Purification and Characterization of Procytotoxin of *Pseudomonas aeruginosa*

**DIMER TO MONOMER CONVERSION OF PROTOXIN BY PROTEOLYTIC ACTIVATION**

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CTX produced by *P. aeruginosa* is inactive and requires proteolytic action for activation. Analyses of the ctx gene and an active toxin purified from the trypsin-treated crude extract of *P. aeruginosa* (PACTX) showed that CTX is produced as a precursor (procytotoxin (proCTX)) of 286 amino acids and is activated by proteolytic cleavage at the C terminus (13). The cleavage occurs at the carboxyl side of Arg266 (13). Among the channel-forming toxins, aerolysin from *Aeromonas* spp. (12) and the alpha toxin of *Clostridium septicum* (14, 15) also require proteolytic cleavages at the C-terminal regions for conversion from inactive precursors to active forms. In the case of proaerolysin, the C-terminal region was seen to mask hydrophobic patches of the toxin molecule and to inhibit oligomerization of the toxin on the membrane (12, 16). Concerning proCTX, nothing is known of the function of the C-terminal sequence or what changes occur on the CTX molecule by the C-terminal cleavage.

Using a method developed for the purification of the recombinant CTX, we have now purified proCTX for the first time. Purified proCTX bound to the erythrocyte membrane but did not form the oligomer on the membrane. In circular dichroic experiments, significant changes in the secondary structure were not observed between the active CTX and proCTX. However, a striking difference was found in the oligomeric states of the proteins. We describe here a novel activation mechanism of the pore-forming toxin, involving a dimer-to-monomer conversion.

**EXPERIMENTAL PROCEDURES**

Materials—Trypsin (Type XIII: 1-tosylamido-2-phenylethylchloromethyl ketone-treated), bovine serum albumin, and apomyoglobin were from Sigma. The molecular size standards for SDS-PAGE were obtained from Bio-Rad and New England Biolabs. All columns used for protein purification were purchased from Pharmacia Biotech.

Site-directed Mutagenesis and Overproduction of the Toxins in *Escherichia coli*—A codon for threonine at position 267 of the ctx gene was replaced with a premature stop codon by changing ACA to TAA. The mutagenesis was performed by the gapped duplex DNA method of Kramer and Frits (17) using a Mutan-G system (Takara). The mutagenized ctx gene was inserted into a broad host range expression plasmid, pMMB22 (18), to construct pMMB(DΔ20), using the same strategy as described for pMMB(CTX) for the overproduction of procytotoxin (13).

*E. coli* SM32 was transformed with pMMB(DΔ20) or pMMB(CTX) and grown at 37 °C in Luria-Bertani broth supplemented with 50 μg/ml ampicillin. When cell cultures reached an *A*_{600} of 0.5, isopropyl-1-thio-β-D-galactopyranoside was added at a final concentration of 1 mM, and incubation was continued for 4 h. The cells were collected by centrifugation, washed twice with saline, and stored at −80 °C.

Purification Procedure—All purification procedures were performed at 4 °C, except that fast protein liquid chromatography procedures (Pharmacia Biotech Inc.) were carried out at room temperature.

For purification of Δ20, the cells derived from a 10-liter culture were suspended in 400 ml of Buffer A (50 mM sodium phosphate buffer, pH 7.2) and disrupted by ten 10-s bursts of sonication with 50 s interval...
between each burst at 4 °C, using a tip sonicator (Branson). The cell lysate was then centrifuged at 20,000 g at 4 °C for 30 min. Because overexpressed ΔC20 was aggregated in E. coli, more than 95% of ΔC20 was recovered in the pellet fraction. The pellet was dissolved in 100 ml of Buffer A containing 2 M urea while stirring for 2 h. After removing solubilized materials by centrifugation (20,000 g at 4 °C for 30 min), the soluble fraction was dialyzed four times against a 20-fold volume of Buffer B (Buffer A with 0.2 M NaCl). The dialyze was clarified by centrifugation at 100,000 g for 1 h, and concentrated by ultrafiltration through a YM10 membrane (Amicon). The concentrated preparation was applied to a HiLoad 26/60 Superdex 200 26/60 column equilibrated with Buffer B by fast protein liquid chromatography. Proteins were eluted with the same buffer at a flow rate of 2 ml/min. Fractions with toxic activity were pooled and dialyzed against Buffer C (Buffer A containing 2 M NaCl). The dialyze was applied to a phenyl-Superose HR 10/10 column equilibrated with Buffer C. Proteins were eluted with a linear NaCl gradient (2.0–0 M) at a flow rate of 1 ml/min.

The procedure used for purification of proCTX was the same as that used for ΔC20 up to the step of hydrophobic chromatography, except for two modifications: proCTX was solubilized with 3 M urea, and Buffer A containing 1 M NaCl was used for the gel filtration. After hydrophobic chromatography, fractions containing proCTX were subjected to a Mono Q HR 5/5 column equilibrated with Buffer A. Proteins were eluted with a linear NaCl gradient (0–1.0 M) at a flow rate of 1 ml/min. During the purification, proCTX was monitored by immunoblotting with anti-CTX serum (4).

Purified proteins were stored at −20 °C in Buffer A containing 20% (v/v) glycerol.

Measurement of Cytotoxic Activity and Oligomer Formation—Cytotoxic activities of the toxins were determined by means of a hemolytic activity assay (10). Oligomer formation of toxins on the rat erythrocyte membrane was detected by immunoblotting as described previously (10). Activation of proCTX was done by incubation with trypsin at 37 °C for 60 min at 1:20 trypsin per proCTX (w/w) ratio. The reaction was quenched by adding phenylmethylsulfonyl fluoride (final concentration, 1 mM).

Preparation of Antisera and Affinity Purification of Antibody—Rabbit anti-ΔC20 serum was prepared as described (4). For the specific detection for proCTX, a 9-mer peptide corresponding to the C-terminal sequence of proCTX (LETRVRSAE) with a cysteine residue at the N terminus was synthesized by the Fmoc (9-fluorenlymethoxycarbonyl) strategy using a peptide synthesizer (Applied Biosystems Model 431A). The peptide (0.1 mg) was cross-linked to 0.1 mg of bovine serum albumin with maleimidobenzoyl-N-hydroxysuccinimide ester and used toimmunize a rabbit.

The antibody specific to the C terminus of proCTX was purified by affinity chromatography using 2-fluoro-1-methylpyridinium toluene-4-sulfonate-activated cellulofine (Seikagaku) coupled with the C-terminal peptide. The antibody obtained reacted to proCTX and not to ΔC20.

Immunoadsorption of Toxins with Immobilized Antibody—The affinity purified antibody was immobilized on Affi-Gel 10 (Bio-Rad) in coupling buffer (0.1 M HEPES, pH 8.0). Toxins (0.5 μg) were incubated with 10 μl of the antibody-coupled gel (1.1 μg of IgG/μl gel) in 40 μl of binding buffer (coupling buffer with 0.1 M NaCl) for 1 h at 37 °C. After unbound materials were separated by brief centrifugation, pellets were washed five times with the binding buffer (200 μl). Bound materials were eluted with SDS loading buffer, and the bound and unbound fractions were analyzed by SDS-PAGE. For immunostaining, rabbit anti-ΔC20 serum and peroxidase-conjugated anti-rabbit IgG antibody specific to the Fc region (Promega) were used.

Measurement of Circular Dichroism (CD) spectra—CD spectra of toxins were measured at 25 °C in a Jasco J-600 spectrometer with a quartz cell of 0.2 mm path length. The protein concentration was 150 μg/ml in 20 mM sodium phosphate buffer, pH 7.2. Contents were calculated using the reference described by Yang et al. (19). Estimates of the secondary structure were made using a SSE-338 program (Jasco).

Measurement of Tryptophan Fluorescence—Fluorescence measurements were made with a Hitachi fluorimeter F3000 with a quartz cuvette. The excitation wavelength was 290 nm, and the range of emission wavelengths was from 300 to 400 nm. The protein concentration was adjusted to 0.6 μM in Buffer B.

Mass Spectrometry—Samples for spectrometry were prepared according to Nakaniishi et al. (20). Briefly, 33 pmol of toxins in 10 μl of Buffer A was incubated with an equal volume of anti ΔC20 serum for 3 h at room temperature, the samples were centrifuged, and the pellets were washed once with 0.6 ml of 0.9% NaCl and twice with 0.6 ml distilled water and lyophilized. Pellets were dissolved in 3.3 μl of distilled water and mixed with an equal volume of saturated sinapinic acid matrix solution in 33% (v/v) acetonitrile/distilled water. Mass measurements were made on a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Voyager Elite XL, PerSeptive Biosystems) according to the manufacturer's instructions. Bovine serum albumin and apomyoglobin were used as standards.

Analytical Gel Filtration Analysis—Analytical gel filtration was performed using a Superox T12 HR 16/60 column equilibrated with Buffer B. Two hundred μl of toxin solution (150 μg/ml) was applied to the column and then eluted at a flow rate of 0.5 ml/min. Molecular weight standards were used: ovotransferrin (76,000–78,000), ovalbumin (45,000), carbonic anhydrase (30,000), and myoglobin (17,200).

Cross-linking—Purified proteins (50 μg/ml in 50 mM triethanolamine, pH 8.0) were incubated with 2.5 mM or 10 mM dimethyl suberimidate for 10 min at 37 °C. Reaction was quenched by incubation with Tris-HCl, pH 8.0 (final concentration, 100 mM), and then SDS loading buffer was added. Aliquots of the reaction mixture were boiled and analyzed by SDS-PAGE.

Analytical Ultrafiltration—Analytical ultrafiltration was carried out using a Beckman Optima XL-A ultraconcentrator equipped with an optical scanning detector. Proteins in Buffer B at a concentration of 150 μg/ml were analyzed. Data were collected in a Beckman An-60Ti rotor with double sector charcoal-filled Epon cells and quartz windows.

Centrifugation velocity run was carried out at 50,000 rpm at 25 °C. Boundaries were recorded at 280 nm. Apparent sedimentation coefficients were standardized to water at 20 °C.

For sedimentation equilibrium experiments, samples were brought to equilibrium at 4 °C for 20 h at 24,000 rpm. Partial specific volumes for proCTX and ΔC20 were calculated for amino acids composition, using data for specific volumes of amino acids as described by Edsall (21). Other Methods—N-terminal amino acid sequences of the purified proteins were determined by Edman degradation using an automated protein sequencer (Applied Biosystems Model 476A). SDS-PAGE was performed as described by Laemmli (22). Proteins were stained with Coomassie Brilliant Blue R-250. Immunoblotting was done as described previously (13). Protein concentrations were assessed according to Lowry et al. (23) for unpurified materials and by absorbance at 280 nm for purified preparations. Absorption coefficients of ΔC20 and proCTX (2.24 and 2.32, respectively) were determined from amino acid compositions of the proteins.
shown for PACTX (10). ΔC20 formed an oligomer of 145 kDa on the rat erythrocyte membrane in a manner correlating with the hemolytic activity (Figs. 2 and 3). The N-terminal sequence of the purified ΔC20 was identical to the sequence deduced from the ctx gene (MNDID) and a mass of ΔC20 assessed by mass spectrometry was 29,341 ± 2 Da, in agreement with a calculated value of 29,367.1 Da. In addition, ΔC20 showed a marked resistance to various proteases, including trypsin, as observed for PACTX (data not shown). Thus, the purified ΔC20 had the same biological and physicochemical properties as PACTX and degradation on the toxin molecules was negligible during the purification process.

Purification of proCTX—We applied the method developed here to purify proCTX because this approach needed no activation procedure. ProCTX produced in E. coli was also insoluble and could be solubilized with urea. The optimal concentration of urea for solubilization of proCTX was 3 M. Solubilized proCTX was purified to apparent homogeneity by the procedure used for the purification of ΔC20 (Fig. 1B), although the extra step of anion exchange chromatography was necessary. From 10 liters of culture, 5.5 mg of proCTX was obtained.

Purified proCTX migrated slightly more slowly than ΔC20 on a SDS-PAGE gel, and after activation by trypsin treatment, it migrated at the same position as ΔC20 (data not shown). The N-terminal sequence was the same as that of ΔC20. The molecular mass determined by mass spectrometry was 31,644 ± 2 Da, close to 31,680.6 Da, a value calculated from the amino acid sequence (13). These data indicated that the purified protein consisted of the full length of the polypeptide encoded by the ctx gene.

ProCTX Does Not Form a 145-kDa Oligomer on the Target Membrane—After trypsin treatment, the purified proCTX exhibited hemolytic activity on rat erythrocytes comparable to the activity of PACTX, as well as that of ΔC20 (Fig. 2). Without activation, proCTX had no hemolytic activity (up to 4 μM). When erythrocytes incubated with proCTX were analyzed by immunoblotting, proCTX at the concentration of 0.06 μM bound to the erythrocytes. Binding of proCTX was in a dose-dependent manner. ProCTX, however, did not form the oligomer of 145 kDa on the erythrocyte membrane (Fig. 3). Formation of the oligomer by proCTX was not detected even at the higher concentration (up to 4 μM; data not shown). Trypsin-treated proCTX formed the oligomer as seen for ΔC20. These data suggested that the inactive nature of proCTX was due to the lack of potential to form a 145 kDa oligomer on the target membrane.

In Fig. 3, in addition to the 145-kDa band of the oligomer, 60-kDa bands were seen for the active toxins. This was also the case for proCTX, albeit in a reduced amount. It appears that CTX forms a dimer on the erythrocyte membrane, even though the dimeric form of active CTX is not seen in solution, as described below. The oligomeric state of CTX may change once CTX encounters the hydrophobic environment of the membrane and the dimer could be an intermediate in the formation of the 145-kDa oligomer. However, because a 60-kDa band was also detected by immunoblotting for the heat-inactivated toxin and the toxin not incubated with cells (10), we cannot exclude the possibility that it was formed after solubilization with SDS.

C-terminal Peptide Is Dissociated from the Active Toxin—Proteolytic cleavage in the C terminus is required for activation of proCTX. It remained to be determined whether the proteolytic cleavage removes the C-terminal peptide from the toxin molecule or simply introduces a nick in the molecule. Using an antibody specific to the C-terminal peptide immobilized on Affi-Gel 10 beads, we estimated the fate of the C-terminal peptide generated by trypsin treatment. As shown in Fig. 4A,
and then stained with Coomassie Blue (no staining with anti-)
and unbound (P) (with anti-C-terminal peptide antibody. Toxins added (P)
were displayed at 337.5 nm, whereas a maximum emission
spectra of the active toxin and proCTX. As shown in Fig. 6, the
incision of the C-terminal region on proCTX, we compared fluorescence
rence is sensitive for microenvironment transition around tryp-
To confirm the results, chemical cross-linking experiments
were done. As shown in Fig. 7, a protein band migrating at the
position of the dimer was detected for proCTX following incubation
with dimethyl suberimidate. On the contrary, the dimer
band was observed for neither ΔC20 nor trypsin-treated
proCTX.
Finally, the exact oligomeric states of the toxins were deter-
mained in analytical ultracentrifugation experiments (Table I).
Sedimentation coefficient was 5.04 S for proCTX or 2.61 S for
ΔC20. Equilibrium ultracentrifugation of the toxins showed
molecular weights of 63,000 for the proteotoxin and 29,000 for the
active toxin. Each value was in good agreement with a calcu-
lated molecular mass of the dimer of proCTX or the monomer of
ΔC20.

**DISCUSSION**

To purify proCTX of *P. aeruginosa*, we used a new method,
which is simple but includes a step of solubilizing the aggregated
CTX by urea. This may raise the possibility that the
purified proteins lost their native structures. However, the

ΔC20 and the trypsin-treated proCTX were not adsorbed by the
immobilized antibody and recovered in the supernatants,
whereas only a small portion of proCTX remained in the
supernatant fraction. Although toxins in the pellet fractions were
not clearly visible by Coomassie Blue staining because of the
presence of broad bands of the IgG light chain co-migrating
with toxins, immunostaining revealed that proCTX was recov-
ered in the pellet fraction, whereas trypsin-treated proCTX, as
well as ΔC20, was not (Fig. 4B). These observations suggested that
the C-terminal peptide left the toxin molecule once it was
generated by proteolytic cleavage. This was further support for
the notion that ΔC20 could serve as a model molecule for the
activated toxin.

**CD Spectra of proCTX and ΔC20 in the Far-ultraviolet**—As a
first step to elucidate molecular events involved in the activation
process, we wanted to know whether activation of proCTX
would alter the structure. For this purpose, we carried out CD
spectroscopic analysis of proCTX and ΔC20. The far-UV CD
spectra of both proteins, presented in Fig. 5, were similar.
ProCTX was estimated to contain 14.3% α-helices, 52.7%
β-sheet, 14.2% β-turn, and 18.8% random structure, and ΔC20
contained 12.5% α-helices, 54.4% β-sheet, 10.3% β-turn,
and 22.9% random structure. These findings indicated that the
secondary structure of proCTX was not affected by proteolytic
removal of the C-terminal 20 amino acid residues and that CTX
was a β-sheet predominant protein.

**Tryptophan Fluorescence**—In some cases, intrinsic fluores-
cence is sensitive for microenvironment transition around tryp-
tophan residues. Because there was no tryptophan residue in the
C-terminal region on proCTX, we compared fluorescence spectra of the active toxin and proCTX. As shown in Fig. 6, the
maximum of the fluorescence emission spectrum of proCTX
was displayed at 337.5 nm, whereas a maximum emission
wavelength of ΔC20 spectrum shifted to 342 nm and the inten-
sity was decreased. The maximum wavelengths of both proteins
were not affected by shifted salt concentration in the range of 0–1.0 M NaCl (data not shown). Tryptsin-treated
proCTX without fractionation of the C-terminal peptide also
showed a red-shifted spectrum with a maximum wavelength
the same as that of ΔC20 (Fig. 6). Although there was a small
difference in intensity between ΔC20 and trypsin-treated
proCTX, this was attributed to slightly different concentrations
of the proteins. These data suggested that an environment
around the tryptophan residue(s) became hydrophilic during the
activation process of proCTX.

**Dimer to Monomer Conversion of proCTX by Proteolytic Ac-
tivation**—The active CTX was suggested to exist as a monomer
in the solution (4), and the oligomeric state of proCTX re-
mained to be characterized. We first did analytical gel filtration
experiments. ProCTX was eluted much earlier than active tox-
ins ΔC20 and PACTX, and the molecular weights of proCTX
and active toxins were estimated to be 52,000 and 24,000,
respectively. Furthermore, the molecular weight of proCTX
was reduced to 24,000 by trypsin treatment (data not shown).
These findings suggested that proCTX existed as a homodimer
in the solution and was converted to monomers by proteolytic
activation.

To confirm the results, chemical cross-linking experiments
were done. As shown in Fig. 7, a protein band migrating at the
position of the dimer was detected for proCTX following incubation
with dimethyl suberimidate. On the contrary, the dimer
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Finally, the exact oligomeric states of the toxins were deter-
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active toxin. Each value was in good agreement with a calcu-
lated molecular mass of the dimer of proCTX or the monomer of
ΔC20.

**FIG. 4.** Immunoadsorption of ΔC20, proCTX, and trypsinized
proCTX. Toxins (0.5 μg) were incubated with Affi-Gel 10 beads coupled
with anti-C-terminal peptide antibody. Toxins added (P) and the bound
(S) fractions were separated on an 11% SDS-PAGE gel
and then stained with Coomassie Blue (panel A) or analyzed by immu-
nostaining with anti-ΔC20 antiserum (panel B). Lanes 1, proCTX; lanes
2, trypsin-treated proCTX; lanes 3, ΔC20; lanes 4, no toxin. Molecular
size markers (New England Biolabs) are indicated on the left.

**FIG. 5.** Circular dichroic spectra of ΔC20 and proCTX. Spectra
of toxins measured in 20 mM sodium phosphate buffer pH 7.2 are shown.

**FIG. 6.** Dimer to Monomer Conversion of proCTX by Proteolytic Ac-
activation.
biological and physicochemical properties of the purified recombinant active CTX, ΔC20, were indistinguishable from those of an active toxin obtained from \textit{P. aeruginosa}. Purified proCTX was also active after trypsin treatment. Thus, the protein refolded correctly during dialysis after being denatured by urea.

When characterizing the purified proCTX, there were four major findings: (a) proCTX binds to the erythrocytes membrane but cannot form the oligomer on the membrane; (b) proteolytic action removes the C-terminal peptide from the toxin molecule; (c) activation of proCTX by the C-terminal cleavage does not induce change in the secondary structure; and (d) by proteolytic removal of the C-terminal peptide, the homodimer of proCTX is converted to monomers.

One of the crucial steps in intoxication by CTX is oligomerization on the target membrane. The oligomer formed by CTX is most likely in a pentameric form (10). From our findings, the activation process of proCTX is proposed to be as follows: proCTX existing in a dimeric form can bind to the membrane but is cytolytically inactive because it does not oligomerize into a pentamer. Once the C-terminal peptide is removed by proteolytic action, proCTX is converted into monomers, which form a pentamer on the membrane and thus are active in cytolytic events. This is in clear contrast with the activation process described for aerolysin, another pore-forming toxin that requires the C-terminal processing for activation. Aerolysin is also produced as an inactive precursor and exists as a homodimer. After activation by C-terminal cleavage, aerolysin exists as a homodimer (26). To form a heptameric oligomer on the membrane, conversion from dimer to monomer is thought to be essential for aerolysin as well. Although such an intermediate has not been detected, the conversion may be induced by a structural change upon active dimeric toxin after binding to the target membrane (12). The process of oligomerization on the membrane is one point that remains to be elucidated for pore-forming toxins.

The C terminus of proCTX contributes to stable dimer formation but the mechanism remains obscure. A synthesized 21-mer peptide corresponding to the C terminus of proCTX did not form the dimer. A direct interaction between the C-terminal regions may not be an event in dimer formation, or such an interaction may be stabilized by other interactions between the two proteins. Another possibility is that the C-terminal region, which contains a stretch of 10 hydrophobic amino acids followed by a sequence potentially forming a coiled-coil helix (residues 277–286), may involve an intermolecular interaction with other parts of the toxin molecule to stabilize the dimeric form of proCTX.

It also remains unclear how the change in intrinsic fluorescence of the toxin occurred by activation. The results suggest that a tryptophan residue(s) buried in the proCTX molecule was exposed to the solvent after activation; it might have been covered directly by the C-terminal peptide and appeared on the surface of the protein by removal of the peptide. Alternatively, the residue(s) might exist on the intersurface of the dimeric proCTX and be exposed to the solvent by dissociation of the toxin molecule into monomers. Identification of the tryptophan residue(s) responsible for change in intrinsic fluorescence will be informative for understanding the structural features of proCTX.

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Another important finding on the structure of CTX is that the toxin consists predominantly of the β-sheet. Increasing numbers of bacterial toxins that form a pore by oligomerization

\begin{table}[h]
\centering
\caption{Sedimentation coefficients and molecular weights of proCTX and ΔC20.}
\begin{tabular}{|c|c|c|}
\hline
& S_{20,w} (S) & Molecular weight & Calculated molecular weight \tabularnewline
\hline
ProCTX & 5.04 & 62,866 \pm 624 & 31680.6 \tabularnewline
ΔC20 & 2.61 & 29,472 \pm 236 & 29367.1 \tabularnewline
\hline
\end{tabular}
\end{table}

\textsuperscript{a} Data are from amino acid compositions.
have been reported to have structures rich in the β-sheet. This was documented most clearly for aerolysin (16) and staphylococcal α toxin (27). The structure of oligomerized α toxin at a resolution of 1.9 Å revealed that a transmembrane domain of the channel is a 14-stranded β-barrel. The β-sheet predominant feature may be essential for oligomerization and channel formation of CTX as well. To understand the structural roles of the β-sheet in CTX, the oligomerized toxin will have to be isolated, and then it will be possible to examine the exact number of CTX protomers constituting the oligomer.

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