Interaction of θ-Toxin (Perfringolysin O), a Cholesterol-Binding Cytolysin, with Liposomal Membranes: Change in the Aromatic Side Chains upon Binding and Insertion

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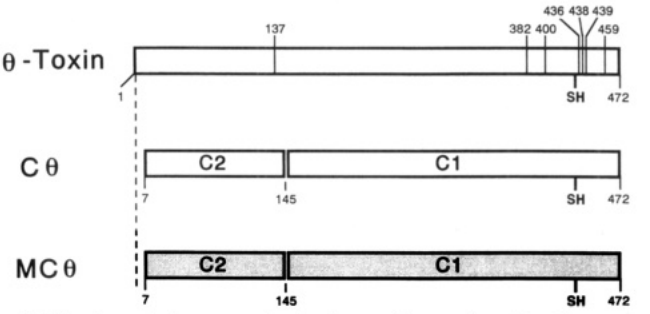
ABSTRACT: To understand the mechanism of membrane lysis by θ-toxin (perfringolysin O) from *Clostridium perfringens*, a cholesterol-binding, pore-forming cytolysin, we undertook a spectroscopic analysis of the structural changes that occur during the lytic process using lipid vesicles. In particular, the spectra were compared with those obtained using a modified θ-toxin, MCθ, that binds membrane cholesterol without forming oligomeric pores, thus bypassing the oligomerization step. The interaction of θ-toxin with liposomes composed of cholesterol and phosphatidylcholine but not with cholesterol-free liposomes caused a remarkable increase in the intensity of the tryptophan fluorescence emission spectra and ellipticity changes in the near- and far-UV CD peaks. A CD peak shift from 292 to 300 nm was specific for θ-toxin, suggesting oligomerization-specific changes occurring around tryptophan residues. Structural changes in the aromatic side chains were detected in the near-UV CD and fluorescence spectra upon MCθ-liposome interaction, although the far-UV CD spectra indicate that the β-rich secondary structure of MCθ is well-conserved after membrane binding. Quenching of the intrinsic tryptophan fluorescence of MCθ by brominated lecithin/cholesterol liposomes suggests that θ-toxin inserts at least partly into membranes in the absence of oligomerization. These results indicate that regardless of oligomerization, the binding of θ-toxin to cholesterol induces partial membrane insertion and triggers conformational changes accompanied by aromatic side chain rearrangement with retention of secondary structure. The spectral changes depend on the cholesterol/toxin molar ratio and pH, with maxima at pH 5−7, correlating with the optima for binding, suggesting that the cholesterol-induced insertion mechanism is distinct from the acid-induced one.

θ-Toxin (perfringolysin O) is a cytolytic toxin produced by *Clostridium perfringens* type A that belongs to a group of oxygen-labile and thiol-activated cytolysins (Mitsui *et al.*, 1973; Smyth, 1975). θ-Toxin is known to bind to cholesterol in membranes and to cause membrane damage by forming oligomeric pores. Its cytolytic mechanism is thought to comprise at least three steps: binding to cholesterol, insertion into the membrane, and pore formation. The characterization of θ-toxin fragments has shown that a C-terminal 25-kDa fragment is responsible for binding to cholesterol (Iwamoto *et al.*, 1990). To analyze the early events in the lytic process separate from the final cytolysis, we previously prepared modified θ-toxins, Cθ[1] and MCθ, shown schematically in Scheme 1. Cθ is produced by limited proteolysis of θ-toxin with subtilisin Carlsberg (Ohno-Iwashita *et al.*, 1986). Cθ is a complex of C1 and C2 fragments that can dissociate only in the presence of denaturants, such as urea (Ohno-Iwashita *et al.*, 1986). MCθ is produced by the reductive methylation of Cθ and also exists as a complex of two fragments (Ohno-Iwashita *et al.*, 1990). Cθ and MCθ have

Scheme 1: Schematic Illustration of Structures of θ-Toxin, Cθ, and MCθ[a]



[a] θ-Toxin contains seven tryptophan residues whose locations are indicated as bars in the θ-toxin structure. MCθ is produced by the reductive methylation of Cθ; both fragments of MCθ are methylated at the ϵ-amino groups of lysine residues.

the same binding activities to erythrocyte membranes as θ-toxin but no hemolytic activity at temperatures of 20 °C or below in the case of Cθ or even at 37 °C in the case of MCθ. MCθ has been shown not to form oligomers as judged by electron microscopy (Ohno-Iwashita *et al.*, 1990) and sucrose-density gradient analysis (Iwamoto *et al.*, 1990). Thus, MCθ seems to be a good probe with which to examine the membrane binding and insertion mechanism of θ-toxin apart from toxin oligomerization.

Using these modified toxins, the mode of θ-toxin binding to cholesterol has been studied extensively under various

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[1] Abbreviations: Cθ, subtilisin Carlsberg-digested θ-toxin; MCθ, reductive methylated Cθ; PC, phosphatidylcholine; EPC, phosphatidylcholine from egg yolk; DOPC, dioleoylphosphatidylcholine; Br-DSPC, bis(bromostearoyl)phosphatidylcholine; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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conditions (Ohno-Iwashita *et al.*, 1988, 1990−1992; Iwamoto *et al.*, 1990). The results suggest that there are two distinct states of cholesterol as revealed by high- and low-affinity toxin-binding sites on the membranes of intact cells and liposomes (Ohno-Iwashita *et al.*, 1988, 1990−1992). It was also demonstrated that the cholesterol content of membranes is a major determining factor for toxin binding. θ-Toxin binds only slightly to liposomes whose cholesterol content is less than 25 mol %, indicating that the packing conditions of cholesterol molecules in membranes affect toxin binding (Ohno-Iwashita *et al.*, 1991, 1992). Chain length and head group variation in the liposomal phospholipids also affect the binding of θ-toxin by influencing the topology of cholesterol in membranes (Ohno-Iwashita *et al.*, 1991, 1992).

On the other hand, there is little information on the changes in the secondary and higher order structures of θ-toxin upon interaction with membranes. Upon insertion into a membrane, soluble proteins, whose hydrophilic side chains are outside and hydrophobic side chains inside, are expected to change their conformations so that the hydrophobic side chains become exposed to the membrane lipid bilayer (van der Goot *et al.*, 1992). Since θ-toxin and related thiol-activated cytolysins remain in a monomer form in solution (Iwamoto *et al.*, 1990; Morgan *et al.*, 1993) and are detected as oligomers in the membrane-inserting form as judged by electron microscopy (Sekiya *et al.*, 1993; Mitsui *et al.*, 1979), they are expected to undergo conformational changes during the membrane insertion process. Spectroscopic approaches, such as CD and fluorescence, are effective methods by which to analyze the structure of θ-toxin in connection with its cytolytic mechanism.

Recently, brominated phospholipids have been used to determine membrane protein topology (Gonzalez-Manas *et al.*, 1992). Bromine atoms are known to quench the intrinsic tryptophan fluorescence of proteins. The introduction of bromine atoms into the fatty acid acyl chain of a phospholipid makes possible the determination of the depth of a fluorophore group of a protein within the membrane.

In the present study, we used modified θ-toxins and brominated phospholipids and examined changes in the secondary structure of θ-toxin and the environment around the aromatic side chains upon interaction of the toxin with liposomal membranes by CD and fluorescence emission spectra measurements. Our data show that vesicles containing cholesterol induce a conformational change of θ-toxin with the rearrangement of aromatic side chains regardless of oligomer formation and that θ-toxin inserts partially into liposomal membranes with conversion of its aromatic side chains. This conformational change occurs at pH 5−7, the same range at which θ-toxin shows optimal binding to erythrocyte membranes.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (PC) from egg (EPC), bis-(bromostearoyl)phosphatidylcholine (Br-DSPC), and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Pelham, AL). Cholesterol was obtained from Sigma (St. Louis, MO). θ-Toxin and MCθ were prepared as described previously (Ohno-Iwashita *et al.*, 1986, 1990).

Preparation of Lipid Vesicles. Cholesterol was mixed with EPC, DOPC, or Br-DSPC (cholesterol:phospholipid 1:1, mol/mol) in chloroform and evaporated in a rotary evaporator. Hepes-buffered saline (pH 7.2) was added to the lipid film to give a final lipid concentration of 1.3 μmol/mL, and the suspension was vigorously vortexed. Small unilamellar vesicles were prepared by sonication with a sonication probe (Branson Sonifier 250). The vesicle suspension was then centrifuged at 9000*g* for 10 min to remove undispersed lipids and particles of titanium released from the probe. Liposomes without cholesterol were prepared similarly and used as controls. Cholesterol concentrations in the liposome preparations were determined by the method of Richmond (1973) with slight modification (Ohno-Iwashita *et al.*, 1988). Phospholipid concentrations in the liposome preparations were determined by quantifying choline release after phospholipase D treatment of the lipid samples.

Circular Dichroism (CD). CD spectra were obtained using a JASCO J-720 spectropolarimeter at 25 °C with quartz cells with a path length of 5 mm (far-ultraviolet) or 20 mm (near-ultraviolet). On the basis of amino acid analysis, the mean residue weight and extinction coefficient ($E_{280}^{0.1\%}$) of θ-toxin were estimated to be 110.8 and 1.6, respectively. The molecular ellipticity ([θ] in deg cm^2 dmol^{-1}) of θ-toxin and MCθ were calculated from these values. Spectra of liposomes alone were obtained as references and subtracted from the spectra obtained of toxins in the presence of liposomes. Neither EPC liposomes nor EPC/cholesterol liposomes produced any obvious light scattering at the concentrations used in our experiments.

Fluorescence. Fluorescence spectra were recorded at 25 °C on a Shimadzu spectrofluorophotometer RF-5000. The excitation wavelength was 280 or 295 nm, and slit widths were 5 nm for both excitation and emission. The Raman scatter contribution was removed by subtraction of blanks.

Determination of Hemolytic Activity of θ-Toxin and Modified Toxins. The hemolysis assay reaction mixture was composed of 0.75 mL of sheep erythrocyte suspension (0.67% hematocrit) and 0.25 mL of toxin solution diluted in phosphate-buffered saline (PBS, pH 7.0), Tris-buffered saline (pH 8.0 and 9.0), or carbonate-buffered saline (pH 10.0) containing 1 mg/mL bovine serum albumin (BSA). The mixture was incubated for 30 min at 37 °C and centrifuged, and the absorbance of the supernatant was determined at 540 nm.

Binding of θ-Toxin to Erythrocytes. θ-Toxin was incubated with erythrocytes in PBS (pH 7.0), Tris-buffered saline (pH 8.0 and 9.0), or carbonate-buffered saline (pH 10.0) containing 0.5−1.0 mg/mL BSA for 60 min at 10 °C. After centrifugation of the mixture, unbound toxin in the supernatant was removed, and the pellet was suspended in PBS (pH 7.0) containing BSA. The suspension was incubated for 30 min at 37 °C and centrifuged, and the absorbance of the supernatant was determined at 410 nm. The amounts of bound toxin were determined from the toxin dose−lysis titration curves at pH 7.0. In another binding experiment, θ-toxin was incubated with erythrocytes at different pH as described above and centrifuged. The pH of the resulting supernatant was adjusted to 7.0, and the aliquots were assayed for hemolytic activity in PBS containing BSA to determine the amounts of unbound toxin.

Sensitivity of θ-Toxin to Protease. θ-Toxin was preincubated with EPC or EPC/cholesterol liposomes at different pH for 30 min, after which time subtilisin Carlsberg was added to the solutions in the ratio 1:14 (w/w). The mixtures were incubated for 4 h at room temperature, and phenylmethanesulfonyl fluoride was added to stop proteolysis.

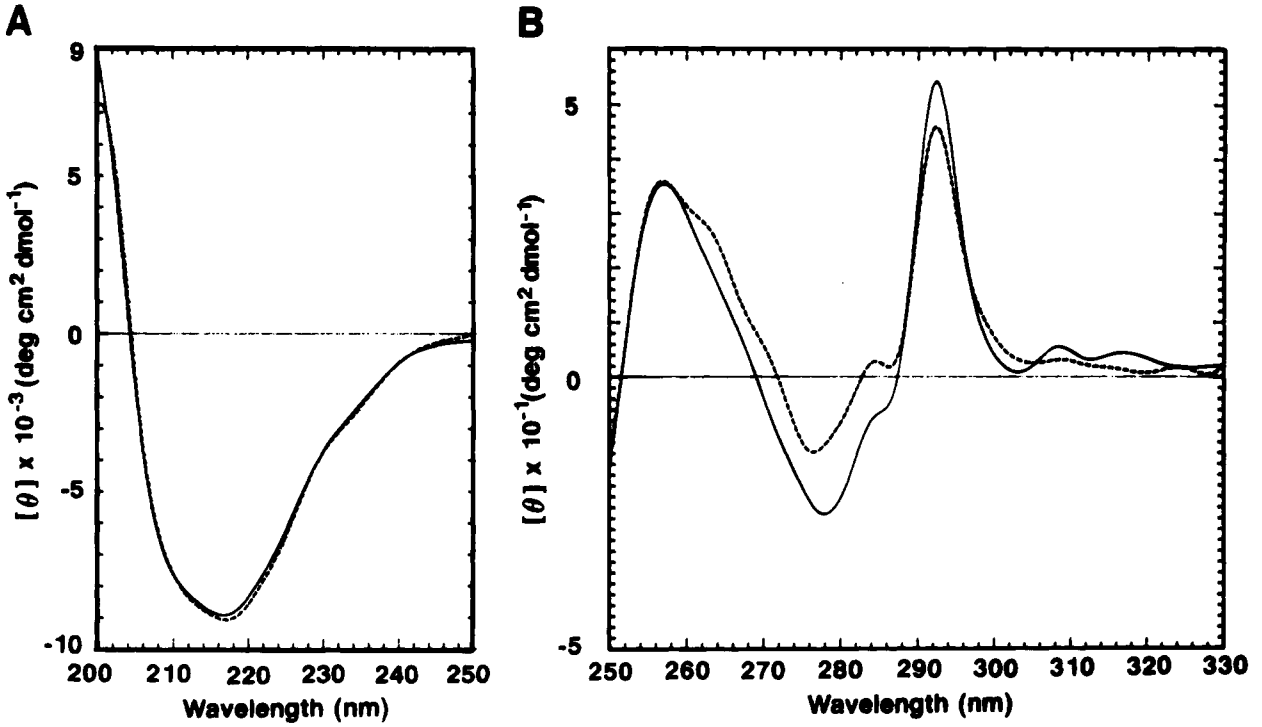

FIGURE 1: Structural similarity between θ-toxin and MCθ. (A) Far-UV CD spectra of θ-toxin (—) and MCθ (- - -). Samples were prepared at a concentration of 0.037 mg/mL in PBS (pH 7.0). Spectra were measured using a 5 mm path length cylindrical cuvette at 25 °C. (B) Near-UV CD spectra of θ-toxin (—) and MCθ (- - -). Samples were prepared at a concentration of 0.21 mg/mL in PBS (pH 7.0). Spectra were measured using a 20 mm path length cylindrical cuvette at 25 °C.

Sample diluter (125 mM Tris-HCl, pH 6.8/4% sodium dodecyl sulfate/10% 2-mercaptoethanol/20% glycerol/0.005% bromophenol blue) was added, and the mixtures were incubated at 100 °C for 3 min. The samples were electrophoresed on 12% acrylamide gels in the presence of SDS according to Laemmli (1970). Proteins were detected by silver staining, and protein bands were traced using a Shimadzu CS-9000 dual-wavelength flying-spot scanner.

RESULTS

Circular Dichroism in the Far-Ultraviolet Region. The secondary and higher order structures of θ-toxin and MCθ were compared based on circular dichroism (CD) measurements (Figure 1). The toxins show typical spectra of proteins rich in β structure with no significant differences observed in far-ultraviolet CD. In the near-ultraviolet region, the CD spectrum of MCθ differed slightly from that of native θ-toxin (Figure 1B).

We examined the CD spectral changes in these toxins caused by interaction with liposomes. θ-Toxin mixed with PC/cholesterol liposomes underwent significant spectral changes with a slight red shift of the minimum peak (Figure 2A). The CD spectrum of lysozyme, which does not bind to cholesterol, underwent no significant changes upon incubation with liposomes, indicating that the change described above is specific for θ-toxin (data not shown). Furthermore, the spectrum remained unchanged upon treatment of θ-toxin with cholesterol-free liposomes (Figure 2A), indicating that the spectral change is induced specifically by interaction of the toxin with cholesterol. Figure 3 shows the CD difference spectra of θ-toxin before and after incubation with liposomes. In the presence of cholesterol, a negative peak at 227 nm with a shoulder at 216 nm is seen in the difference spectrum. There was no CD spectral change seen in the reaction with cholesterol-free liposomes.

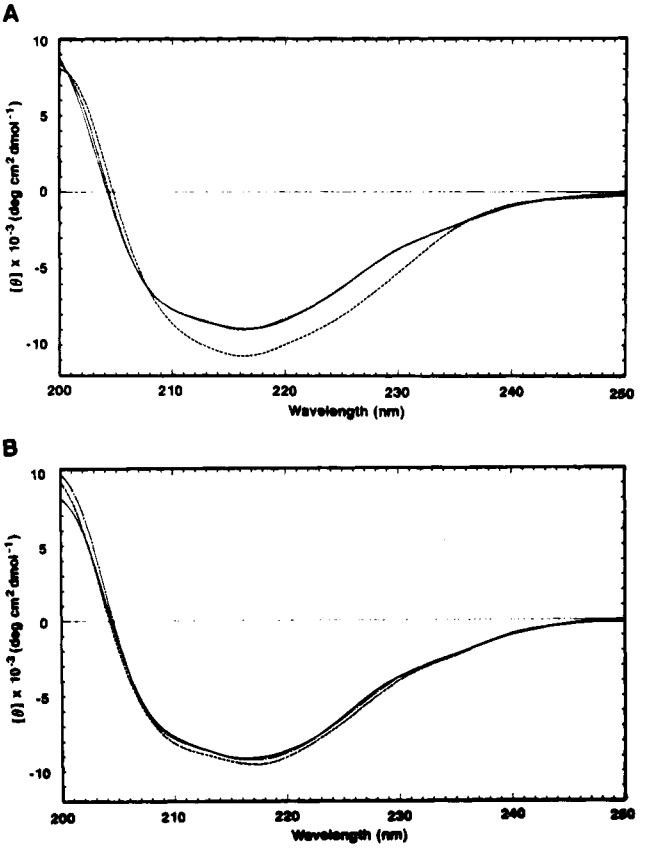

FIGURE 2: Far-UV CD spectra of θ-toxin and MCθ in the presence of liposomes at pH 7.0. Samples contained 0.3 nmol of θ-toxin (A) or MCθ (B) in 0.45 mL of PBS (pH 7.0). θ-Toxin or MCθ alone (—), θ-toxin or MCθ in the presence of EPC liposomes (— · —) (toxin/EPC 1:43, mol/mol), and θ-toxin or MCθ in the presence of EPC/cholesterol liposomes (- - -) (toxin/cholesterol 1:43, mol/mol). Spectra were measured using a 5 mm path length cylindrical cuvette at 25 °C.

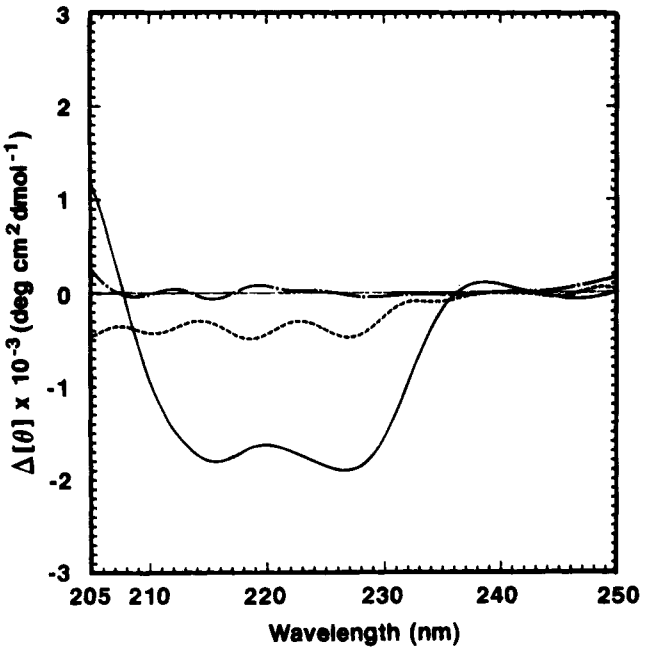

FIGURE 3: CD difference spectra for toxins in the presence and absence of liposomes. Each difference spectrum was obtained by subtracting the spectrum of the toxin alone from that of the toxin in the presence of liposomes using 0.037 mg/mL θ-toxin or MCθ. θ-Toxin incubated with EPC/cholesterol liposomes at 25 °C for 10 min (—). MCθ incubated with EPC/cholesterol liposomes at 25 °C for 10 min (- - -) (toxin/cholesterol 1:43, mol/mol). θ-Toxin or MCθ incubated with EPC liposomes (— • —) (toxin/EPC 1:43, mol/mol).

Additionally, the difference at 227 nm increased with both reaction time (Figure 4A) and the molar ratio of liposomal cholesterol to θ-toxin (Figure 4B). The reaction appeared complete by 20 min (Figure 4A). The decrease in molar ellipticity at 227 nm was proportional to the ratio of cholesterol to θ-toxin (mol/mol) up to a molar ratio of 30 and saturated at a molar ratio of about 60 (Figure 4B). The degree of ellipticity change shown in Figure 4B might reflect the amount of bound toxin.

The interaction of MCθ with PC/cholesterol liposomes produced essentially the same changes, but they were smaller than those produced by θ-toxin (Figures 2B and 3). In addition, the time course of the spectral changes for MCθ showed that the reaction was complete within 3 min, suggesting that the structural changes that accompany membrane binding are rapidly completed. The spectral changes in θ-toxin continued up to 15 min (Figure 4A), perhaps reflecting additional structural changes in θ-toxin following binding. These results suggest that the change in the secondary structure caused by toxin binding to membranes is very small and that a large part of the CD spectral change in the far-UV region can be ascribed to steps following binding.

It is not known whether the CD spectral changes at 227 nm are really attributable to changes in the secondary structure of the toxin or whether they reflect changes in the aromatic side chains, since a contribution of aromatic residues to CD peaks around 230 nm has been reported for proteins with low α-helix contents (Green & Melamed, 1966; Coleman, 1968). Since the time courses for the changes in the far-UV and tryptophan fluorescence intensity resemble each other as described later, the changes in the far-UV region seem to accompany structural changes around tryptophan residues.

Effect of pH on the Interaction of θ-Toxin with Liposomes. Several proteins, such as granulocyte colony stimulating factor and *Pseudomonas* exotoxin, require conformational changes induced at low pH for translocation across membranes (Jiang & London, 1990; van der Goot *et al.*, 1991; Collins & Cha, 1994). Thus, we examined the pH dependence of the interaction of θ-toxin with liposomes. The CD spectra of θ-toxin measured in the absence of liposomes showed no significant differences between pH 5 and pH 10 (data not shown), indicating that θ-toxin retains its secondary structure within the above pH range. However, the spectrum changed below pH 4. Figure 5 shows that the same CD spectral changes obtained in the reaction with liposomes at pH 7 were exhibited between pH 5 and pH 7. At pH 8, the spectral changes were smaller than at pH 5−7. Above pH 9, there were no spectral changes observed regardless of the presence of EPC or EPC/cholesterol liposomes.

To determine whether any relationship exists between hemolysis and the secondary structure of θ-toxin, we also measured hemolytic activity, binding activity, and sensitivity to proteases at various pH's. To examine the pH dependence of hemolytic activity, the amounts of θ-toxin required to cause 50% hemolysis of 1 mL of 0.5% sheep erythrocytes in 30 min at 37 °C (HD_{50}) were determined at pH 5−10. The HD_{50} values were 0.2−0.27 ng at pH 5−7 and 0.63 ng at pH 8, indicating that hemolytic activity at pH 8 is one-half to one-third that at pH 5−7. At pH 9−10, no hemolysis was detected even with the addition of 1.6 ng of θ-toxin.

Next, we examined the pH dependence of θ-toxin binding to erythrocyte membranes. After incubation of θ-toxin with erythrocytes at 10 °C for 60 min at various pH values, the unbound toxin was removed by centrifugation, and hemolysis by the bound toxin was determined as described in Materials and Methods (Figure 5). In parallel experiments, the amounts of unbound toxin in the supernatant were obtained by titration. Figure 5 shows that toxin binding is at a maximum at pH 6−7. In contrast, no hemolysis was detected in samples where toxin was preincubated with erythrocytes at pH 9 or above. In parallel with this result, titration of the hemolytic activity of θ-toxin in the centrifuged supernatant showed that almost 100% of the toxin was recovered in the supernatant, indicating that θ-toxin does not bind to erythrocyte membranes above pH 9. At pH 8.0, the binding was less than that at pH 7.0. These results indicate that the pH optima for toxin binding and for the change in ellipticity coincide closely.

In another experiment, we examined the effect of liposomes on the proteolysis of θ-toxin by a serine protease, subtilisin Carlsberg. This protease cleaves θ-toxin into two fragments, C1 (38 K) and C2 (15 K) (see Cθ in Scheme 1) at pH 5−10. The proteolysis was inhibited by preincubating θ-toxin at pH 5−7 with EPC/cholesterol liposomes (Figure 5) but not with cholesterol-free liposomes (data now shown), suggesting that θ-toxin is protected from proteolysis by binding to the liposomal membrane. In contrast, preincubation with liposomes at pH 8 only partially protected θ-toxin from proteolysis, and at pH 9−10 no protection was observed (Figure 5). These results suggest that θ-toxin binds only slightly to liposomes above pH 8. Thus, the pH dependency of the liposome-induced structural changes in θ-toxin parallels that of toxin binding to membranes. This strongly suggests that the structural change is caused by the binding of the toxin to membrane cholesterol.

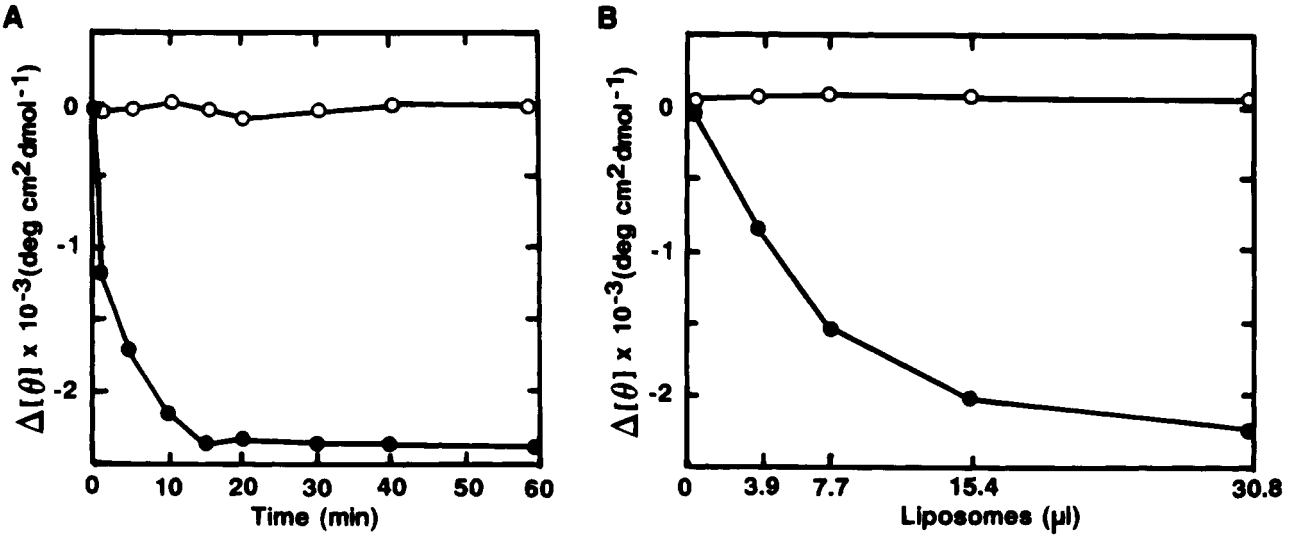

FIGURE 4: Difference in ellipticity of θ-toxin at 227 nm upon interaction with EPC or EPC/cholesterol liposomes. (A) CD spectral change at 227 nm as a function of reaction time at 25 °C. The reaction mixture (0.465 mL) contains 0.025 mg of θ-toxin and 15.4 μL of liposomes. (B) Effect of cholesterol/θ-toxin molar ratio on the spectral change. To the indicated amounts of liposomes, 0.055 mg/mL θ-toxin was added, and incubation was carried out at 25 °C for 20 min. Closed circles, difference in ellipticity of θ-toxin incubated with EPC/cholesterol liposomes from that of θ-toxin alone. Open circles, control experiments using liposomes without cholesterol. When 3.9, 7.7, 15.4, or 30.8 μL of EPC/cholesterol liposomes were added, the molar ratio of liposomal cholesterol to θ-toxin was 15, 30, 60 or 120, respectively.

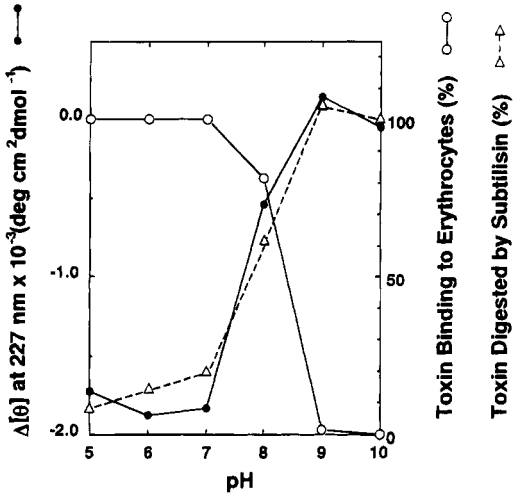

FIGURE 5: pH Dependence of CD spectral changes and toxin binding. θ-Toxin (0.3 nmol) was incubated with either EPC/cholesterol (13 nmol) or control EPC liposomes for 10 min at 25 °C at pH 5−10, and the far-UV CD difference spectrum was obtained for each pH (●). In control experiments, no spectral differences were observed between the far-UV CD spectra of θ-toxin alone and θ-toxin incubated with EPC liposomes at pH 5−10. For binding experiments, θ-toxin was incubated with sheep erythrocytes at the indicated pH for 60 min at 10 °C; the amount of bound toxin was determined as described in Materials and Methods and expressed relative to the amount bound at pH 7.0 (○). The amount of θ-toxin digested with subtilisin Carlsberg in the presence of EPC/cholesterol liposomes was determined as described in Materials and Methods and expressed as a relative value to that digested in the absence of liposomes (△).

Circular Dichroism in the Near-Ultraviolet Region. A drastic change in the CD spectrum of the near-ultraviolet region was observed when θ-toxin was incubated with EPC/cholesterol liposomes as shown in Figure 6A. The peak at 292 nm decreased remarkably and then shifted to 300 nm, suggesting a contribution by tryptophan residues to the spectral change. In parallel, when we examined the CD spectra of the same samples in the far-ultraviolet region using a cylindrical cuvette with a 1 mm path length, the same spectral patterns as shown in Figure 2A were obtained

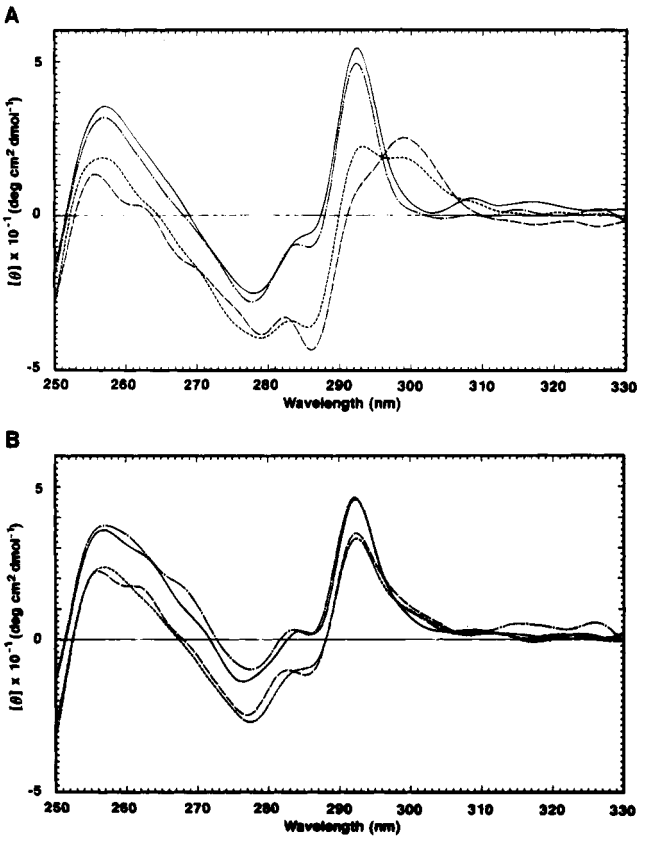

FIGURE 6: Change in the near-UV CD spectra of θ-toxin and MCθ upon interaction with liposomes. Each sample contained 6.8 nmol of θ-toxin (A) or MCθ (B) in Hepes-buffered saline (pH 7.0). Liposomes were added, and the mixtures were incubated at 25 °C. A path length of 2 cm was employed: (—) toxin alone; (— • —) toxin incubated with EPC liposomes; (- - -) and (— —) toxin incubated with EPC/cholesterol (280 nmol) liposomes for 2 and 20 min, respectively.

regardless of the 23-fold difference in the sample concentration. These results suggest that the interaction of θ-toxin with cholesterol in liposomal membranes leads to a dramatic change in the structure of the aromatic side chains of θ-toxin, although the overall secondary structure rich in β-sheet is

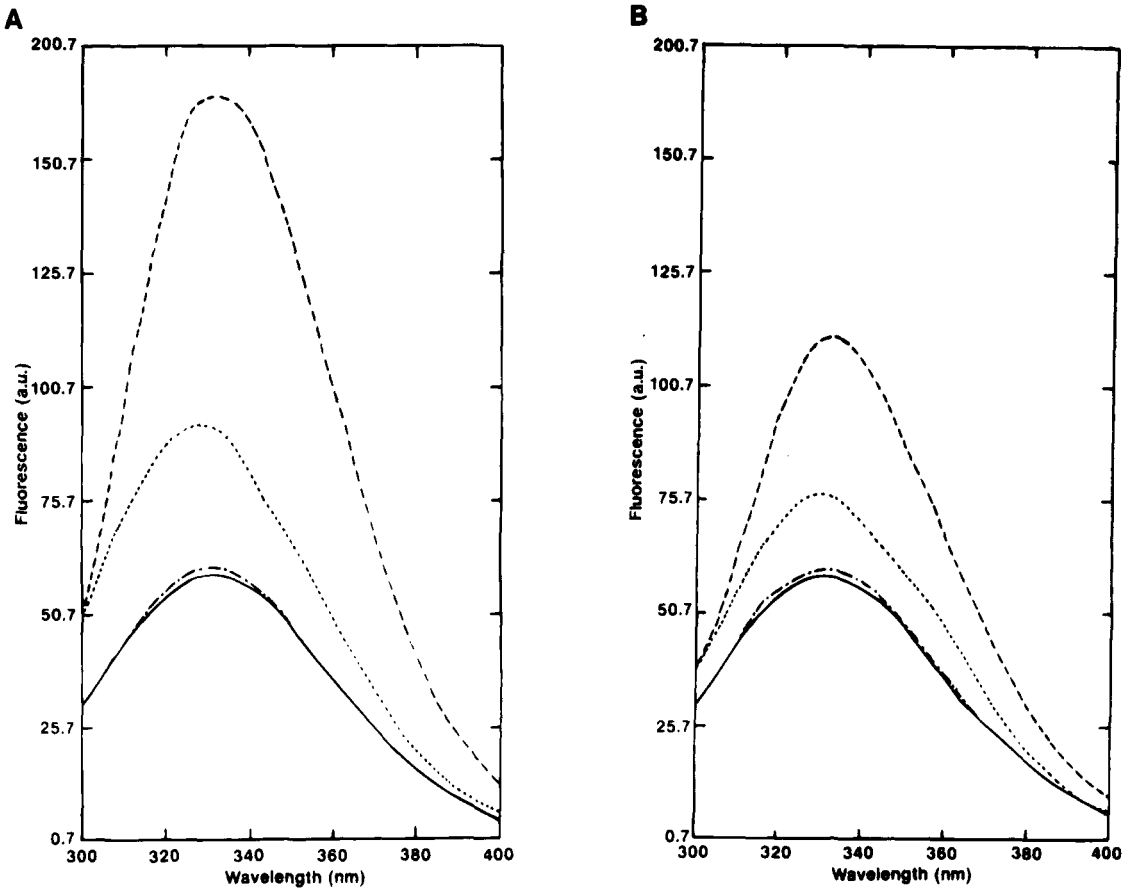

FIGURE 7: Fluorescence emission spectra of θ-toxin and MCθ in the presence or absence of liposomes. DOPC/cholesterol (— —), Br-DSPC/cholesterol (- - -), or Br-DSPC (— • —) liposomes were added to θ-toxin (A) or MCθ (B) in Hepes-buffered saline, and the mixtures were incubated for 10 min at 25 °C. The samples were excited at 280 nm for measurement of the intrinsic tryptophan fluorescence spectra. The molar ratio of toxin to liposomal cholesterol was 1:27. The solid lines indicate the fluorescence emission spectrum of θ-toxin (A) or MCθ (B) in the absence of liposomes; a.u. is arbitrary unit.

well-conserved. MCθ also showed changes in ellipticity of the near-UV peaks, although some peaks showed smaller changes than the corresponding θ-toxin peaks after incubation with EPC/cholesterol liposomes (Figure 6B). In particular, the peak at 292 nm did not change so much upon interaction of MCθ with liposomes, in contrast to the dramatic change in the θ-toxin peak (Figure 6). Because MCθ does not form oligomers, the spectral change in MCθ can be assigned to the structural changes in θ-toxin that occur during the binding and/or insertion processes, which could be induced by toxin monomer—membrane interaction. On the other hand, the θ-toxin-specific spectral changes at 292 and 300 nm might reflect the oligomerization process.

Fluorescence. The CD spectral data described above suggest an alteration in the local environmental conditions around aromatic side chains, especially tryptophan residues. The intrinsic tryptophan fluorescence was measured by exciting samples in Hepes-buffered saline at 280 or 295 nm and scanning the emission from 300 to 400 nm. The fluorescence spectra of θ-toxin (Figure 7A, solid line) and MCθ (Figure 7B, solid line) were the same, each molecule showing an emission maximum at 330 nm, indicating that most of the tryptophan residues are located in a hydrophobic environment. The addition of liposomes composed of both DOPC and cholesterol to θ-toxin induced a 3-fold maximum increase in fluorescence intensity but no peak shift (Figure 7A). Similarly, the fluorescence intensity of MCθ doubled upon incubation with DOPC/cholesterol liposomes (Figure 7B). The enhancement in fluorescence intensity was dependent on the cholesterol/toxin molar ratio, reaction time, and pH, showing similar patterns as described in Figures 4 and 5 for the ellipticity changes in the far-UV CD spectra. Since experiments at an excitation wavelength of 295 nm gave essentially the same results, the enhancement in intensity could be assigned to tryptophan residues. EPC/cholesterol liposomes produced similar results, but liposomes without cholesterol caused no fluorescence enhancement, indicating that no interaction takes place with liposomal membranes in the absence of cholesterol. These results together with the near-UV CD spectral data strongly suggest that significant changes in the toxin microstructure around tryptophan residues are induced by interaction with cholesterol.

Membrane Insertion. It remains unknown whether oligomerization is essential for the insertion of θ-toxin and related thiol-activated cytolysins into membranes. In experiments using Cθ and MCθ, which bind membrane cholesterol with affinities as high as native θ-toxin (Ohno-Iwashita *et al.*, 1988, 1990), we previously demonstrated that the θ-toxin monomer can bind to membrane cholesterol without oligomer formation. Now using MCθ, we examined whether the toxin monomer inserts into membranes following binding. For this purpose, we examined the quenching of the intrinsic fluorescence of tryptophan residues in the θ-toxin molecule by liposomes containing brominated lecithin, since bromine atoms are known to quench the intrinsic tryptophan fluorescence of proteins located in their vicinity (Bolen & Holloway, 1990). If tryptophan residues in the toxin

molecule insert into the membrane, bromine atoms located at the midpoint of the phospholipid acyl chains will quench their fluorescence. The reaction of θ-toxin and MCθ with Br-DSPC/cholesterol liposomes produced a smaller increase in fluorescence intensity with a slight blue shift in the fluorescence emission maximum (Figure 7), indicating the quenching of the enhanced tryptophan fluorescence. The degree of quenching depended on the amount of bromine contained in the lecithin/cholesterol liposomes (data not shown). θ-Toxin incubated with Br-DSPC/cholesterol liposomes gave essentially the same far-UV CD spectrum as θ-toxin incubated with EPC/cholesterol liposomes (data not shown), indicating that bromination of liposomal lecithin affects neither the binding of θ-toxin to liposomes nor the structural changes. These results suggest that the unquenched tryptophan residues are located in a more hydrophobic environment than the quenched ones and that some of the tryptophan residues of MCθ as well as θ-toxin are at least partially inserted into liposomal membranes. Thus, it appears that the monomer form of the toxin undergoes changes in microstructure around tryptophan residues and inserts into liposomal membranes.

DISCUSSION

Many bacterial toxins interact with the membranes of target cells and then insert into the lipid bilayer to exert their toxicity. In the light of recent research, the insertion of toxins into membranes requires the partial unfolding of the protein and conformational changes (Verner & Schatz, 1988; Parker *et al.*, 1990). For example, colicin A is a well-characterized toxin that consists of three domains; the N-terminal domain is responsible for protein translocation, the central domain is the receptor-binding domain, and the C-terminal domain forms the pore (Pattus *et al.*, 1990). Spectroscopic studies on colicin A associated with membranes revealed that its pore-forming domain does not undergo drastic unfolding but that the environment around the aromatic side chains changes markedly upon insertion into membranes (van der Goot *et al.*, 1991; Lakey *et al.*, 1991; Gonzalez-Manas *et al.*, 1992).

Although thiol-activated cytolysins have been reported to form membrane-spanning oligomeric pores, there is little information on the membrane insertion mechanism of θ-toxin or other thiol-activated cytolysins. In this study, we used a modified θ-toxin (MCθ) to analyze binding and insertion steps separately from the oligomerization step. MCθ binds to membrane cholesterol with the same affinity as θ-toxin, but causes no membrane lysis (Ohno-Iwashita *et al.*, 1990). MCθ does not form oligomeric structures, which is probably why MCθ has no cytolytic activity. We speculate that at least one of the sites for oligomerization might be damaged in MCθ by methylation of the protein. Methyl groups introduced near the oligomerization sites could inhibit toxin assembly through steric hindrance. There is also the possibility that methylation might cause a conformational change in θ-toxin structure, resulting in the loss of oligomerization activity. Although MCθ has almost the same secondary structure as θ-toxin (Figure 1A), its side chain structure is slightly different (Figure 1B). This conformational difference in side chains might be related to the loss of oligomerization activity in MCθ.

Using θ-toxin and MCθ, we demonstrated that the binding of θ-toxin to membrane cholesterol triggers membrane insertion and conformational changes. These conformational changes accompany rearrangement of the aromatic side chains as shown by changes in ellipticity in the near-UV region (Figure 6) and enhancement of the fluorescence emission spectra of tryptophan residues (Figure 7). Upon interaction with liposomes, tryptophan fluorescence is more enhanced in θ-toxin than in MCθ (Figure 7). This difference probably reflects the oligomerization-specific change around tryptophan residues along with the θ-toxin-specific change observed in the near-UV CD spectra. Our results also suggest that the membrane-inserted form of θ-toxin has a different conformation from the soluble form with respect to side chain structure, although the secondary structure, as shown by the far-UV CD spectra, is well-conserved (Figure 2). These observations suggest a 'molten globule' state (Ohgushi & Wada, 1983) in which the protein retains a secondary structure comparable to the native protein but lacks a rigid tertiary structure. Recently, this transition state has been implicated in the membrane insertion of several proteins (Bychkova *et al.*, 1988; van der Goot *et al.*, 1991; Collins & Cha, 1994). Some of these proteins are shown to undergo conformational changes upon reaction with membranes at low pH together with increases in helicity. A model has been proposed where acid-induced conformational changes cause exposure of hydrophobic α-helices, resulting in insertion into membranes (Parker *et al.*, 1990). In contrast, the largest conformational changes in θ-toxin were obtained at neutral pH upon the addition of EPC/cholesterol liposomes but not cholesterol-free liposomes. In addition, θ-toxin is rich in β-structure, and no enhancement of helicity was detected. In the case of colicin A, negatively charged lipids in addition to acidic pH are required for membrane insertion (Lakey et al., 1991; van der Goot et al., 1991; Gonzalez-Manas et al., 1992). Negatively charged phospholipids are known to promote a lower surface pH and electrostatic interaction of colicin A with lipid vesicles. Neutral lipids, such as phosphatidylcholine used in our experiments, do not produce this electrostatic effect. θ-Toxin binds to EPC/cholesterol liposomes without the aid of negative charges on the cell surface. Furthermore, our data clearly demonstrate that cholesterol is indispensable for binding and conformational changes of θ-toxin. No other factors are required for the structural change. Thus, the membrane insertion process of θ-toxin is distinctly different from the low pH-induced insertion mechanism, such as that of colicins, and cholesterol molecules are postulated to play a key role in the process.

The present fluorescence emission spectral data on intrinsic tryptophan confirm that the microenvironment around tryptophan residues is apparently altered during the interaction of θ-toxin with cholesterol molecules in liposomal membranes. θ-Toxin denaturated by 6 M guanidine hydrochloride undergoes a similar enhancement in fluorescence, but the peak shifts to 350 nm (data not shown), indicating that tryptophan residues become exposed in the hydrophilic atmosphere produced by the denaturant. In contrast, both soluble and membrane-associated forms of θ-toxin and MCθ show an emission maximum at around 330 nm (Figure 7). This indicates that most of the tryptophan residues are in a hydrophobic environment, either buried in the hydrophobic interior of the protein or exposed in the hydrocarbon core moiety of the lipid bilayer. Since brominated lecithin in cholesterol-containing liposomes quenches the tryptophan fluorescence of the membrane-associated forms of the toxins (Figure 7), it is likely that some of the tryptophan fluoro-

phores that are buried in the protein interior of the soluble forms become exposed to the lipid bilayer upon membrane insertion. In addition, the enhancement of fluorescence upon membrane insertion implies that membrane-associated forms of the toxins might undergo a loosening of their tertiary structures, during which tryptophan residues might be released from close packing with intramolecular quenchers or energy acceptors as in the case of fluorescence enhancement upon toxin denaturation.

A comparison of the DNA and predicted amino acid sequences of θ-toxin (Tweten, 1988; Shimizu *et al.*, 1991) with those of streptolysin O (Kehoe *et al.*, 1987), listeriolysin O (Mengaud *et al.*, 1987), pneumolysin (Walker *et al.*, 1987), and alveolysin (Geoffroy *et al.*, 1990) shows considerable homology among these proteins. Each of these thiol-activated cytotoxins has a single cysteine residue near the C terminus lying within an 11 amino acid sequence (ECTGLAWEWWR; residues 430−440) that is conserved in these thiol-activated cytolysins. Three of the seven tryptophans in θ-toxin are contained in this consensus sequence. It has been pointed out that the amino acids in this region have the potential to form an amphiphilic helix (Mitchell *et al.*, 1992), making the region a candidate for the membrane insertion domain. In addition, the four other tryptophans also lie at sites that are invariable among other toxins in this family.

We have previously reported that modification of the thiol group causes a decrease in the binding affinity for membranes (Iwamoto *et al.*, 1987). Furthermore, the C-terminal 25-kDa fragment of trypsin-nicked θ-toxin, T2 (residues 277−472), has the same binding activity to erythrocyte membranes and specificity for cholesterol as intact θ-toxin (Iwamoto *et al.*, 1990). These findings suggest that the C-terminal region of θ-toxin is important for binding to membranes. Interestingly, six of the seven tryptophan residues are located in the C-terminal region (residues 382−472, see Scheme 1), which comprises about one-fifth of the total protein length. In contrast, the N-terminal region contains only one tryptophan residue (Trp-137). We have recently prepared a mutant θ-toxin in which Trp-137 is replaced by Phe and examined its tryptophan fluorescence upon interaction with cholesterol-containing liposomes. The Trp-137 mutant shows fluorescence patterns similar to those of wild-type θ-toxin with respect to fluorescence enhancement by DOPC/cholesterol liposomes and quenching by liposomes containing brominated lecithin and cholesterol, suggesting that the dramatic change in tryptophan fluorescence shown in Figure 7 is mainly ascribed to the other six tryptophan residues located in the C-terminal region (Nakamura, M., Sekino, N., and Ohno-Iwashita, Y., unpublished results). This suggests that at least some part in the C-terminal region undergoes rearrangement upon binding and insertion into membranes. Although the exact regions of θ-toxin involved in the lytic process are unknown, these results suggest that tryptophan residues in the C-terminal region play an important role in the interaction of θ-toxin with membranes.

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