyme-linked immunosorbent assay plate reader. Percentage of cell viability was calculated as follows: the mean absorbance value of a toxin-group/that of a control.
Construction and Characterization of e-Protoxin Derivatives—Both an N-terminal peptide of 13 amino acids and a C-terminal peptide of 22 amino acids are removed upon tryptic activation of e-protoxin. To determine which peptide inactivates e-protoxin, we constructed recombinant e-protoxin derivatives without the N- or C-terminal peptide using an E. coli expression system. The recombinant e-protoxins with and without the N-terminal peptide were inactive but activated by treatment with trypsin plus chymotrypsin, as shown by the mouse lethality test (Table I). The LD50 of their activated forms was 50 ng/kg of body weight, which coincided with the value reported for e-toxin from C. perfringens cultures. When the recombinant e-protoxin without the C-terminal peptide was expressed in E. coli, it precipitated as inclusion bodies, which could be solubilized with 6 M urea, but it could not be recovered in a soluble form after dialysis (data not shown). Thus, we constructed two e-protoxin derivatives, with and without the N-terminal peptide, respectively, in which IEGR, a coagulation factor Xa recognition sequence, was inserted between Lys-273 and Lys-274 (trypsin cleavage site), as shown in Fig. 1A. This construction enabled the recovery of these proteins, i.e. PD and ΔN-PD, from the periplasmic space and also allowed cleavage at the relevant site with factor Xa.

PD and ΔN-PD were purified from E. coli cultures to homogeneity, as demonstrated by SDS-PAGE (Fig. 1B). Another two derivatives, ΔC-PD and ΔNC-PD, were obtained by treatment of the purified PD and ΔN-PD, respectively, with factor Xa (Fig. 1B). The identities of all the constructs were confirmed by nucleotide sequencing of the recombinant plasmids, N-terminal amino acid sequencing (data not shown), and molecular mass determination by MALDI-TOF mass spectrometry (Fig. 1A). CD analysis revealed that all the derivatives gave a negative ellipticity band at 215 nm, indicating a predominance of α-structure. Some minor differences were observed in the CD spectra, which may be due to a minor change in the conformation arising from the insertion of the factor Xa recognition sequence or removal of the N- and/or C-terminal sequence(s). However, there were no remarkable differences in the CD spectra, strongly suggesting neither the insertion nor the removal(s) affects the overall structure of e-protoxin (Fig. 2).

Toxicity of e-Protoxin Derivatives—The toxicity of the e-protoxin derivatives was determined by means of a mouse lethality test (Table I). The LD50 of PD with an insertion of the IEGR sequence was 31,000 ng/kg of body weight, slightly lower than that previously reported for e-protoxin purified from C. perfringens cultures (70,000 ng/kg of body weight). The LD50 of ΔN-PD was the same as that of PD, being higher than that of ΔC-PD by a factor of about 60. The LD50 of ΔNC-PD was almost the same as that of ΔC-PD, although both were slightly higher than that reported for trypsin-activated e-toxin (320 ng/kg). This might be due to the difference in the C-terminal region: ΔNC-PD and ΔC-PD have four extra amino acids after the trypsin cleavage site. To eliminate the possibility that N-terminal processing has an additional activating effect, the toxicities of the four derivatives were determined by means of an in vitro assay method using MDCK cells, which is less sensitive but more accurate than the mouse lethality test (Fig. 3). ΔC-PD and ΔNC-PD exhibited almost the same toxicity toward MDCK cells, and there was no significant difference in the 50% cytotoxicity dose between the two derivatives. No cytotoxicity was detectable for ΔN-PD and PD at the highest concentration (600 ng/ml) used in this study. These results clearly indicate that only cleavage at the C-terminal region is responsible for activation of e-protoxin.

Large Complex Formation in Synaptosomal Membranes—Activated e-toxin has been shown to form an SDS-resistant 174-kDa complex in the membranes of MDCK cells (6), and the complex has also been reported in brain homogenates incubated with the toxin (5). Such a complex may also be formed in synaptosomal membranes, which could be responsible for the neuronal cell death in the hippocampus observed in e-toxin injected mice (2). In order to assess this possibility, membranes were incubated with 125I-labeled toxin derivatives and ana-
cells. PD. When equal amounts of 125I-labeled D fore, the 200- or 180-kDa large complex should consist of five percentage of the value for a control culture. described under “Experimental Procedures,” and is expressed as a sequences. Panel A, e-protoxin; panel B, PD; panel C, ΔN-PD; panel D, ΔC-PD; panel E, ΔNC-PD. lyzed by SDS-PAGE (Fig. 4A). In the samples incubated with 125I-labeled ΔC-PD and ΔNC-PD, large SDS-resistant complexes were detected, with apparent molecular masses of ~200 and 150 kDa, respectively. No SDS-resistant complex was detected in membranes incubated with 125I-labeled PD and ΔN-PD. When equal amounts of 125I-labeled ΔC-PD and unlabeled ΔNC-PD or vice versa were incubated with the membranes, a smeared band corresponding to 200–180-kDa complexes was detected on a SDS-PAGE gel (Fig. 4B), indicating that both active forms are equally capable of forming the complex.

Heptamerization of Active e-Toxin—The molecular masses of ΔC-PD and ΔNC-PD are 31 and 29 kDa, respectively. Therefore, the 200- or 180-kDa large complex should consist of five toxin molecules and an intrinsic membrane protein, or at maximum seven toxin molecules, if they are unprocessed in the membrane. The result of an experiment involving a mixture of the two active forms suggested that the smeared band corresponds to heteromultimers consisting of the two forms in various ratios, and that the number of toxin molecules per complex is independent of the ratio. If this is the case, complexes of different molecular sizes will form, which can be separated to determine the number of toxin molecules in a complex. The heteromeric complexes formed by 125I-labeled toxins were not well separated even with a SDS-PAGE gel system for large polypeptides. To circumvent this problem, we constructed GST fusion proteins containing a protein kinase recognition site (RRXS) at the N termini of the two toxin derivatives (Fig. 5A). These constructs were cleaved by factor Xa not only at the C-terminus specific site but also between the two arginine residues in the RRXSV sequence. Thus, the fusion proteins were purified (Fig. 5B, lanes 1 and 2), phosphorylated, and cleaved with factor Xa (Fig. 5B, lanes 3 and 4). The resultant phospho-rylated ΔC-PD and ΔNC-PD were designated as P-ΔC-PD and P-ΔNC-PD, respectively. The identities of these protein derivatives were verified by N-terminal amino acid sequencing (data not shown), and molecular mass determination by MALDI-TOF mass spectrometry (Fig. 5A). There was no significant difference in mouse lethality between these two constructs, although their lethalities were slightly lower than those of ΔN-PD and ΔNC-PD (Table 1). They also retained the ability to form an SDS-resistant complex in synaptosomal membranes (Fig. 5C). The two 32P-labeled proteins were mixed in appropriate ratios, incubated with membranes, and then electrophoresed on a 5% SDS-PAGE gel in a DNA sequencing gel apparatus (Fig. 5D). Six distinct bands were observed between those of the P-ΔC-PD and P-ΔNC-PD homopolymers, indicating that the large complex contains seven monomers.

Inhibition of Complex Formation by e-Protoxin—The effect of e-protoxin on the ability of ΔC-PD to form an SDS-resistant complex was examined. Complex formation was inhibited in a dose-dependent fashion by e-protoxin and completely blocked...
The kinase recognition sequence is underlined. Calculated from amino acid sequences are given on the arrow. The N-terminal region is indicated by an asterisk. The factor Xa-sensitive site in the N-terminal region is indicated by an arrow. The molecular masses calculated from amino acid sequences are given on the right, and those determined by MALDI-TOF mass spectrometry are given in parentheses. Purification of recombinant GST fusion proteins and their cleavage by factor Xa. The purified proteins (3 μg each) were heated in SDS-sample buffer and then electrophoresed on a 12.5% SDS-PAGE gel, followed by staining with Coomassie Brilliant Blue R. The sizes of the molecular mass markers are indicated on the left. Lane 1, GST-PD; lane 2, GST-ΔN-PD; lane 3, factor Xa-cleaved GST-PD; lane 4, factor Xa-cleaved GST-ΔN-PD. C, formation of a large SDS-resistant complex by P-ΔN-PD and P-ΔNC-PD. Purified GST-PD and GST-ΔN-PD were labeled with 32P and then cleaved with factor Xa. 32P-Labeled P-ΔC-PD and P-ΔNC-PD were purified as described under “Experimental Procedures.” Twenty nanograms of each of these active toxins were incubated with and without synaptosomal membranes (2.2 μg of protein) in 10 μl of TBS. The samples were heated in SDS-sample buffer, electrophoresed on a 4–20% gradient SDS-PAGE gel, and autoradiographed. Lane 1, 32P-labeled P-ΔC-PD; lane 2, 32P-labeled P-ΔNC-PD; lane 3, 32P-labeled P-ΔC-PD with membranes; lane 4, 32P-labeled P-ΔNC-PD with membranes. D, heptamer complex formation through copolymerization of [32P]ΔC-PD and [32P]ΔNC-PD. [32P]ΔC-PD and [32P]ΔNC-PD were mixed in different proportions: 1:0 (lane 1), 2:1 (lane 2), 1:1 (lane 3), 1:2 (lane 4), and 0:1 (lane 5). Each mixture (12 ng of protein) was incubated with synaptosomal membranes (2.2 μg of protein) in 12 μl of TBS. Samples were heated in SDS-sample buffer and separated on a 5% SDS-PAGE gel (800 × 170 × 0.4 mm), followed by autoradiography. The predicted molar ratios of [32P]ΔC-PD to [32P]ΔNC-PD are given on the left.

Fig. 5. Construction and heptamerization of phosphorylated ΔN-PD and ΔNC-PD. A, schematic diagrams of phosphorylated ΔN-PD and ΔNC-PD. The filled, hatched, and dotted boxes are as in Fig. 1A, and the gray box indicates the GST-linker peptide. The protein kinase recognition sequence is underlined, and the phosphorylated residue is indicated by an asterisk. B, purification of recombinant GST fusion proteins and their cleavage by factor Xa. The purified proteins (3 μg each) were heated in SDS-sample buffer and then electrophoresed on a 12.5% SDS-PAGE gel, followed by staining with Coomassie Brilliant Blue R. The sizes of the molecular mass markers are indicated on the left. Lane 1, GST-PD; lane 2, GST-ΔN-PD; lane 3, factor Xa-cleaved GST-PD; lane 4, factor Xa-cleaved GST-ΔN-PD. C, formation of a large SDS-resistant complex by P-ΔN-PD and P-ΔNC-PD. Purified GST-PD and GST-ΔN-PD were labeled with 32P and then cleaved with factor Xa. 32P-Labeled P-ΔC-PD and P-ΔNC-PD were purified as described under “Experimental Procedures.” Twenty nanograms of each of these active toxins were incubated with and without synaptosomal membranes (2.2 μg of protein) in 10 μl of TBS. The samples were heated in SDS-sample buffer, electrophoresed on a 4–20% gradient SDS-PAGE gel, and autoradiographed. Lane 1, 32P-labeled P-ΔC-PD; lane 2, 32P-labeled P-ΔNC-PD; lane 3, 32P-labeled P-ΔC-PD with membranes; lane 4, 32P-labeled P-ΔNC-PD with membranes. D, heptamer complex formation through copolymerization of [32P]ΔC-PD and [32P]ΔNC-PD. [32P]ΔC-PD and [32P]ΔNC-PD were mixed in different proportions: 1:0 (lane 1), 2:1 (lane 2), 1:1 (lane 3), 1:2 (lane 4), and 0:1 (lane 5). Each mixture (12 ng of protein) was incubated with synaptosomal membranes (2.2 μg of protein) in 12 μl of TBS. Samples were heated in SDS-sample buffer and separated on a 5% SDS-PAGE gel (800 × 170 × 0.4 mm), followed by autoradiography. The predicted molar ratios of [32P]ΔC-PD to [32P]ΔNC-PD are given on the left.

by a 10-fold excess of e-toxin (Fig. 6A). Such dose-dependent inhibition by e-toxin was also observed for complex formation by ΔNC-PD (Fig. 6B). The inhibition of SDS-resistant complex formation seems to be due to competition for receptor binding, but it may also involve a subsequent complex formation step. The labeled toxins were each detected as a monomer in the membranes to a similar extent, independent of the amount of e-toxin added. If they represent monomers dissociated from a receptor but associated with the membrane, e-toxin would inhibit complex formation by these monomers.

DISCUSSION

This paper demonstrates that removal of the C- but not the N-terminal peptide is responsible for the activation of e-toxin. In a previous study, we showed that e-toxin is cleaved not only at an N-terminal region but also a C-terminal region by trypsin, chymotrypsin, and a-protease, and that e-toxin activated by such proteases differs in both its N and C termini (19). The different forms also differ in toxicity, with the trypsin plus chymotrypsin-activated e-toxin being the most potent. Since the presence or absence of the N-terminal peptide did not affect toxicity, the potency of e-toxin is likely to be determined solely by the difference in the C-terminal cleavage site. This paper also provides evidence of the ability of active e-toxin to form a large complex in rat synaptosomal membranes. We previously demonstrated that the toxin is neurotoxic to the brains of mice and rats (2, 3). The finding that the toxin forms a large complex in the synaptosomal membrane strongly suggests that the neurotoxicity is due to large complex formation, which also is implicated in membrane permeabilization and cell death of e-toxin-treated MDCK cells.

Aerolysin has been proposed to form heptameric oligomers, based on the results of SDS-PAGE, scanning transmission electron microscopic mass measurement of single oligomers, and image analysis of two-dimensional crystals, which were obtained by reconstitution of purified aerolysin and E. coli phospholipids (13, 25). To solve the problem that low resolution electron crystallography gave artifactual data for other pore-forming toxins, Moniatte et al. (26) determined the molecular masses of oligomers formed in a solution by MALDI-TOF mass spectrometry, providing further evidence of heptamerization. However, analysis of the complex in biological membranes is a prerequisite for proving heptamerization and for elucidating its molecular mechanism, since aerolysin polymerizes at higher concentrations (27). Taking advantage of the fact that the N-terminally processed and unprocessed e-toxins both form the large complex, we have demonstrated that seven monomers are present in the synaptosomal membrane complex. This is the first demonstration of a heptameric toxin complex in a biological membrane, and also supports the validity of the heptamerization suggested for aerolysin and other pore-forming toxins.

The inhibition of SDS-resistant complex formation by e-pro-
toxin shown in this study can be explained simply by competition for receptor binding. Alternatively, ε-protoxin associated with the membrane may exert its inhibitory effect by sequestering active ε-toxin, making it unavailable to assemble into a productive complex. Whichever of these possibilities is true, the results unequivocally show the ability of ε-protoxin to bind to a receptor and its inability to form the productive large complex. Thus, it seems very likely that a receptor binding site is exposed on the surface of ε-protoxin, and that the C-terminal peptide sterically hinders exposure of a site or conformational change required for complex formation.

ε-Protoxin exerts no toxicity unless it encounters proteases such as trypsin, chymotrypsin, and λ-protease. This may imply that the C-terminal peptide functions as an intramolecular chaperone. It should be noted that C-terminally truncated forms of ε-protoxin were produced as inclusion bodies, and that several attempts to obtain active forms through solubilization of the precipitate with urea or guanidine HCl and subsequent terminal propeptide of C. septicum lysin in 7M urea was reversible upon dilution in urea-free buffer. These observations suggest that these C-terminal peptides aid proper folding of toxin molecules. Another likely chaperonic function of the C-terminal peptide is to prevent the active ε-toxin from aggregating in solution. The 5.1-kDa C-terminal propeptide sterically hinders exposure of a site or conformational change required for receptor binding. Alternatively, the toxin shown in this study can be explained simply by competition for receptor binding. Alternatively, ε-protoxin associated with the membrane may exert its inhibitory effect by sequestering active ε-toxin, making it unavailable to assemble into a productive complex. Whichever of these possibilities is true, the results unequivocally show the ability of ε-protoxin to bind to a receptor and its inability to form the productive large complex. Thus, it seems very likely that a receptor binding site is exposed on the surface of ε-protoxin, and that the C-terminal peptide sterically hinders exposure of a site or conformational change required for complex formation.

ε-Protoxin exerts no toxicity unless it encounters proteases such as trypsin, chymotrypsin, and λ-protease. This may imply that the C-terminal peptide functions as an intramolecular chaperone. It should be noted that C-terminally truncated forms of ε-protoxin were produced as inclusion bodies, and that several attempts to obtain active forms through solubilization of the precipitate with urea or guanidine HCl and subsequent terminal propeptide of C. septicum lysin in 7M urea was reversible upon dilution in urea-free buffer. These observations suggest that these C-terminal peptides aid proper folding of toxin molecules. Another likely chaperonic function of the C-terminal peptide is to prevent the active ε-toxin from aggregating in solution. The 5.1-kDa C-terminal propeptide sterically hinders exposure of a site or conformational change required for receptor binding. Alternatively, the toxin shown in this study can be explained simply by competition for receptor binding. Alternatively, ε-protoxin associated with the membrane may exert its inhibitory effect by sequestering active ε-toxin, making it unavailable to assemble into a productive complex. Whichever of these possibilities is true, the results unequivocally show the ability of ε-protoxin to bind to a receptor and its inability to form the productive large complex. Thus, it seems very likely that a receptor binding site is exposed on the surface of ε-protoxin, and that the C-terminal peptide sterically hinders exposure of a site or conformational change required for complex formation.

Acknowledgments—We thank Yuki Taniguchi for excellent technical assistance. We also thank Drs. Hiroshi Tokumitsu, Ryoji Kobayashi (Department of Chemistry, Kagawa Medical University, Kagawa, Japan), Ryuichi Moriyama, Shio Makino (Department of Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan), and David B. Wilson (Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY) for valuable discussions.

REFERENCES

1. Sakurai, J. (1995) Rec. Med. Microbiol. 6, 175–185
2. Miyamoto, O., Minami, J., Terashita, T., Nakamura, T., Masada, T., Nagao, S., Negi, T., Itano, T., and Okabe, A. (1998) Infect. Immun. 66, 2501–2508
3. Miyamoto, O., Sumitani, K., Nakamura, T., Yamagami, S-I., Miyata, S., Itano, T., Negi, T., and Okabe, A. (1999) J. Comp. Pathol. 120, 415–420
4. Nagahama, M., Ochi, S., and Sakurai, J. (1998) J. Nat. Toxins 7, 291–302
5. Petri, L., Gibert, M., Gillet, D., Laurent-Winter, C., Bouquet, P., and Marais, M. R. (1997) J. Bacteriol. 179, 6480–6487
6. Garland, W. J., and Buckley, J. T. (1988) Infect. Immun. 56, 1249–1253
7. Sellman, B. R., and Tweten, R. K. (1997) Mol. Microbiol. 25, 429–440
8. Ohnishi, M., Hayashi, T., and Terawaki, Y. (1998) J. Biol. Chem. 273, 453–458
9. Habeel, A. F., Lee, C. L., and Atassi, M. Z. (1973) Biochim. Biophys. Acta 322, 245–250
10. Rossjohn, J., Feil, S. E., McKinstry, W. J., Tweten, R. K., and Parker, M. W. (1997) Cell 89, 685–692
11. Li, J. D., Carroll, J., and Ellar, D. J. (1991) Nature 353, 815–821
12. Parker, M. W., Buckley, J. T., Postma, J. P., Tucker, A. D., Leonard, K., Pattus, F., and Tseng, D. (1994) Nature 367, 292–295
13. Song, L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J. E. (1996) Science 274, 1859–1866
14. Petosa, C., Collie, R. J., Klimpel, K. R., Leppa, S. H., and Liddington, R. C. (1997) Nature 385, 833–838
15. Lesueur, C., Vecsey-Semenj, B., Abrami, L., Fivaz, M., and Gisou van der Goot, F. (1997) Mol. Membr. Biol. 14, 45–64
16. Hunter, S. E. C., Clarke, I. N., Kelly, D. C., and Titball, R. W. (1992) Infect. Immun. 60, 102–110
17. Okabe, A., Matsushita, O., Katayama, S., Minami, J., and Okabe, A. (1996) Infect. Immun. 64, 230–237
18. Minami, J., Katayama, S., Matsushita, O., Matsushita, C., and Okabe, A. (1997) Microbiol. Immunol. 41, 527–535
19. Kaelin, W. G. J., Krek, W., Sellers, W. R., DeCaprio, J. A., Achtenhous, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., Livingston, D. M., and Flemington, E. K. (1992) Cell 70, 351–364
20. Okabe, A., Matsushita, O., Katayama, S., and Hayashi, H. (1986) Antimicrob. Agents Chemother. 30, 82–87
21. Gray, E. G., and Whitaker, V. P. (1962) J. Nat. Toxins 1, 273, 668–685
22. Fivaz, M., Velluz, M. C., and van der Goot, F. G. (1999) J. Biol. Chem. 274, 36722–36728
23. Kaelin, W. G. J., Krek, W., Sellers, W. R., DeCaprio, J. A., Achtenhous, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., Livingston, D. M., and Flemington, E. K. (1992) Cell 70, 351–364
24. Okabe, A., Matsushita, O., Katayama, S., Minami, J., and Okabe, A. (1998) J. Biol. Chem. 273, 3643–3648
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Wilmsen, H. U., Leonard, K. R., Tichelaar, W., Buckley, J. T., and Pattus, F. M. (1992) EMBO J. 11, 2457–2463
27. Minami, J., Katayama, S., Matsushita, O., Matsushita, C., and Okabe, A. (1997) Microbiol. Immunol. 41, 527–535
28. Lesueur, C., Fritiger, S., Hughes, G., Kellner, R., Pattus, F., and van der Goot, F. G. (1999) J. Biol. Chem. 274, 36722–36728