C-terminal Amino Acid Residues Are Required for the Folding and Cholesterol Binding Property of Perfringolysin O, a Pore-forming Cytolysin*

(Received for publication, March 16, 1999, and in revised form, April 20, 1999)

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Perfringolysin O (θ-toxin) is a pore-forming cytolyisin whose activity is triggered by binding to cholesterol in the plasma membrane. The cholesterol binding activity is predominantly localized in the β-sheet-rich C-terminal half. In order to determine the roles of the C-terminal amino acids in θ-toxin conformation and activity, mutants were constructed by truncation of the C terminus. While the mutant with a two-amino acid C-terminal truncation retains full activity and has similar structural features to native θ-toxin, truncation of three amino acids causes a 40% decrease in hemolytic activity due to the reduction in cholesterol binding activity with a slight change in its higher order structure. Furthermore, both mutants were found to be poor at in vitro refolding after denaturation in 6 M guanidine hydrochloride, resulting in a dramatic reduction in cholesterol binding and hemolytic activities. These activity losses were accompanied by a slight decrease in β-sheet content. A mutant toxin with a five-amino acid truncation expressed in Escherichia coli is recovered as a further truncated form lacking the C-terminal 21 amino residues. The product retains neither cholesterol binding nor hemolytic activities and shows a highly disordered structure as detected by alterations in the circular dichroism and tryptophan fluorescence spectra. These results show that the C-terminal region of θ-toxin has two distinct roles; the last 21 amino acids are involved to maintain an ordered overall structure, and in addition, the last two amino acids at the C-terminal end are needed for protein folding in vitro, in order to produce the necessary conformation for optimal cholesterol binding and hemolytic activities.

Thiol-activated cytolsins (1) comprise a family of bacterial protein toxins that are produced by Gram-positive bacteria. They share a high degree of homology in their amino acid sequences (40–70%) (2–7) and have common biological characteristics, cholesterol binding and the formation of oligomeric pores on plasma membranes. Perfringolysin O (472 amino acids), known as θ-toxin, is such a toxin produced by Clostridium perfringens type A. Its cytolytic mechanism is thought to comprise at least four steps: binding to cholesterol in membranes, insertion into the membrane, oligomerization, and pore formation. θ-Toxin binds specifically to cholesterol on plasma membranes with high affinity (Kd ~ 10⁻⁸ M) (8). By forming oligomeric pores on plasma membranes (9), θ-toxin causes cell disruption.

After several attempts to crystallize θ-toxin (10, 11), its three-dimensional structure was recently revealed by x-ray diffraction (12). This analysis showed θ-toxin to be an elongated rod-shaped molecule rich in β-sheets and to consist of four discontinuous domains. Domain 4 (Fig. 1b) (residues 363–472), the C-terminal domain, is an autonomous structure comprising a continuous amino acid chain. Six of the seven total tryptophan residues reside in domain 4, and three are located in the sequence of ECTGLAWEWR (residues 430–440), the longest conserved sequence among theta-activated cytolsins (2, 3). From many efforts to achieve mutagenesis of this toxin family (13–17), it was shown that all mutations that inhibit cell binding activity reside in domain 4, suggesting that some region in domain 4 binds to membrane cholesterol upon binding to cells. This is consistent with our previous findings that a C-terminal tryptic fragment that contains predominantly domain 4 binds to cholesterol and to cholesterol-containing membrane (18). Our findings that the toxin binding to cholesterol in liposomal membrane triggers a conformational change around tryptophan residues in domain 4 also support this view (19, 20). Recently, possible roles of the C-terminal region in cell binding were suggested by a report that a monoclonal antibody thought to bind near the C terminus specifically blocks cell binding, although the exact epitope was not identified (21). Despite this finding, it is not known whether the C-terminal region plays a role in cholesterol binding or membrane insertion activity, inasmuch as either one could affect toxin binding to cells. Recent x-ray crystallographic analysis showed that there are two β-strands in antiparallel orientation in the C-terminal end and that one of them is composed of 7 amino acids in the C-terminal end (12).

Here, we constructed and analyzed toxin mutants truncated in the C terminus to define the role of the C-terminal region on cholesterol binding activity. Using an ELISA* assay for quantitative analysis of cholesterol binding activity, we show that the C-terminal end is essential for folding of θ-toxin into the membrane.

* This work was supported in part by grants from the Hayashi Memorial Foundation for Female Natural Scientists (to Y. O.-I.) and the Memorial Foundation for Female Natural Scientists (to Y. S.) and the Naito Foundation (to Y. Naito). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; CD, circular dichroism; GdnHCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; BisTris, 2-[bis(2-hydroxyethyl)amino]–2-(hydroxymethyl) propane-1,3-diol; ESI, electrospray ionization; ESI-MS, electrospray ionization mass spectrometry.
native conformation, thus ensuring activities of cholesterol binding and hemolysis.

EXPERIMENTAL PROCEDURES

Materials—Anti-θ-toxin antibody was obtained by immunizing rabbits as described previously (18). Cholesterol, subtilisin Carlsberg, and isopropylthioβ-D-galactoside were purchased from Sigma. DEAE-Sephacel was from Amersham Pharmacia Biotech (Uppsala, Sweden). Horseradish peroxidase-conjugated anti-rabbit IgG serum was purchased from Seikagaku (Tokyo, Japan).

Site-directed Mutagenesis—Plasmid pNSP10 containing the perfringolysin O gene (pfoA) (13) was used to construct six pfoA derivatives encoding truncated θ-toxins. Stop codons were introduced at appropriate sites in the pfoA gene by a site-directed mutagenesis kit (CLONTECH) based on the unique site elimination method (22). The 5′-deoxyadenosine oligodeoxynucleotide dCATTTTTTACTTTATGaTcTaATAGAATCTAG (D471), dCTTTATGTTATATTcAtAATcGTcGATCGGcGTT; D468, dGTTTATAGTtCAtAGGTcGATCGGcGTT. Lowercase letters represent bases changed for mutagenesis. The DNA sequences in the resulting plasmids were confirmed by means of the dideoxynucleotide chain-termination method (23). Predicted amino acid sequences in the C-terminal ends of the mutant toxins are shown in Fig. 1.

Protein Production and Purification—Protein production and purification were performed as described previously (13) with slight modifications. Escherichia coli strains BL21(DE3) and BL21(DE3) harboring pLYS S (24) (Novagen, Madison, WI) were used for the overexpression of wild type θ-toxin and mutant toxins. Wild type θ-toxin and mutant toxins were purified from the periplasmic fraction by a series of DEAE-Sephacel chromatographies. In the case of mutant toxins having no hemolytic activity, the fractions eluted from the first DEAE-Sephacel column were analyzed by immunostaining with anti-θ-toxin antibody after SDS-PAGE. Then, the toxin fractions were loaded onto a second DEAE-Sephacel column equilibrated with 20 mM sodium phosphate buffer, pH 7.5, and eluted with 40 mM NaCl. For further purification, the toxin fractions were applied to a hydroxyapatite column equilibrated with 20 mM sodium phosphate buffer, pH 7.5, and the toxins were eluted with 100 mM sodium phosphate. Then, the toxins were loaded onto a butyl-agarose column equilibrated with 20 mM sodium phosphate buffer, pH 7.5, and the toxins were eluted with 100 mM sodium phosphate. Then, the toxins were loaded onto a butyl-agarose column equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1.7 mM (NH4)2SO4 and eluted with 0.5 M (NH4)2SO4. The purity of the toxins was checked by SDS-PAGE (25).

Determination of the Hemolytic Activity of Toxins—Hemolytic activity was determined as described previously (26). The amount of toxin required for 50% hemolysis of 1 ml of 0.5% sheep erythrocytes in 30 min at 37°C (HD50) was determined using the von Krogh equation (27). The HD50 served as molecular mass of the protein recovered from the cells. SDS-PAGE gel by the method of Nakayama et al. (29). After SDS-PAGE, the gel was washed with distilled water and stained with 0.3% CuCl2 for 3 min. The toxin spot was excised from the SDS-PAGE gel and subjected to electrospray ionization mass spectrometry (ESI-MS) using a Fourier transform ion cyclotron resonance mass spectrometer BioAPEX47E (Bruker Instruments) equipped with an external ESI source (Analytica of Branford). Before being injected into the source by a syringe pump operated at 30 μl/h, the samples were desalted on a reverse-phase high pressure liquid chromatography column (Senshu Pak CS-1251-N) eluted with a 10–60% gradient of acetoni trile, 0.1% trifluoroacetic acid. In some cases, the samples were prepared from the SDS-PAGE gel by the method of Nakayama et al. (29). After SDS-PAGE, the gel was washed with distilled water and stained with 0.3% CuCl2 for 3 min. The toxin spot was excised from the SDS-PAGE gel and sent successively in 25 mM Tris-HCl, pH 8.3, and 12.5 mM Tris-HCl, pH 8.3. Then, the toxin was excised from the gel in 50 mM Tris-HCl, pH 8.8, containing 50 mM EDTA and 0.1% SDS. To remove SDS and other impurities, the extracted toxin was applied to a Phenyl-5PW RP column (Tosoh, Tokyo, Japan) and recovered with 90% acetonitrile in 0.1% trifluoroacetic acid. When this method was used, the molecular mass of θ-toxin was determined by subtracting the mass of the adduct of copper, 63.4, from the observed mass.

RESULTS

Characterization of Truncated θ-Toxin—θ-Toxin mutants truncated at the C terminus were constructed and expressed in E. coli as described under “Experimental Procedures” (Figs. 1 and 2). θ-Toxin has an intrinsic signal sequence at its N terminus and is secreted into the periplasm when expressed in E. coli (Fig. 2 and Ref. 13). A similar expression profile was observed for D471 and amounts comparable to wild type θ-toxin were recovered from the periplasmic fraction (Fig. 2). A slightly smaller amount was recovered in the case of D470. Upon exposure of the DNA construct for D468 and mutants with larger truncations, the amounts of proteins with molecular sizes close to that of intact θ-toxin decreased with concomitant increases in the amounts of degradation products with sizes around 38 kDa (Fig. 2 and data not shown). The results suggest that truncations at the C terminus affect the biosynthesis of θ-toxin and/or its secretion into the periplasm.

To further characterize the mutant toxins, the expressed proteins were purified from the periplasmic fraction by DEAE-Sephacel chromatographies and their molecular masses were determined by ESI-MS (Table 1). The observed molecular masses of wild type D471, and D470 are within the range of the predicted molecular masses. In contrast, the observed molecular mass of the protein recovered from the cells harboring the constructed plasmid for D468 is smaller than the predicted mass for D468 (Table 1). This indicates that the
production of the protein with a five-amino acid C-terminal truncation brings about a further truncated form. N-terminal sequence analysis revealed that the product has the same N-terminal sequence as the wild type toxin. From the results of N-terminal and molecular mass analyses, we conclude that the product comprises residues 1–451 (predicted $M_r$, 50,375.6), with a 21-amino acid truncation at the C terminus. We designate the product as $D_{452}$ hereafter. The elution profile of $D_{452}$ is different from those of wild type $\theta$-toxin and two mutants, $D_{471}$ and $D_{470}$; the former eluted from DEAE-Sephacel column at 110 mM NaCl, whereas the latter two mutants eluted at 60 mM.

The relative hemolytic activities of purified wild type and three mutant toxins were determined (Fig. 3, upper part). No differences in hemolytic activity were detected between wild type $\theta$-toxin and $D_{471}$, indicating that the deletion of two amino acids from the C terminus of $\theta$-toxin does not affect hemolytic activity. In contrast, $D_{470}$ showed a lower hemolytic activity, 40% that of wild type, while $D_{452}$ showed no hemolytic activity. These results indicate that truncation of the C terminus by 21 amino acids causes a loss of hemolytic activity.

Hemolysis by $\theta$-toxin involves two important steps, binding and insertion into membranes, prior to pore formation. The binding activity to cells was measured and compared among the wild type and mutant toxins (Fig. 3, lower part). $D_{471}$ showed high-affinity binding to sheep erythrocytes similar to the wild type, but $D_{470}$ showed only very weak binding. $D_{452}$, which has no hemolytic activity, never bound to the cells. These results show a good correlation between cell binding activity and relative hemolytic activity.

Cholesterol on plasma membranes serves as a receptor for $\theta$-toxin. Fig. 4a shows the cholesterol binding activity of mutant toxins on TLC plates as detected by immunostaining with...
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Fig. 4. Cholesterol binding activity of toxins. a, detection of specific binding of toxins to cholesterol on TLC plates. Cholesterol (PC), phosphatidylcholine (PC), and esterified cholesterol (CE) were spotted on TLC plates at the indicated doses. Toxins were incubated with these lipids on the TLC plates, and bound toxins were detected by immunostaining. b, cholesterol binding activity was determined by ELISA. Details are described under “Experimental Procedures.” The activities of three mutant toxins are expressed relative to the activity of the wild type at 10,000 pmol cholesterol. Wild type, open circles; Δ471, closed circles; Δ470, closed triangles; Δ452, open triangles; W438F, closed squares; W439F, open squares.

anti-α-toxin antibody. Δ471 and Δ470 were found to bind to cholesterol on TLC plates and to specifically recognize free cholesterol but not phosphatidylcholine or esterified cholesterol. Their manner of binding was the same as that of wild type toxin, although Δ470 shows weaker spots. On the other hand, Δ452 did not bind to cholesterol at all. Fig. 4b shows the quantitative analysis of the cholesterol binding activity of the toxins by ELISA. Δ471 shows an activity comparable to the wild type toxin. The activity of Δ470 is about 40% of the wild type, while no activity could be detected for Δ452. The results show that the cholesterol binding activity of the toxins correlates well with their cell binding and hemolytic activities.

We previously reported that mutants with Trp to Phe substitutions within the tryptophan-rich consensus sequence show decreased binding affinity for erythrocytes (13). We examined the cholesterol binding activity of two such mutants, W438F and W439F, by ELISA and compared the results with the cholesterol binding activity of the C-terminal truncation mutants (Fig. 4b). Mutants with Trp to Phe substitutions show cholesterol binding activity similar to that of the wild type toxin (Fig. 4b), showing that mutations of Trp in the consensus sequence have little effect on the cholesterol binding activity. The decrease in cell binding activity of the mutants should be attributable to step(s) other than cholesterol binding. This makes a distinct difference from the results for the mutants with C-terminal truncations.

Effect of C-terminal Truncation on the Structure of α-Toxin—The defects in the cholesterol binding activities of Δ470 and Δ452 can be attributed to either the deletion of cholesterol-binding sites or conformational changes around the binding sites. To assess these possibilities, we first examined the susceptibility of mutant toxins to a protease (Fig. 5). Digestion of wild type α-toxin and Δ471 by subtilisin Carlsberg produced a distinctive 39-kDa fragment assigned as the C-terminal fragment (28); a smaller amount of this fragment was detected when Δ470 was digested. In contrast, Δ452 was digested over time into undetectable pieces showing no distinctive bands. Trypsin digestion also produced proteolytic fragments of 28 and 25 kDa (18) from Δ471 and Δ470, but not from Δ452 (data not shown). The results indicate that the secondary or tertiary structures of Δ452 has been changed by C-terminal truncation of 21 amino acid residues.

Because six out of the seven tryptophan residues in α-toxin are located in the C-terminal region (see Fig. 1), it is reasonable to measure tryptophan fluorescence in order to monitor the conformational alterations of α-toxin induced by truncation. When the toxins were excited at 295 nm, no differences in the peak emission wavelength at 338 nm were detected between wild type and two mutants, Δ471 and Δ470 (Table II), indicating that the environmental changes around the Trp residues are not significant in those two mutants. However, environmental changes around some fluorophores other than tryptophan appear to have occurred, since the mutants exhibited a red shift in the maximal emission wavelength when excited at 280 nm (Table II). On the other hand, a distinctive red shift of the maximal emission wavelength was observed for Δ452 as compared with the wild type toxin (Table II), indicating that the environment of the tryptophan residues in these mutants is more hydrophilic than in the wild type. Simultaneously, the intensity of the tryptophan fluorescence in Δ452 excited at 295 nm was enhanced to 3.2 times that of the wild type α-toxin. The results suggest that the inactive mutant Δ452 has significant alteration in its tertiary structure around tryptophan residues, and that this leads to the loss in hemolytic activity.

When α-toxin interacts with cholesterol on dioleoylphosphatidyl choline/cholesterol liposomes, there is an increase in the intensity of the tryptophan fluorescence (19). The two truncated toxins, Δ471 and Δ470, also showed increases in the intensity when incubated with dioleoylphosphatidyl choline/cholesterol liposomes. In contrast, no enhancement of fluorescence intensity was detected for Δ452 (data not shown). There-
fore, this mutant lacks an appropriate structure for interaction with cholesterol in membranes.

In order to determine whether the deletion of C-terminal amino acids affects the secondary structure of the toxin, far ultraviolet CD spectra were measured. As shown in Fig. 6a, wild type θ-toxin has a β-sheet-rich structure and the spectra of Δ471 and Δ470 closely resemble that of the wild type toxin. On the other hand, drastic difference was detected in the spectrum of Δ452 as compared with the wild type toxin. A significant increase in negative ellipticity was observed at 208 nm and shorter wavelengths. The CD difference spectrum obtained by subtracting the wild type spectrum from the Δ452 spectrum exhibited a deep minimum at 200 nm or a shorter wavelength and a shoulder at around 225 nm. This difference spectrum resembles that usually taken to indicate an unfolded conformation (30). This observation suggests a large disorder in the secondary structures of Δ452.

**Effect of C-terminal Truncation on in Vitro Refolding**—The structural analysis suggests that several amino acids at the C terminus play essential roles in in vivo protein folding and/or the maintenance of protein conformation. We carried out in vitro refolding experiments on the truncated mutants to define the function of C-terminal amino acids during folding. Wild type θ-toxin and mutant Δ471 (truncated by two amino acids) were denatured in 6 M GdnHCl, renatured by dialysis, and their hemolytic activities were measured. As shown in Fig. 7a (upper part), wild type θ-toxin recovered 81% of full hemolytic activity while Δ471 displayed only 13% recovery, even though Δ471 has an activity comparable to the wild type before denaturation. The refolded Δ471 hardly bound to sheep erythrocytes as shown in Fig. 7a (lower part), showing a good correlation with relative hemolytic activity. To investigate whether the refolded Δ471 recognizes cholesterol, the cholesterol binding activity was measured by ELISA. The refolded Δ471 shows much less cholesterol binding activity than native Δ471, while the activity of the wild type toxin is not changed by the denaturation-refolding treatment (Fig. 7b). Although we could not judge whether all the refolded Δ471 molecules have lower binding affinities than in the native state or whether a small population of Δ471 refolds to the native form with full activity, it is clear that Δ471 easily loses its ability to bind cholesterol during the denaturation-renaturation process. A decrease in the cholesterol binding activity was also observed after denaturation-refolding of Δ470 (data not shown).

To rule out the possibility that there might be a minor contaminating protease that cleaves the Δ471 protein during the refolding treatment and causes it to lose activity, the relative molecular masses of native and refolded Δ471 were determined by ESI-MS (Table I). The relative molecular masses determined for the refolded Δ471 and native Δ471 are the same and within the range of the predicted one (Table I), indicating that no proteolytic cleavage occurs during the refolding process. Wild type toxin also maintains its intact size during refolding treatment, as shown by the relative molecular masses before and after treatment (Table I). These results indicate that the loss of Δ471 activity after refolding is not caused by the

| Toxin  | Emission maximum (λem) | Relative intensity (nM) |
|--------|------------------------|-------------------------|
|        | 280 nm | 295 nm | 280 nm | 295 nm |
| Wild type | 332 | 338 | 100 | 100 |
| Δ471   | 334 | 338 | 106 ± 3 | 127 ± 3 |
| Δ470   | 336 | 338 | 115 ± 2 | 135 ± 3 |
| Δ452   | 340 | 342 | 241 ± 3 | 319 ± 4 |

**FIG. 6.** Far-ultraviolet CD spectra of wild type and truncated mutants. a, spectra for the wild type and three truncated toxins were measured in a 5-mm pathlength cuvette at room temperature. Samples were prepared at a toxin concentration of 30 μg/ml in 20 mM phosphate buffer, pH 7.0, containing 150 mM NaCl. 1, wild type (solid line); 2, Δ471 (dotted line); 3, Δ470 (long dashed line); 4, Δ452 (dot-dashed line). b, comparison of the far-ultraviolet CD spectra of Δ471 before and after denaturation-refolding. Measurements of the far-ultraviolet CD spectra were performed in a 1-mm pathlength cuvette at room temperature. Native Δ471 (1, dot-dashed line) and Δ471 after denaturation-refolding (2, long dashed line) were prepared at a toxin concentration of 150 μg/ml in 10 mM phosphate buffer, pH 7.0.
action of protease.

The above results show that even just two amino acid residues at the C terminus are involved in the correct folding of θ-toxin. To assess whether the inactivation of Δ471 by denaturation-refolding is accompanied by a conformational alteration, the structural properties of wild type and Δ471 after denaturation-refolding treatment were studied by CD and fluorescence analyses. Compared with native Δ471, the far-ultraviolet CD spectrum of refolded Δ471 shows a slight alteration in the secondary structure (Fig. 6b), a 3% decrease in β-sheet content and a concomitant increase in random coil in the refolded Δ471 as estimated by the algorithm program, CONTI

Fig. 6a and Table II, suggesting that the changes in refolded Δ471 occur in a limited region of the toxin molecule.

**DISCUSSION**

The crystallographic study of θ-toxin showed that domain 4, the C-terminal domain supposed to contain the cholesterol-binding region, has nine β strands folded into a compact β-sheet sandwich (12). Two β strands in antiparallel orientation are located within the C-terminal 20 amino residues and form a part of one β sheet (Fig. 1). In this study, focusing on the two C-terminal β strands, we constructed several C-terminal truncated θ-toxin mutants to investigate how C-terminal amino acids contribute to the folding of the protein and its toxic action.

We first demonstrated that amino acids in the C-terminal β strand play an important role in correct folding of the toxin. When Δ471 was refolded after denaturation in 6 M GdnHCl, it lost its membrane binding and hemolytic activities with the reduction in cholesterol binding activity (Fig. 7), indicating the importance of the two C-terminal amino acids for correct folding in vitro into the conformation required for cholesterol binding. However, Δ471 showed essentially the same hemolytic activity and secondary structure as wild type θ-toxin. This indicates that the mutant folds into the native conformation in vivo. Taking the difference between in vivo and in vitro folding into consideration, probably chaperone-like molecules help to achieve correct folding in vivo (32, 33). As shown in Δ470, a three-amino acid truncation affects folding both in vivo and in vitro.

The truncation of five amino acids from the C terminus leads to a further truncation of the protein in host E. coli, indicating that the C-terminal β strand protects the protein against proteolytic cleavage in host cells. For toxin production, we used E. coli B strain, BL21(DE3), as a host, because it lacks both lon and ompT proteases. Some other minor protease(s) in E. coli may contribute to cleaving the product during synthesis or secretion into the periplasm (34). The product, Δ452, lacks the two β strands in the C terminus and completely loses its cell binding and hemolytic activities due to its inability to recognize cholesterol (Fig. 4). It is likely that the molecular structure required for the specific binding of cholesterol molecules is absent or not correctly organized in Δ452. Spectroscopic data indicate its partially unfolded secondary structure and an environmental alteration around tryptophan residues to a more hydrophilic and unrestricted state (Fig. 6e and Table II). Since the elimination of the two β strands from the C-terminal end causes this remarkable disorder in structure, the C-terminal two β strands are suggested to play key roles in constructing the overall structure of the toxin.

There are two distinct steps in cell binding by θ-toxin, cholesterol recognition and membrane insertion. In this study we demonstrated that truncation of the C terminus abolishes cholesterol-recognition ability, resulting in the loss of cell binding activity. This is in contrast to mutants with Trp to Phe substitutions within the tryptophan-rich consensus sequence (residues 430–440), which show a loss in cell binding activity despite their ability to bind cholesterol (Fig. 4b and Ref. 13). We previously suggested that the tryptophan-substituted mutants have some deficiency in membrane insertion activity (13, 20) that could cause them to lose cell binding activity. The tryptophan-rich consensus sequence locates in close proximity to one of the two β sheets in domain 4, and distant from the other β sheet to which the C-terminal β strand belongs (Fig. 1b). The crystallographic data indicate that there are some amino residues in the proximal β sheet that are possible quenchers of the fluorescence of Trp-436 and Trp-439, Trps within the consensus sequence. Thus, a change in tryptophan fluorescence intensity could be a sensitive marker for a change in three-dimensional arrangement among these Trp residues and quenchers. Since either Δ470 or refolded Δ471 shows no significant change
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in tryptophan fluorescence compared with the wild type toxin, the microenvironment around the Trp residues remains intact in these mutants. This strongly suggests that the C-terminal truncation does not affect the structural features around the tryptophan-rich consensus sequence despite its significant effect on cholesterol binding activity. Probably the site of cholesterol binding is in different region from that of membrane insertion.

Since the molecular mass of \( \Delta 471 \) remains unchanged by the unfolding-refolding treatment (Table I), the activity loss in cholesterol binding should be ascribed to a conformational change. It is likely that the molecular structure required for the specific binding of cholesterol molecules is not correctly organized after treatment. An approximately 3% decrease in \( \beta \)-sheet content in \( \Delta 471 \) was detected after treatment. No change occurs in tryptophan fluorescence as discussed above. This implies that the change occurs within a limited region upon refolding of \( \Delta 471 \) and that this local change in structure directly affects the cholesterol-binding site. Since the C-terminal \( \beta \) strand interacts directly with the next \( \beta \) strand (residues 453–460) by hydrogen bonding to form part of an antiparallel \( \beta \) sheet (Fig. 1 and Ref. 12), it is likely that the decrease in \( \beta \)-sheet content produced by treatment occurs near these strands. It has been reported that C-terminal truncation of pneumolysin, another cholesterol-binding cytotoxin, causes a loss of cell binding activity (17). It would be interesting to know whether this loss in the cell binding activity of pneumolysin is due to the loss of cholesterol binding activity, although conformational studies and molecular mass determination of the truncated species of pneumolysin would be required to draw a conclusion.

There have been several reports showing that C-terminal residues are important for the correct folding and maintenance of the native protein conformations (35, 36). Among them, \( \theta \)-toxin is a distinct example since the deletion of only two amino acids from the C terminus out of a total of 472 residues seriously affects its folding and the maintenance of the functional conformation. We have reported chemically modified \( \theta \)-toxin as a new probe for cholesterol (37). If the relationships between binding activity and the conformation of the C-terminal region are clarified and the minimum binding unit is identified, further design of useful probes can be realized.

Acknowledgments—We thank Drs. M. Inomata and M. Hayashi for technical advice and helpful discussion; Drs. S. Mizuno, H. Morisawa, and K. Shibazaki for technical advice concerning the computer analysis of the secondary structure; and Dr. H. Nakayama for technical advice in preparing the samples for ESI. We thank Drs. S. Iwashita and K. Murakami-Murofushi for critical reading of the manuscript and helpful discussion. We thank Dr. M. M. Dooley-Ohito for reading the manuscript.

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