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Contribution of Tryptophan Residues to the Structural Changes in Perfringolysin O during Interaction with Liposomal Membranes

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Perfringolysin O (θ-toxin) is a cholesterol-binding and pore-forming toxin that shares with other thiol-activated cytolysins a highly conserved sequence, ECTGLAWEWWR (residues 430-440), near the C-terminus. To understand the membrane-insertion and pore-forming mechanisms of the toxin, we evaluated the contribution of each Trp to the toxin conformation during its interaction with liposomal membranes. Circular dichroism (CD) spectra of Trp mutant toxins indicated that only Trp436 has a significant effect on the secondary structure, and that Trp436, Trp438, and Trp439 make large contributions to near-UV CD spectra. Quenching the intrinsic Trp fluorescence of the wild-type and mutant toxins with brominated lecithin/cholesterol liposomes revealed that Trp438 and probably Trp436, but not Trp439, contributes to toxin insertion into the liposomal membrane. Near-UV CD spectra of the membrane-associated mutant toxins indicated that both Trp438 and Trp439 are required for the CD peak shift from 292 to 300 nm, a signal related to θ-toxin oligomerization and/or pore formation, suggesting a conformational change around Trp438 and Trp439 in these processes.

Key words: cholesterol-binding cytolysin, membrane insertion, perfringolysin O, pore-forming toxin, tryptophan.

θ-Toxin (perfringolysin O; M_r, 52,700) is a cytolytic toxin produced by *Clostridium perfringens* type A and one of a group of oxygen-labile and thiol-activated cytolysins (*1, 2*). θ-Toxin contains seven tryptophan residues (Scheme 1), three of which (Trp436, Trp438, Trp439) are located within an 11 amino acid sequence (ECTGLAWEWWR; residues 430-440) near the C-terminus (*3, 4*). This sequence is the longest conserved sequence among thiol-activated cytolysins and contains a unique cysteine, thus is called as the common Cys-containing sequence. The lytic process triggered by θ-toxin is thought to comprise at least four steps: binding to cholesterol in the cellular membrane, insertion into the membrane, oligomerization, and pore formation. Although the mechanism of θ-toxin binding to membrane cholesterol has been studied extensively (*5, 6*), studies on membrane insertion and changes in toxin structure during interaction with membranes have been limited.

Our recent study involving circular dichroism and fluorescence emission spectroscopy demonstrated that liposomes containing cholesterol induce a conformational change in θ-toxin with rearrangement of aromatic side chains, especially tryptophan residues (*7*). We also demonstrated that a tryptophan-containing region of θ-toxin inserts into liposomal membranes following toxin binding to the membrane (*7*). This conformational change appears to be similar to those of several proteins that cross membranes *via* a molten globular state (*8, 9*). This indicates that a partially unfolded, compact state with a native-like secondary structure is required for the membrane translocation of these proteins. Parker and Pattus (*10*) reported that a change in pH is the factor most commonly observed to trigger such conformational change leading to membrane insertion of bacterial toxins *in vivo*. In the case of θ-toxin, cholesterol is essential for the conformational change that occurs at pH 5-7, the same range as that in which θ-toxin shows optimal binding to erythrocyte membranes and hemolytic activity (*7*). The membrane insertion process is postulated to be distinctly different from the low pH-induced insertion mechanism, such as that seen in colicins (*11*). Therefore, to understand the insertion mechanism of θ-toxin, it is interesting to assess the contribution of each individual tryptophan residue to the conformational change that occurs during the interaction with the membrane. However, the CD and fluorescence emission spectra of θ-toxin exhibit the summed profiles of the seven tryptophans, making it difficult to sort out the contributions of the individual tryptophans. To circumvent this problem, we have recently prepared mutant toxins in

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Abbreviations: Br-DSPC, 1,2-distearoyl(9,10-dibromo)phosphatidylcholine; Cθ, subtilisin Carlsberg-digested θ-toxin; DOPC, dioleoylphosphatidylcholine; EPC, phosphatidylcholine from egg yolk; MCθ, reductive methylated Cθ; PC, phosphatidylcholine; PBS, phosphate-buffered saline.

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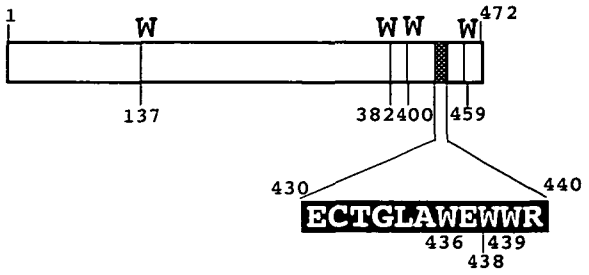

Scheme 1. **Structure of θ-toxin.** θ-Toxin contains seven tryptophan residues. An 11 amino acid sequence (residues 430-440) is the longest conserved sequence among thiol-activated cytolysins. This region is called the common Cys-containing region in the present study.

which tryptophan residues are replaced by phenylalanine and examined the hemolytic, binding, and oligomerization activities of these Trp mutant toxins (*12*). The results indicate that mutations of Trp436, Trp438, or Trp439 in the common Cys-containing region cause a marked decrease in the binding activity to erythrocyte membranes and hemolytic activity.

Recently, Rossjohn *et al.* (*13*) reported the 3D structure of perfringolysin O. According their data on the crystal structure, the toxin molecule consists of four domains and adopts an unusually elongated shape. Six of the total of seven Trp residues reside in Domain 4 and only one, Trp137, is located in Domain 1. Domain 4 is postulated to be a cholesterol-binding and membrane-spanning domain. Among the Trp residues in Domain 4, Trp436, Trp438, and Trp439 are located near the end of the molecule, at which site membrane binding and insertion are postulated to occur. These data on the crystal structure are consistent with our observation showing the importance of the Trp residues in the common Cys-containing region. However, the roles of individual Trp residues in each step of cytolysis remain unclear.

In the present study involving CD and fluorescence emission spectroscopy, we used these Trp mutant toxins to examine the contribution of individual tryptophan residues to the conformational change of θ-toxin during interaction with liposomal membranes. Our results reveal the importance of three tryptophans, Trp436, Trp438, and Trp439, and their distinct roles from each other in retaining the conformation of θ-toxin and in each step of toxin action. Trp436 is shown to make the largest contribution to the secondary structure and the microenvironment around the side chains of θ-toxin. Trp438 seems to be essential for efficient insertion of the θ-toxin molecule into liposomal membranes. Our results also suggest that Trp438 and Trp439 are related to the oligomerization and/or pore formation of θ-toxin molecules.

MATERIALS AND METHODS

Materials—Phosphatidylcholine (PC) from egg (EPC), 1,2-distearoyl(9,10-dibromo)phosphatidylcholine(Br-DSPC), and dioleoylphosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids (Pelham, AL). Cholesterol was obtained from Sigma (St. Louis, MO). θ-Toxin was prepared as described previously (*14, 15*).

Mutagenesis and Protein Purification—Oligonucleotide-directed mutagenesis and the preparation of wild-type and mutant θ-toxin proteins were performed as described previously (*12*). The following mutant toxins were used: W137F, W382F, W400F, W436F, W438F, W439F, W459F, T432V, and W438.439F having double mutations at W438 and W439. For the production of these protein variants, *Escherichia coli* BL21 (DE3) harboring the mutant plasmid was used. The mutant toxins were purified as described previously (*12*). The purities of the proteins were verified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Laemmli (*16*). With the exception of W436F, all mutant and wild-type toxin preparations showed a single band with no detectable impurities on the gel. The SDS-PAGE of the W436F preparation showed an additional minor band at around 60 kDa.

Preparation of Lipid Vesicles—Small unilamellar vesicles were prepared by sonication with a sonication probe (Branson Sonifier 250) as described previously (*7*). Briefly, EPC, DOPC, or BrDSPC was codissolved with cholesterol in chloroform in a ratio of 1:1 (mol/mol) and dried. The dried lipid film was suspended in HEPES-buffered saline and the lipid dispersions were sonicated. The resulting vesicle suspensions were centrifuged at 9,000×*g* for 10 min. Cholesterol and phospholipid concentrations in the liposome preparations were determined by the method of Richmond (*17*) and by quantifying choline release after phospholipase D treatment, respectively.

Circular Dichroism—CD spectra were recorded on a JASCO J-720 spectropolarimeter at 25°C using quartz cells with a path length of 5 mm (far-ultraviolet) or 20 mm (near-ultraviolet). Each CD spectrum represents the average of three or five scans. On the basis of amino acid analysis, the mean residue molecular weights of the wild-type and mutant toxin proteins were estimated to be 110.8. The extinction coefficients ($E^{0.1\%}_{280}$) of the wild-type and mutant toxin proteins were as follows: 1.6 for wild-type, W382F, W436F, W438F, and W439F; 1.5 for W137F, W400F, and W459F; 1.4 for W438.439F. The molecular ellipticity ($[\theta]$ in deg·cm^2·dmol^{-1}) of the wild-type and mutant toxins was calculated from these values. Spectra of liposomes alone were obtained as references and subtracted from the spectra 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 spectrofluorometer RF-5000. All measurements were made in 1-cm quartz cells with excitation at 295 or 280 nm. Spectral bandwidths were 5 nm for both excitation and emission. The Raman scatter contribution was removed by subtracting blanks.

Electron Microscopy—EPC/cholesterol liposomes were incubated with wild-type or mutant toxin in 50 μl of PBS for 20 min at 25°C. The same concentration of toxin protein (0.7-3.8 nmol) and molar ratio of toxin to liposomal cholesterol (1:33 or 1:43) were used as those for CD spectroscopy. Aliquots of toxin-treated liposomal membranes were negatively stained with 1% phosphotungstic acid (pH 6.5-7.2). Observations were made on a JEM-200CX electron microscope operating at 80 kV (*18*).

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RESULTS

Effect of Mutations on the Structure of the θ-Toxin Molecule—The global conformation of recombinant wild-type θ-toxin was compared with that of native θ-toxin purified from *Clostridium perfringens* type A by CD spectroscopy. The CD spectrum of the recombinant toxin was identical with the previously reported spectrum of native θ-toxin (7) in the wavelength region 200-330 nm (data not shown). Both toxins gave the broad peak of negative ellipticity centered at 216 nm, indicating that they are rich in β-structure. The data on crystal structure also show that θ-toxin is rich in β sheets with 40% β sheet made up of 25 β strands contributing to five sheets (*13*). The CD spectrum of each toxin in the near-UV region contains typical positive bands at 256 and 292 nm and a negative band at 277 nm.

A comparison of the far-UV region (200-250 nm) of the CD spectra of mutant toxins with the wild-type toxin showed the CD spectra to be indistinguishable in this spectral range, except for the W436F mutant toxin (*12*). There was an increase in the negative ellipticity around 208 nm in the far-UV CD spectrum of the W436F mutant toxin, suggesting a higher α-helix content than in the wild-type or other mutant toxins. These results suggest that Trp436 makes a large contribution to the secondary structure of the θ-toxin molecule and that mutations of the other six tryptophan residues do not perturb the overall conformation of the molecule.

To estimate the influence of Trp mutations on the microenvironment around the side chains of θ-toxin, we recorded the CD spectra in the near-UV region for all of the Trp mutant toxins. Wild-type and all mutant toxins showed a sharp peak of positive ellipticity at 292 nm (Fig. 1 and data not shown). The W459F mutant toxin gave a CD spectrum similar to that of the wild-type θ-toxin with

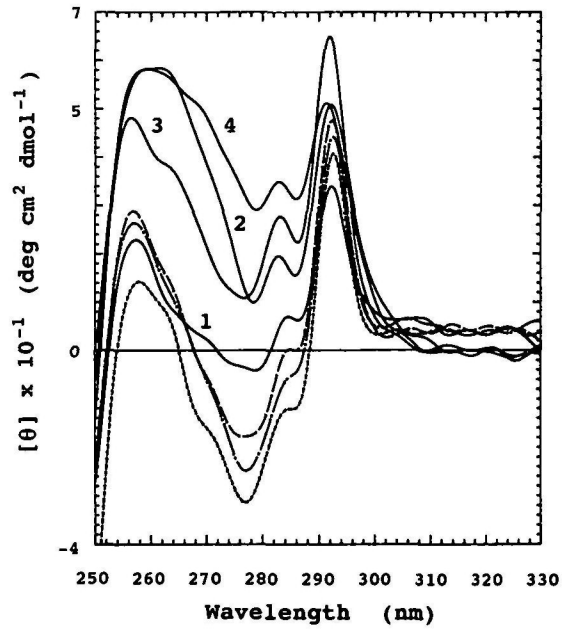

Fig. 1. **CD spectra of mutant toxins and wild-type θ-toxin in the near-UV region.** Near-UV CD spectra of wild-type (·····), W436F (¹——), W438F (²——), W439F (³——), W438.439F (⁴——), W459F (····), and T432V (----). Samples were prepared at a concentration of 3.8 nmol/ml in PBS (pH 7.0). Spectra were measured using a 20 mm path length cylindrical cuvette at 25°C.

TABLE I. **Fluorescence emission[a] at pH 7.0 measured as the maximal intensity of each mutant.**

Mutant toxin	Wavelength emission maximum (nm)		Relative intensity (%)	
	$\lambda_{ex}=280$	$\lambda_{ex}=295$	$\lambda_{ex}=280$	$\lambda_{ex}=295$
Wild-type	330	336	100	100
W137F	333	338	103	97
W382F	330	337	86.7	83
W400F	332	336	102	103
W436F	339	341	119	137
W438F	328	335	102	97
W439F	331	335	204	186
W438.439F	326	333	123	115
W459F	332	336	124	126

[a]Measurements were carried out in HEPES-buffered saline (pH 7.0) at 25°C using 280 nm ($\lambda_{ex}=280$) or 295 nm ($\lambda_{ex}=295$) excitation.

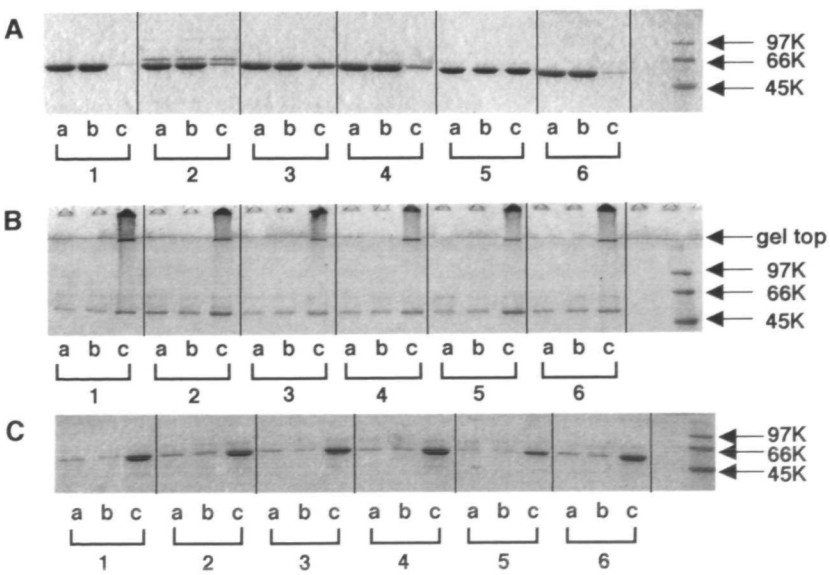

Fig. 2. **Detection of liposome-bound toxin by SDS-PAGE.** (A) Wild-type toxin (1), W436F (2), W438F (3), W439F (4), W438.439F (5), and T432V (6) were incubated with or without liposomes at 25°C for 20 min. After incubation, the samples were centrifuged 350,000×*g* for 30 min, and their supernatants were mixed with an equal volume of the sample buffer for SDS-PAGE. Aliquots were electrophoresed on a 12% slab gel in the presence of SDS. (B and C) The pellets were suspended in PBS, and the suspension was mixed with an equal volume of the sample buffer. The mixture was divided into two fractions, and aliquots of each fraction were electrophoresed on a 12% slab gel in the presence of SDS either without heat treatment (B) or after heating at 100°C for 10 min (C). a, b, and c represent toxin alone, toxin with EPC liposomes, and toxin with EPC/cholesterol liposomes (toxin/cholesterol=1 : 33, mol/mol), respectively.

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positive bands at 255 and 292 nm and a negative band at 277 nm (Fig. 1). Similar results were obtained with W137F, W382F, and W400F mutant toxins (data not shown). In these mutant toxins, the replacement of Trp by phenylalanine has little effect on the near-UV CD spectra. In contrast, there are significant changes in the near-UV region of the CD spectra of W436F, W438F, W439F, and W438.439F mutant toxins, which have mutations within the common Cys-containing region (Fig. 1). Positive ellipticity around 257 nm greatly increased in W438F, W439F, and W438.439F mutants. This change might be ascribed to phenylalanine introduced at 438 and 439 positions. On the other hand W436F shows no change around 257 nm but a decreased negative CD signal around 277 nm. The contribution of each Trp residue was evaluated by CD difference spectra obtained by subtracting the mutant spectrum from the wild-type spectrum. Comparison of the CD difference spectra of W438F, W439F, and W438.439F shows that the spectral change caused by double mutation at Trp438 and Trp439 is just the sum of the changes caused by the individual Trp mutations (data not shown). To examine whether mutations of any other amino acids in the common Cys-containing region cause changes in the near-UV CD spectrum, we recorded the CD spectrum of the T432V, a mutant toxin with lower hemolytic activity than W439F (*12*). The spectrum was almost identical to that of the wild-type θ-toxin (Fig. 1), indicating that this mutation within the common Cys-containing region does not cause any changes of microenvironment around aromatic residues despite causing the loss of hemolytic activity.

Effect of Mutations on the Fluorescence Properties of θ-Toxin—The fluorescence emission spectrum of the recombinant wild-type toxin in HEPES-buffered saline (pH 7.0) is similar to that of native θ-toxin purified from

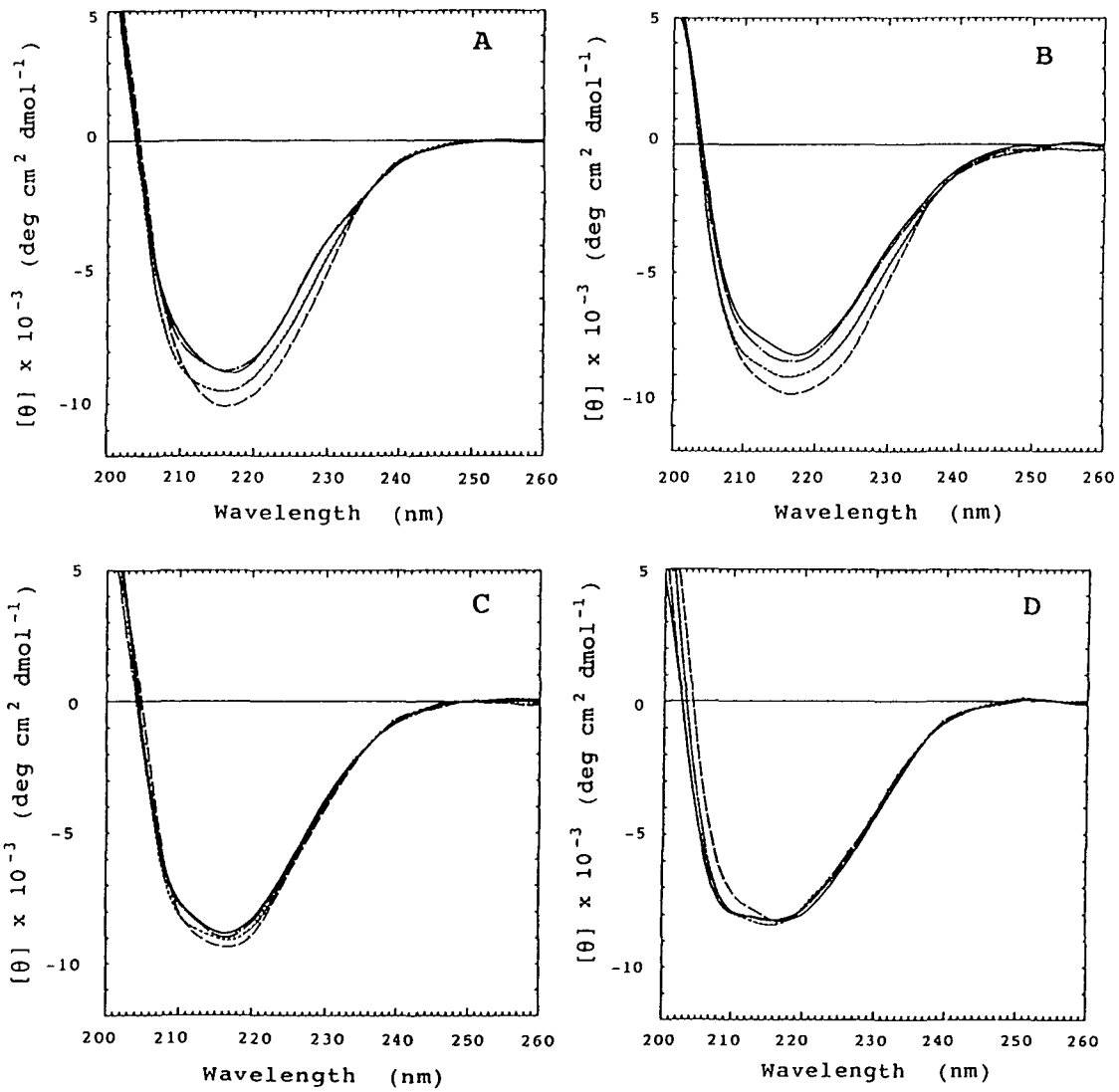

Fig. 3. **Far-UV CD spectra of wild-type and Trp mutant toxins in the presence of liposomes at pH 7.0.** Samples contained 0.7 nmol/ml of the wild-type θ-toxin (A), W137F (B), W438.439F (C), or W436F (D) mutant toxins in PBS (pH 7.0). Spectra were measured using a 5 mm path length cylindrical cuvette at 25°C. Wild-type or mutant toxins alone (——), wild-type or mutant toxins incubated with EPC liposomes at 25°C for 20 min (-·-·-), and wild-type or mutant toxins incubated with EPC/cholesterol liposomes (toxin/cholesterol = 1:43, mol/mol) at 25°C for 2 min (·····) or 20 min (----).

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Clostridium perfringens with a maximum at 330 nm (excitation at 280 nm) or at 336 nm (excitation at 295 nm), indicating that the Trp residues are located in a hydrophobic environment (Table I). The profiles of W436F, W438F, and the double mutant W438.439F differ from that of the wild-type toxin under both 280 and 295 nm excitation. The fluorescence emission spectra of the W438F and W438.439F mutant toxins show a blue shift, while that of the W436F mutant toxin is red-shifted relative to the wild-type toxin. The emission wavelength maximum of the T432V mutant toxin is red-shifted to 339 nm (data not shown), like that of the W436F mutant toxin. The blue shift of W438F was detected in five measurements. Therefore we conclude that it is a significant difference, although it is within the wavelength accuracy of the fluorometer (±2 nm). The emission intensities were measured at the λ_{max} of each mutant. The results are summarized in Table I. Trp439 makes a negative contribution to the fluorescence intensity, because the W439F mutant toxin exhibits a remarkable increase in fluorescence compared with the wild-type θ-toxin under both 280 and 295 nm excitation. The results also indicate that the Trp residues must be the primary fluorophore under 280 nm excitation.

Binding of Mutant Toxin Proteins to Liposomal Membranes—We have previously shown that W439F, W436F, W438F, and W438.439F mutant toxins have lower binding affinities to erythrocytes than the wild-type toxin in this order (*12*). Therefore, we firstly examined whether these mutant toxins interact with liposomal membranes in the same manner as with erythrocytes. Wild-type and mutant toxins were incubated with EPC/cholesterol liposomes (toxin/cholesterol=1:33, mol/mol) and the mixture was centrifuged at 350,000×*g* for 30 min. The supernatant (Fig. 2A) and pellet (Fig. 2, B and C) fractions of each toxin were analyzed by SDS-PAGE. Almost all of the wild-type toxin (53 kDa band) in the supernatant fraction disappeared after association with EPC/cholesterol liposomes (Fig. 2A, lane 1c), and the liposome-bound toxin appeared as SDS-resistant oligomers in the top of the gel (Fig. 2B), which formed a 53 kDa band after heat treatment (Fig. 2C). In the case of W436F and W439F mutant toxins, not all but large amounts of the toxins bind to liposomes (Fig. 2, lanes 2c and 4c). W438F and W438.439F mutant toxins also bind to liposomes (Fig. 2, lanes 3c and 5c), although in smaller amounts than that detected in W439F sample. No binding was detected by the incubation of any mutant toxin with cholesterol-free liposomes (Fig. 2, lanes b), indicating cholesterol-specific binding. These results suggest that W438.439F, W438F, W436F, and W439F have lower affinity to liposomal membrane containing cholesterol than the wild-type toxin, as shown in the experiments with erythrocytes. These Trp mutant toxins are useful to examine structural changes of the θ-toxin molecule during cytolysis.

Far-UV CD Spectra of Mutant Toxin Proteins in the Presence of Liposomes—To determine the changes in the secondary structures of various mutant toxins induced by association with liposomal membranes, we examined their CD spectra in the far-UV region. Figure 3 shows typical results of wild-type (Fig. 3A) and mutant toxins having Trp mutations in the common Cys-containing region (Fig. 3, C and D) or outside the region (Fig. 3B). After interaction with EPC/cholesterol liposomes (toxin/cholesterol=1:43, mol/mol), recombinant wild-type θ-toxin gave similar results to those of native θ-toxin described previously, that

TABLE II. **Changes in molecular ellipticity at 227 nm during interaction of wild-type and mutant toxins with liposomal membranes.**

Toxin variant	Control[a] (toxin only) (%)	+EPC[b] 25°C, 20 min (%)	+EPC/cholesterol[c] 25°C, 2 min (%)	+EPC/cholesterol[c] 25°C, 20 min (%)
Wild-type	100	100	116	133
W137F	100	101	110	133
W382F	100	100	113	127
W400F	100	101	118	130
W436F	100	102	101	100
W438F	100	101	108	116
W439F	100	100	123	128
W438.439F	100	100	105	109
W459F	100	102	109	121

[a]The molecular ellipticity at 227 nm of each toxin in the absence of liposomes was set as 100%. [b,c]These values were calculated from the far-UV CD spectrum of each toxin interacting with liposomes.

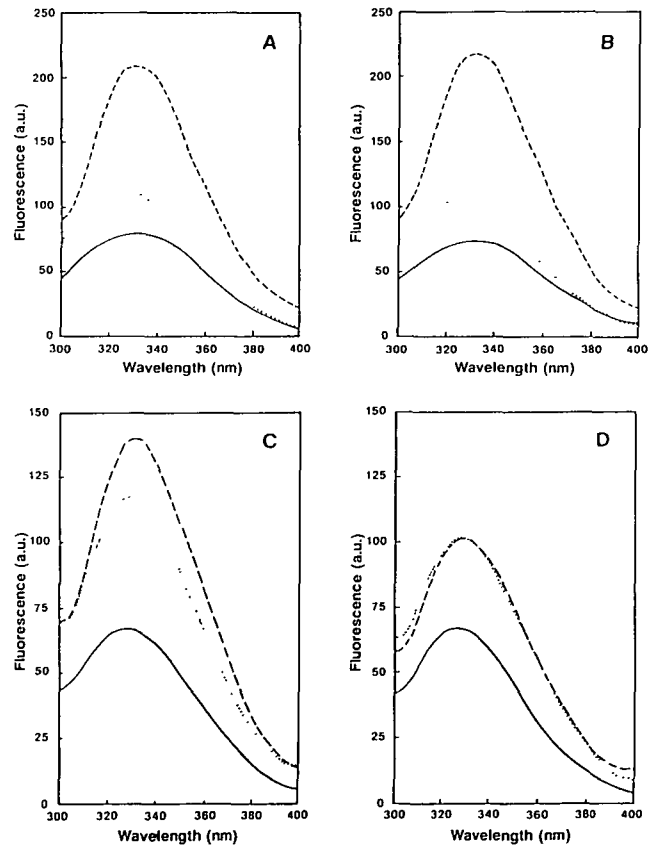

Fig. 4. **Fluorescence emission spectra of wild-type and Trp mutant toxins interacting with liposomes.** Samples contained 0.2 nmol/ml of the wild-type θ-toxin (A), or W400F (B), W438F (C), or W438.439F (D) mutant toxins in HEPES-buffered saline (pH 7.0). Liposomes were added and the mixtures were incubated for 10 min at 25°C. The molar ratio of toxin to liposomal cholesterol was 1:27. The excitation wavelength was 280 nm. Solid lines represent fluorescence spectra of toxins in the absence of liposomes. Broken and dotted lines represent spectra of toxins in the presence of DOPC/cholesterol and BrDSPC/cholesterol liposomes, respectively. As a control, the addition of DOPC or BrDSPC liposomes to the wild-type and mutant toxins produced the same profiles as those of the toxins alone shown as solid lines.

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is, an increase in negative ellipticity at 227 nm and a slight red shift of the minimum peak at 216 nm (7). Mutant toxins mixed with EPC/cholesterol liposomes (toxin/cholesterol = 1:43, mol/mol) also showed similar changes with the exception of the W436F mutant toxin, indicating that interaction with liposomes causes no drastic changes in their secondary structures. The relative ellipticities at 227 nm in the spectra of membrane-associated mutant toxins compared with the corresponding toxin in the native state are summarized in Table II. The ellipticity of W436F at 227 nm does not change after interaction with liposomal membrane containing cholesterol. The increase in the negative ellipticities at 227 nm of the W438F and W438.439F mutant toxins are small, probably due to their weaker binding. Other mutant toxins show changes at 227 nm comparable to that of the wild-type toxin.

Enhancement of Trp Fluorescence by Liposomes—We previously showed that Trp fluorescence of θ-toxin is greatly enhanced by the interaction with cholesterol-containing liposomes (7). To evaluate the effect of each Trp residue on the fluorescence enhancement, fluorescence spectral changes of Trp mutants were examined. Figure 4 shows the fluorescence emission spectra of wild-type, W400F, W438F, and W438.439F mutant toxins in the presence or absence of DOPC/cholesterol liposomes. The molar ratio of each toxin to liposomal cholesterol was 1:27. The results clearly indicate that DOPC/cholesterol liposomes produce an enhancement in the fluorescence intensity of all four proteins without a peak shift (compare dashed and solid lines in Fig. 4). Similar results were obtained for all other mutant toxins (Table III, 2nd column). Among the mutant toxins, W436F exhibits the smallest enhancement of fluorescence intensity by the incubation with DOPC/cholesterol liposomes (Table III, 2nd column), in spite of its higher binding activity than W438F and W438.439F (Fig. 2). This may suggest that Trp 436 is one of the major fluorophores in θ-toxin molecule interacting with the liposomes, although the structural difference between W436F and wild-type toxin should be taken into account. Smaller enhancement of fluorescence in W438F and W438.439F mutants than wild-type toxin is partly ascribed to their lower binding to the liposomes. In the case of W439F, the enhancement by the addition of the liposomes is apparently small (Table III, 2nd column), but this is because the soluble form of W439F mutant already has two times higher fluorescence intensity than wild-type toxin (Table I). The absolute value of fluorescence intensity of W439F in the presence of the liposomes is comparable to that of wild-type toxin.

Insertion of Mutant Toxins into Liposomal Membranes—It remains unknown which Trp residues are essential for the insertion of the θ-toxin molecule into membranes. To analyze this problem, we examined the quenching of the intrinsic tryptophan emission fluorescence of wild-type and mutant toxins by liposomes containing brominated lecithin with bromines at the 9- and 10-positions, since bromine atoms are known to quench the intrinsic tryptophan fluorescence of proteins (*19, 20*). If tryptophan residues in the toxin molecule insert into the membrane, then bromine atoms located at the midpoint of the phospholipid acyl chains will quench their fluorescence. Bromine atoms introduced into phospholipid are known not

TABLE III. **Changes in fluorescence intensity[a] during interaction of wild-type and mutant toxins with liposomal membranes.**

Toxin variant	F/F_0[b]		Quenching efficiency[c]
	+DOPC/chol	+BrDSPC/chol	
Wild-type	2.5	1.3	0.47
W137F	2.6	1.4	0.47
W382F	2.8	1.4	0.51
W400F	2.7	1.3	0.52
W436F	1.3	0.93	0.26
W438F	2.1	1.7	0.16
W439F	1.4	0.85	0.41
W438.439F	1.5	1.5	0.03
W459F	2.3	1.3	0.43

[a]Fluorescence emission spectra were measured by exciting samples in HEPES-buffered saline (pH 7.0) at 280 nm and scanning the emission from 300 to 400 nm. [b]Relative fluorescence intensity of membrane-bound toxins as compared to soluble forms. F_0 represents the integrated fluorescence intensity from 308 to 392 nm of each toxin in the absence of liposomes. F represents the corresponding intensity after incubation with liposomes at 25°C for 10 min. [c]Quenching efficiency was calculated from the equation: $1-F$(BrDSPC/chol)/F(DOPC/chol). F(BrDSPC/chol)/F(DOPC/chol) is the integrated fluorescence intensities of each toxin in the presence of BrDSPC/cholesterol liposomes relative to those induced by DOPC/cholesterol liposomes.

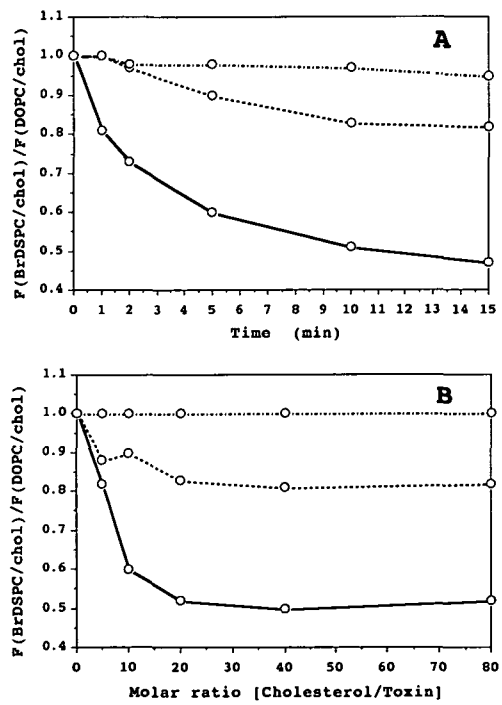

Fig. 5. **Insertion of wild-type and mutant toxins into liposomal membranes.** Samples contained 0.2 nmol/ml of wild-type toxin (——), W438F (·····), or W438.439F (-----) in HEPES-buffered saline (pH 7.0). (A) Each sample was incubated with DOPC/cholesterol or BrDSPC/cholesterol liposomes (toxin/cholesterol = 1:27, mol/mol) at 25°C for the time indicated on the abscissa. Fluorescence emission spectra were measured at an excitation wavelength of 280 nm. (B) Samples were incubated with DOPC/cholesterol or BrDSPC/cholesterol liposomes at different molar ratios of toxin to liposomal cholesterol at 25°C for 10 min. After incubation, the fluorescence emission spectra were recorded as described above. The ordinate represents the ratio of the integrated fluorescence intensity from 308 to 392 nm in the presence of BrDSPC/cholesterol liposomes to that induced by DOPC/cholesterol, indicating the degree of toxin insertion into liposomal membranes.

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to affect the binding efficiency of θ-toxin (7). The enhancement in the fluorescence intensity produced by BrDSPC/cholesterol liposomes (dotted lines in Fig. 4) is remarkably smaller than that produced by DOPC/cholesterol liposomes (dashed lines in Fig. 4) in the wild-type and W400F mutant toxins (Fig. 4, A and B), showing that tryptophan fluorescence is quenched by brominated phospholipids. In contrast, the W438.439F and W438F mutant toxins exhibited no quenching and only slight quenching by BrDSPC/cholesterol liposomes, respectively (Fig. 4, D and C). The time course of quenching shows that the insertion reaction is complete within 10 min (Fig. 5A). The degree of quenching is proportional to the ratio of cholesterol to mutant toxin molecules (mol/mol) up to a molar ratio of 10 and saturates at a molar ratio of about 40 (Fig. 5B). The addition of more liposomes to the W438F and to W438.439F mutant toxin preparations or prolonged incubation leads to no further enhancement or quenching than that shown in Fig. 4, C and D. In addition to these two mutant toxins, the W436F mutant toxin showed less quenching than the wild-type θ-toxin. The last column of Table III summarizes the degree of quenching of each toxin by brominated liposomes. Experiments at an excitation of 295 nm gave essentially the same results. For example, the degree of quenching of wild-type, W436F, W438F, W439F, and W438.439F toxins was 0.50, 0.30, 0.15, 0.46, and 0, respectively, at an excitation wavelength of 295 nm. Each value is similar to the corresponding value obtained at 280 nm (Table III, last column). These results indicate that the fluorescence quenching described above can be assigned to Trp residues. Fluorescence emission spectra of all mutant and wild-type toxins are blue-shifted by interaction with brominated liposomes, suggesting that other Trp residues than the quenched one(s) are in a more hydrophobic environment.

W137F, W382F, W439F, W459F, and T432V mutant toxins, in addition to W400F, showed essentially the same quenching as the wild-type θ-toxin by brominated liposomes (Table III), indicating that these mutant toxins insert into membranes. The fact that the replacement of any residue among Trp137, 382, 400, 439, and 459 has no or little effect on the quenching suggests that the other two tryptophans, Trp436 and 438, are candidates for quenching during insertion into brominated liposomes. This is consistent with the results showing that the replacement of either Trp438 or Trp436 by phenylalanine causes a remarkable decrease in quenching (Table III), suggesting the insertion

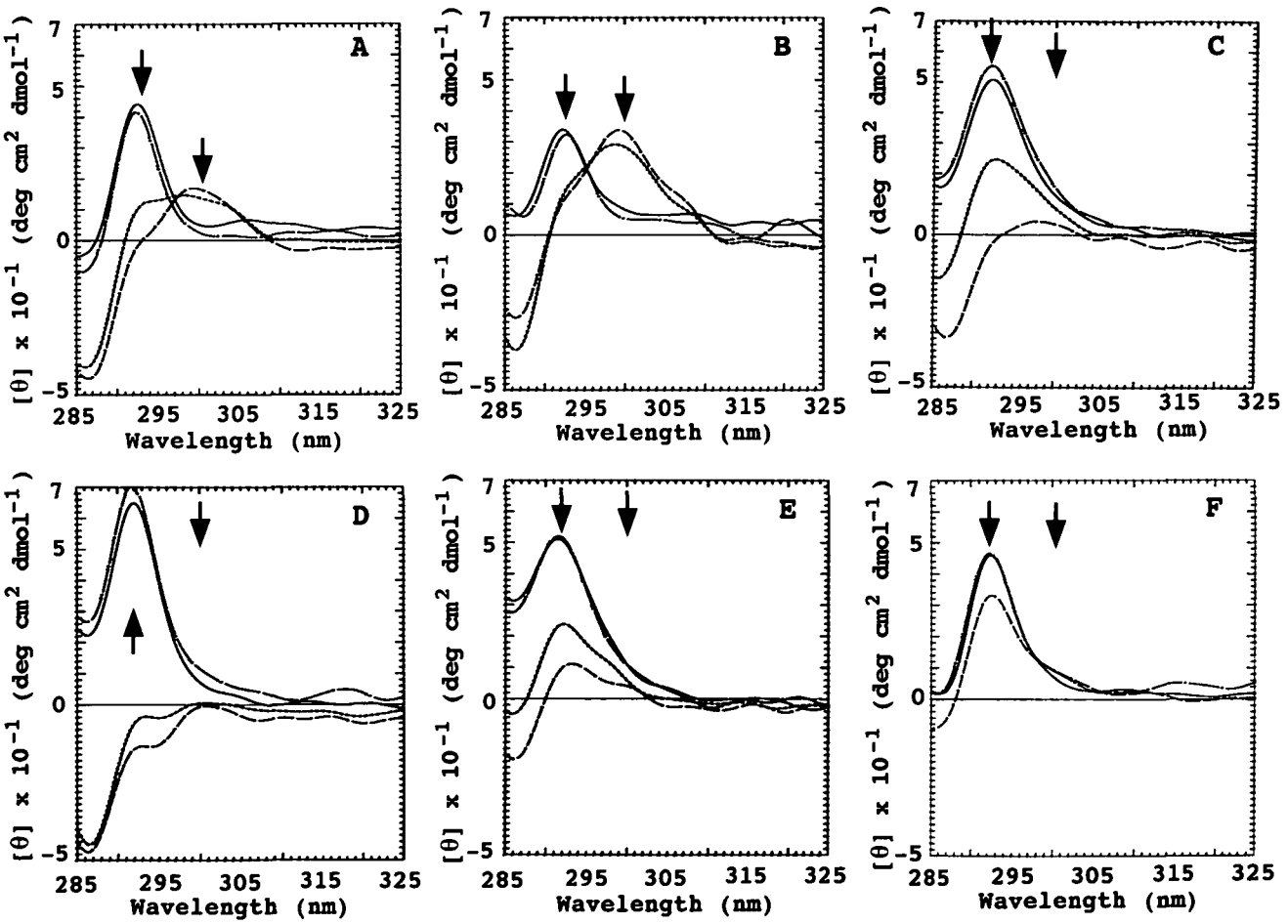

Fig. 6. **Near-UV CD spectra of wild-type and Trp mutant toxins in the presence of liposomes at pH 7.0.** Each sample contained 3.8 nmol/ml of wild-type θ-toxin (A), W436F (B), W438F (C), W439F (D), W438.439F (E), or MCθ (F) in PBS (pH 7.0). Spectra were measured using a 20 mm pathlength cylindrical cuvette at 25°C. Toxin only (——); toxin interacting with EPC liposomes (·····); toxin interacting with EPC/cholesterol liposomes (toxin/cholesterol = 1:33, mol/mol) at 25°C for 2 min (·····) and 20 min (- - -). Arrows indicate peak positions at 292 and 300 nm.

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of Trp438 and 436 into the liposomal membrane. It is also possible, however, that the substitution of Trp438 and 436 decreases toxin insertion. It is difficult at present to decide which of these views is correct.

To determine whether the insertion process is irreversible, we performed two separate experiments. First, we added DOPC/cholesterol liposomes to the wild-type, W439F, or W438F toxins at a cholesterol/protein ratio of 30, and recorded the fluorescence emission continuously with excitation at 280 nm and emission at 330 nm. After approximately 15 min, when the fluorescence had stabilized, BrDSPC/cholesterol liposomes were added to the mixtures. However, no quenching of the enhanced fluorescence was observed for either the wild-type or mutant toxins. The second experiment was the reverse of that described above. The addition of BrDSPC/cholesterol liposomes causes a slight increase in the fluorescence of the wild-type toxin as shown in Fig. 4A. After the fluorescence had stabilized, DOPC/cholesterol liposomes were added to the mixture. No further enhancement in fluorescence occurred and the intensity did not reach the signal induced by incubation of the toxin with DOPC/cholesterol liposomes alone. The W438F and W439F mutant toxins gave the same results as the wild-type. These results suggest that the insertion of θ-toxin into membranes is an irreversible process.

Near-UV CD Spectral Changes of Mutant Toxins by Interaction with Liposomes—First, we recorded the near-UV CD spectra of the wild-type θ-toxin interacting with liposomal membranes. In the presence of cholesterol, the near-UV CD spectrum of the wild-type toxin shows a decrease in positive ellipticity at 292 nm and the appearance of a positive peak at 300 nm (Fig. 6A). This peak shift is similar to that observed in native θ-toxin (*7*). The peak shift from 292 to 300 nm indicates that θ-toxin causes a distinct conformational change around Trp residues upon interaction with liposomes. Previously, we showed that MCθ, a protease-nicked, methylated θ-toxin, shows little decrease in a positive peak at 292 nm and no positive ellipticity at 300 nm when interacting with EPC/cholesterol liposomes (Fig. 6F and Ref. 7). Since MCθ has the same binding activity to erythrocyte membranes as θ-toxin but lacks the ability to form oligomers (*15*), the appearance of the positive peak at 300 nm is probably related to the formation of oligomers of θ-toxin molecules or the subsequent formation of membrane-spanning pores. To assign Trp residues contributing to the peak shift, we next recorded the near-UV CD spectra of mutant toxins in the presence of cholesterol-containing liposomes (toxin/cholesterol=1:33, mol/mol). The positive peak at 300 nm appears along with the decrease in the peak at 292 nm in the spectra of the membrane-associated Trp mutant toxins with mutations outside the common Cys-containing region (data not shown). Among mutant toxins having mutations within the Cys-containing region, W436F (Fig. 6B) and T432V (data not shown) also showed a decrease in positive ellipticity at 292 nm and the appearance of a peak at 300 nm. In contrast, there was no appearance of the positive peak at 300 nm in the spectra of the W438F (Fig. 6C), W439F (Fig. 6D), or W438.439F (Fig. 6E) mutant toxins after reaction with EPC/cholesterol liposomes. In the presence of cholesterol-free liposomes, the molecular ellipticity of all toxins at 292 and 300 nm remained unchanged, indicating that the peak shift is a cholesterol-dependent phenomenon. These results suggest that both Trp438 and Trp439 are essential for the appearance of positive ellipticity at 300 nm. In order to determine the contributions of Trp438 and Trp439 to the 300 nm peak, we compared the summed spectrum of the individual Trp438 and Trp439 spectra with the spectrum of the wild-type toxin interacting with EPC/cholesterol liposomes (Fig. 7). The contribution of the deleted Trp residue can be obtained by subtracting the spectrum of membrane-associated W438F or W439F from that of wild-type, because the substituted phenylalanine residue should not contribute to the CD spectrum in this wavelength range. As shown in Fig. 7, the composite spectrum obtained after the summation of the individual spectra of Trp438 and Trp439 gave a peak at 300 nm qualitatively similar to that of wild-type θ-toxin interacting with EPC/cholesterol liposomes, indicating the large contribution of Trp438 and Trp439 to the spectral change. These results strongly suggest that the conformational change of θ-toxin upon membrane association, which is detected as the CD peak shift from 292 to 300 nm, occurs at around Trp439 and Trp438.

Oligomerization and Ring Formation of θ-Toxin on Liposomal Membranes—As shown in Fig. 2B, the major portions of bound form were detected as SDS-resistant oligomers in the case of all the mutant toxins. The results indicate that all the mutant toxins form oligomers on liposomal membrane once they bind to liposomes.

Previously, we gave evidence for the ability of Trp mutants to form arc-shaped and ring-shaped structures on erythrocyte ghosts by electron microscopic studies (*12*). To examine the formation of these structures on liposomal membranes, wild-type and mutant toxins were incubated with EPC/cholesterol liposomes (toxin/cholesterol=1:33 or 1:43, mol/mol) under the same conditions used for CD spectroscopy and the samples were fixed, negatively stained, and analyzed by electron microscopy. Figure 8

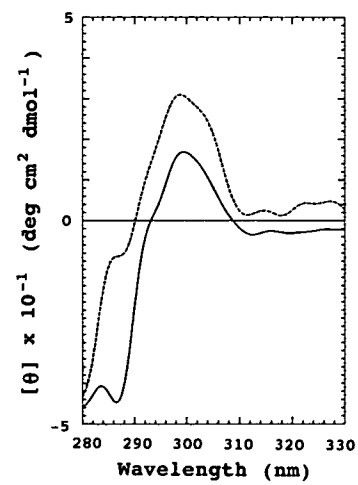

Fig. 7. **Comparison of the CD spectrum obtained after summation of the individual Trp438 and Trp439 spectra with the wild-type CD spectrum in the presence of EPC/cholesterol liposomes.** Trp438 and Trp439 spectra were obtained as difference spectra after subtraction of the near-UV CD spectra of W438F and W439F in the presence of EPC/cholesterol from that of wild-type, respectively. Summed Trp CD spectrum (·····); wild-type (——).

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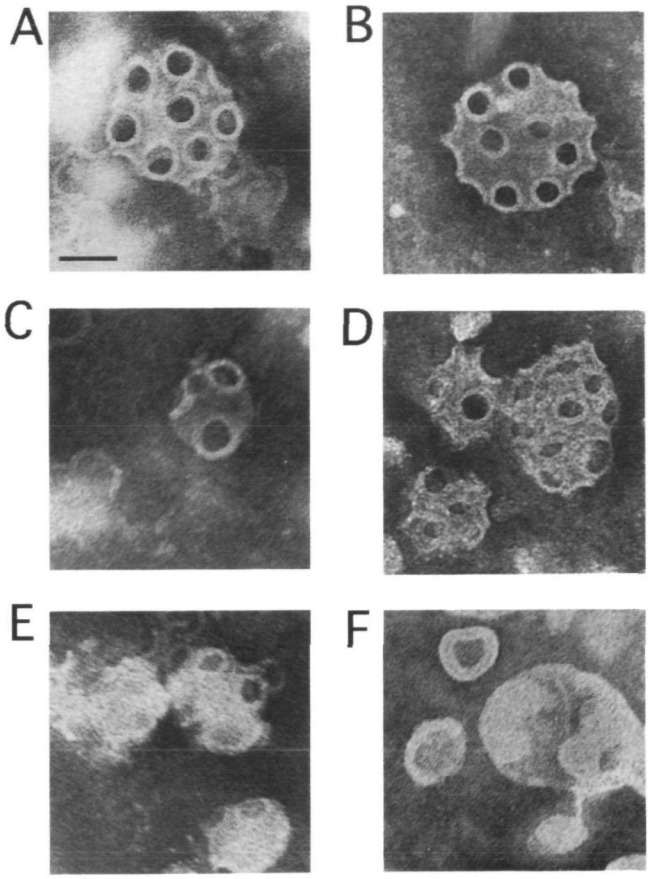

Fig. 8. **Electron micrographs of rings and arcs formed by wild-type and mutant toxins on liposomal membranes.** Liposomal membranes were treated with wild-type (A), W436F (B), W438F (C), W439F (D), and W438.439F (E) mutant toxins as described in "MATERIALS AND METHODS." Untreated liposomes are shown in (F) as a control. Bar represents 50 nm.

clearly shows the formation of ring-shaped, pore-like structures by wild-type toxin on the liposomal membranes. The interaction of W436F, W438F, and W439F mutant toxins with liposomal membranes also results in the formation of such arcs and rings as those of wild-type toxin (Fig. 8, B, C, and D). The ring formation by W438.439F mutant toxin is less than others (Fig. 8E), which probably reflect its lower binding activity. The results are consistent with those by SDS-PAGE shown in Fig. 2. These results confirmed that wild-type and mutant toxins, even those with substitutions within the Cys-containing region, form toxin oligomers on artificial membranes.

DISCUSSION

In the present work, the role of individual tryptophan residues in the cytolytic process of θ-toxin was analyzed spectroscopically using various Trp mutant toxins, focusing on three aspects: conformational changes in the secondary structure and microenvironment around side chains; insertion into liposomal membranes; and the formation of oligomers. In line with the essential role of Trp residues within the common Cys-containing region of thiol-activated cytolysins in the hemolytic activity and binding affinity with erythrocyte membranes (*12*), a large contribution of these Trp residues to the conformation, insertion into membranes, and oligomerization of θ-toxin molecules is shown. It is demonstrated that these Trp residues play not the same but distinctively different roles from each other in these processes.

The mutation of Trp to phenylalanine has no effect on the secondary structure of θ-toxin in the free state, except in the case of the W436F mutant toxin. Recently, we reported that the W436F mutant toxin exhibits a marked decrease in hemolytic activity and binding affinity to erythrocyte membranes (*12*). This mutant toxin was also shown to be more sensitive to proteases than wild-type or other mutant toxins, indicating that its higher order structure is different from that of the wild-type toxin (*12*). Additionally, the near-UV CD spectrum of Trp436 obtained by subtracting the W436F spectrum from the wild-type spectrum qualitatively resembled that of the wild-type toxin. The replacement of Trp436 by phenylalanine must result in a defect in the structure and local environment of θ-toxin. W438F, W439F, and W438.439F mutant toxins showed near-UV CD spectra different from those of the wild-type and other mutant toxins with Trp mutations outside the common Cys-containing region. This suggests that Trp436, Trp438, and Trp439 are important for maintaining the microenvironment around the aromatic side chains of θ-toxin. Human carbonic anhydrase II (HCAII), which is a monomeric zinc-containing enzyme with a molecular weight of 29,300, also contains seven tryptophans. In contrast to θ-toxin, the near-UV CD spectra of Trp mutant HCAII proteins are qualitatively similar to that of wild-type HCAII (*21*), and their reductions in activity are at most 66% (*22*). In the case of θ-toxin, Trp mutations at 436 and 438 induce a decrease in hemolytic activity to 0.8 and 2%, respectively (*12*), and cause qualitative changes in the near-UV CD spectra. Thus, mutations of Trp residues located within the common Cys-containing region of θ-toxin produce larger effects on the activity and local environment around Trp residues than in the case of HCAII. These Trp residues may play a key role in the function of θ-toxin.

The intrinsic fluorescence of Trp residues is also useful as a spectroscopic probe of the properties of the surroundings in solution. Fluorescence measurements of Trp mutant toxins show the spectrum of the W436F mutant toxin to be red-shifted, while those of the W438F and W438.439F mutant toxins are blue-shifted relative to the wild-type toxin. In other words, the replacement of Trp436 or Trp438 results in a more polar or more hydrophobic environment around other Trp residues, respectively. The fluorescence spectrum of the T432V mutant toxin shows a red-shifted peak, although the near-UV CD spectrum is almost identical to that of the wild-type toxin. This suggests that the mutation of Thr432 to valine has some effect on the surroundings of the Trp residues in the θ-toxin molecule. This may be one reason why the hemolytic activity and binding to erythrocyte membranes of T432V are smaller than those of the wild-type toxin (*12*). On the other hand, the mutation of Trp439 to phenylalanine led to a marked increase in fluorescence emission without peak shift, indicating that Trp439 might act as a quencher and that energy is transferred to Trp439 from a nearby Trp.

The membrane-associated θ-toxin retains most of its native secondary structure regardless of the mutation of

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Trp residues. Even the W436F mutant toxin, whose structure in the native state is different from that of the wild-type and other mutant toxins, tends to assume a wild-type-like structure after interaction with EPC/cholesterol liposomes, as judged by far-UV CD spectroscopy. It is known that colicin, diphtheria toxin, and exotoxin A are converted to a membrane-bound conformation *via* a molten globule intermediate (*10, 11, 23, 24*). The molten globular state is characterized by a native-like secondary structure with the unfolding of side chains, and this would lower the energy barrier of the conversion. The θ-toxin molecule is likely to form some similar lower energy state during interaction with membranes.

Examination of the brominated liposome-quenched spectra of various mutant and wild-type toxins reveals that Trp438 and/or the region around Trp438 is the most important for insertion (Fig. 4 and Table III) as well as for toxin binding (Fig. 2 and Ref. *12*). The degree of quenching shown in Table III matches exactly the binding affinity with erythrocyte membranes, which is decreased in the case of the W439F, W436F, W438F, and W438.439F mutant toxins in that order (*12*). This suggests that insertion might be required for the tight binding of the toxin to membranes. Our results showing contribution of Trp438 and 436 to insertion are consistent with recent crystal structure data of θ-toxin, which showed that the Cys-containing region having these Trps is located near the tip of the fourth domain, a postulated membrane-spanning domain (*13*). On the other hand, replacement of Trp439 has little effect on membrane insertion (Table III), although Trp439 is located near Trp436 and 438 in 3D structure.

In the process following binding, θ-toxin undergoes a conformational change around Trp residues, which is shown by the CD peak shift from 292 to 300 nm. We demonstrated that Trp438 and Trp439 are involved in the signal at 300 nm (Fig. 6). Our previous results with MCθ, which lacks the ability to form oligomers (*7, 15*), showed that the positive peak at 300 nm does not appear in the toxin having no oligomerization activity. However, W438F and W439F give no signal at 300 nm although they have the ability to form oligomers (Figs. 2 and 8). This indicates the spectral contribution of Trp438 and Trp439 to the signal at 300 nm. These findings suggest that a conformational change occurs at around Trp439 and Trp438 in the oligomerization and/or subsequent pore-forming process.

In conclusion, the present results demonstrate that Trp436 contributes to maintaining the secondary structure of θ-toxin and that Trp436, Trp438, and Trp439 are important for providing the appropriate microenvironment around side chains. The results also suggest that Trp438 and probably Trp436 are essential for efficient insertion into membranes and that Trp439 and Trp438 make large contributions to the CD signal at 300 nm. The present evidence, showing that tryptophan residues in the highly conserved region play critical roles in toxin action with respect to both structure and activity, together with 3D structural data, will provide further insight into the cytolytic mechanism of θ-toxin.

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