Conformational Changes Due to Membrane Binding and Channel Formation by Staphylococcal α-Toxin*

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Conformational changes occurring upon membrane binding and subsequent insertion of staphylococcal α-toxin were studied using complementary spectroscopic techniques. Experimental conditions were established where binding could be uncoupled from membrane insertion but insertion and channel formation seemed to be concomitant. Binding led to changes in tertiary structure as witnessed by an increase in tryptophan fluorescence, a red shift of the tryptophan maximum emission wavelength, and a change in the near UV CD spectrum. In contrast to what was observed for the soluble form of the toxin, 78% of the tryptophan residues in the membrane-bound form were accessible to the hydrophilic quencher KI. At this stage, the tryptophan residues were not in the immediate vicinity of the lipid bilayer. Upon membrane insertion, a second conformational change occurred resulting in a dramatic drop of the near UV CD signal but an increase of the far UV signal. Tryptophan residues were no longer accessible to KI but could be quenched by brominated lipids. In the light of the available data on channel formation by α-toxin, our results suggest that the tryptophan residues might be dipping into the membrane in order to anchor the extramembranous part of the channel to the lipid bilayer.

Although no crystal structure is yet available for either the soluble or the heptameric form of the toxin, structural data based on mutagenesis, biochemical, and biophysical studies are rapidly accumulating. The soluble monomer is composed of at least two domains as illustrated by the fact that the protein unfolds in several steps upon acidification of the medium (5). It has been speculated that the N- and C-terminal halves of the polypeptide chain may constitute these domains based mainly on the observation that a central glycine-rich segment is very sensitive to proteases and most likely forms an interdomain loop (3, 6–8). Amino acids implicated in membrane binding were found in both halves of the molecule (9–11). Upon binding, the central loop becomes protected toward proteases (3, 4, 6), and finally channel formation involves penetration of this loop into the membrane (4, 8, 9, 12–15). Using single cysteine mutants labeled with an environment-sensitive probe, Valeva and colleagues (16) have recently narrowed down the membrane inserting sequence to amino acids 118–124 and suggested that these residues might line the channel. It has been speculated that the loop might fold into a β-hairpin upon membrane insertion (2, 4). By the contribution of each monomer in the heptamer, the transmembrane domain of α-toxin would be a 14-stranded β-barrel resembling the barrel of a porin monomer (17, 18). The existence of this barrel has recently been confirmed by x-ray crystallography (19). It is unclear at the present time whether parts of the protein other than the central loop penetrate into the membrane. Certain amino acids near the C terminus were shown to be accessible to the hydrophobic probe 2-[3H]diazofluorene suggesting that they might penetrate into the membrane (20), but this remains to be confirmed using complementary methods.

In the present paper, we have analyzed the conformational changes that are associated with membrane binding and membrane insertion. Using brominated phospholipids, we have investigated whether the tryptophan residues in α-toxin reached the vicinity of the hydrophobic core of the bilayer upon membrane interaction. Brominated lipids have indeed been useful in determining the topology of membrane proteins (21, 22) as well as studying the membrane interaction of the pore-forming toxin colicin A (23–25). Tryptophan fluorescence spectroscopy, circular dichroism, and protease sensitivity were used to further compare the structure of the soluble, the membrane-bound, and the membrane-inserted forms of α-toxin.

EXPERIMENTAL PROCEDURES

Protein Purification—α-Toxin was produced by strain Wood 46 and purified as described previously (26). The protein concentration was determined by measuring the absorption at 280 nm based on an OD of 1.8 for a 1 mg/ml solution.

Purification of the α-Toxin Heptamer—Purified α-toxin was concentrated to about 2 mg/ml and dialyzed overnight against 10 mM NaCl, 20 mM HEPES, pH 7.0, at 4 °C. The toxin was then incubated at 37 °C for 2 h and vortexed several times. The heptamers were separated from the remaining monomers by gel filtration on a Sephadex G-75 (Pharmacia...
Preparation of Liposomes—Large unilamellar liposomes were prepared by reverse phase evaporation as described by Szoka and Papahadjopoulos (27, 28). To calibrate the liposomes in size, the vesicle suspension was extruded through 0.2-µm polycarbonate filters (Nucleopore, Pleasanton, CA). Liposomes were either formed of pure dioleoylphosphatidylglycerol (DOPG) or of a one to one mixture of DOPG and dioleoylphosphatidylcholine (DOPC). The corresponding lipids brominated at positions 9-10 of the two acyl chains were also used, Br-DOPG and Br-DOPC (Avanti Polar Lipids, Alabaster, AL). Bromide addition was shown not to alter the physicochemical properties of DOPG and DOPC (25). Liposomes were prepared in a buffer containing 150 mM NaCl, 20 mM HEPES, pH 7.4. In order to measure chloride efflux, 1.5 mg/ml 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ, Molecular Probes) was encapsulated into the liposomes as described previously (5) in a buffer containing 100 mM KCl, 20 mM HEPES, pH 7.4.

Fluorescence Measurements—All fluorescence experiments were carried out using a PTL spectrofluorimeter with a temperature-controlled sample holder (25 °C unless specified). Chloride efflux and tryptophan fluorescence experiments were carried out as described previously (5).

To monitor the insertion of α-toxin into brominated phospholipids, the excitation and emission wavelengths were set at 295 and 330 nm, respectively, with 5-nm slit widths. α-Toxin was initially diluted to 6 µg/ml (180 nM) in 150 mM NaCl, 20 mM MES at the desired pH. Liposomes were then added to reach a final lipid concentration of 0.24 mM, which corresponds to approximately 10¹¹ liposomes per ml (considering that the liposomes had an average diameter of 0.2 µm and that the surface of a lipid head group was 70 Å²).

In order to determine the temperature dependence of membrane insertion and channel formation, the kinetics of quenching of tryptophan fluorescence by Br-DOPG and the kinetics of chloride release were measured at 3 °C intervals between 10 and 40 °C. For each curve, the maximal slope was determined and plotted in semi-log as a function of 1/T.

The amount of α-toxin bound to liposomes was quantified as follows. The tryptophan emission spectrum was measured for α-toxin in solution. Vesicles were then added, and the samples were allowed to equilibrate for 60 min in order to reach a steady state. The samples were then centrifuged using a TL100.3 Beckman rotor for 40 min at 60,000 rpm, and the tryptophan fluorescence spectrum of the supernatant (corresponding to the unbound toxin) was measured. The fraction of bound toxin was calculated as being (Fsol − Fsup)/Fsol, where Fsol is the area of the tryptophan emission spectrum of α-toxin in solution and Fsup the area of that of the supernatant.

In order to determine the extent of quenching of tryptophan fluorescence by brominated phospholipids, the area of the tryptophan emission spectrum of α-toxin after 60 min incubation with brominated liposomes was compared with that of α-toxin in the absence of liposomes, unless specified otherwise.

In order to determine the extent of tryptophan quenching by KI of α-toxin in the membrane-associated form, α-toxin was first incubated with the desired vesicles at the appropriate pH for 60 min to allow binding and/or insertion. The vesicles were then pelleted as described above, resuspended in the appropriate buffer containing either 150 mM KI or 150 mM NaCl, and the tryptophan emission spectra were measured.

Circular Dichroism (CD)—Circular dichroic experiments were carried out at room temperature on a Jasco 715 spectrometer. Quartz cells of 0.1 and 1 cm path lengths were used for measurements in the far (205-250 nm) and near (260-325 nm) ultraviolet (UV), respectively. Small unilamellar vesicles were prepared by sonication as described previously (23) in 150 mM NaCl, 20 mM MES, at pH 4.75 and pH 5 for DOPG and DOPG/DOPC (50:50, w/w), respectively. α-Toxin (10 µM) was added to the vesicles (7.5 µl lipids) and allowed to incubate for 60 s at room temperature. Samples were then briefly sonicated on ice (3 times for 5 s), and their near UV CD spectrum was measured. For far UV CD measurements, the samples were diluted 2-fold with their respective buffers. The amount of unbound toxin was determined by comparing the tryptophan fluorescence spectra of the sample before and after sedimentation of the vesicles as described above.

Protease Treatment—Vesicles were prepared as described for the CD experiments. α-Toxin was incubated with the liposomes for 60 min at room temperature prior to addition of the Promase (1:50, protease/toxin ratio, w/w). After 30 min of incubation at 37 °C, sample buffer was added, and samples were boiled unless specified otherwise. Samples were then analyzed by SDS-PAGE as described by Laemmli (29). The same cleavage patterns were obtained whether liposomes had been made by reverse phase evaporation or by sonication.

RESULTS

Quenching of Tryptophan Fluorescence by Br-DOPG upon Channel Formation by α-Toxin—We have previously shown that α-toxin is able to form channels in egg phosphatidylglycerol vesicles at acidic pH (5). As illustrated Fig. 1A, α-toxin is
able to induce chloride efflux from Br-DOPG vesicles in a similar pH-dependent manner. In parallel experiments, kinetics of changes in tryptophan fluorescence were measured to investigate whether brominated lipids were able to quench the intrinsic fluorescence of α-toxin on membrane insertion. The lipids used had bromines attached at positions 9 and 10 of the acyl chains. At pH 4.75, where channel formation occurred (Fig. 1A), the intrinsic fluorescence dramatically decreased upon addition of Br-DOPG vesicles (Fig. 1B). Insertion was irreversible since further addition of non-brominated DOPG vesicles did not lead to dequenching of the tryptophan fluorescence (not shown). Note that the time scales are different in Fig. 1, A and B. The differences in kinetics are due to the fact that one to two channels are sufficient to empty one vesicle from its chloride content (short time scale, 5); however, in order to detect quenching of tryptophan fluorescence, a significant proportion of the total toxin population must insert into the brominated bilayer (longer time scale). At pH 6 or 7, channels did not form, and tryptophan fluorescence was not affected by addition of brominated DOPG vesicles.

These experiments indicate that quenching of the intrinsic fluorescence by brominated lipids can be used to monitor membrane insertion of α-toxin. Moreover, they show that upon channel formation, tryptophan residues become accessible to quenchers that are located within the lipid bilayer. The interpretation of this observation will be discussed later (see “Discussion”).

Membrane Insertion and Channel Formation Are Concomitant Events—Having established that quenching of tryptophan residues by brominated phospholipids can be used to follow membrane insertion of α-toxin, we have investigated whether we could uncouple insertion from channel formation. We were unable to establish experimental conditions where insertion occurred without channel formation. We have then analyzed the temperature dependence of both membrane insertion and channel formation into Br-DOPG vesicles (pH 4.75). As shown in Fig. 1C, the temperature dependence was the same for both events. These observations suggest that the two techniques used here, fluorescence quenching by brominated phospholipids and release of entrapped markers, measure the same event and that membrane insertion and channel formation occurred concomitantly under our experimental conditions.

Accessibility of Tryptophan Residues in the α-Toxin Channel to Hydrophilic and Hydrophobic Quenchers—The decrease in tryptophan fluorescence observed upon addition of Br-DOPG vesicles to α-toxin at pH 4.75 indicated that, upon channel formation, certain tryptophan residues became accessible to quenching by bromine (Fig. 1B). Based on the tryptophan fluorescence spectra shown in Fig. 2A, the fluorescence was quenched by 60 ± 4% (n = 5). The residual 40% could be due to a fraction of unbound toxin, a fraction of inaccessible tryptophans, or to the inefficiency of bromine as a quencher. The first possibility could be ruled out since all the toxin was bound to the vesicles as indicated by the fact that the supernatant obtained after sedimentation of the membranes showed no fluorescence (Fig. 2A). In order to investigate the second possibility, we have tested whether the fraction of tryptophans that had not been quenched by the brominated lipids were accessible to a soluble quencher such as KI.

α-Toxin was first allowed to insert into Br-DOPG vesicles in the absence of KI; the vesicles were then pelleted and resuspended in buffer (20 mM MES, pH 4.75) containing either 150 mM KI or 150 mM NaCl. Experiments were performed at 150 mM KI in order to reach maximal quenching while maintaining the ionic strength at 150 mM (at lower KI concentrations quenching was lower, data not shown). As illustrated in Fig. 2B, 48 ± 5% (n = 3) of the residual fluorescence (which had not been quenched by bromines) could still be quenched by KI, a value that corresponds to quenching of about 19% of the initial fluorescence of α-toxin in solution (compare Fig. 2A, B). It seems unlikely that higher KI concentrations would lead to significantly more quenching. Most of the fluorescence remaining after quenching by both Br-DOPG and KI, i.e., 21% of the initial fluorescence, is likely due to the limited efficiency of tryptophan quenching by bromine. Indeed, this value agrees well with the estimate of Boilen and Holloway (22) that the quenching efficiency of dibrominated hydrocarbons with bromines located on adjacent carbons is about 80%.

It is noteworthy that the maximum emission wavelength (λ_max) shifted dramatically upon addition of Br-DOPG vesicles as well as upon subsequent addition of KI (Fig. 2). Initially α-toxin in solution had a λ_max of 330.5 nm corresponding to that of the native toxin (5). After addition of Br-DOPG vesicles, λ_max underwent a 9 nm red shift (λ_max = 339 nm) indicating that a significant fraction of the unquenched tryptophan residues were exposed to a hydrophilic environment. Upon addition of KI, λ_max shifted back to lower wavelengths (331.5 nm) indicating that KI had, as expected, quenched solvent-exposed tryptophans and that the remaining fraction that is still fluorescent in the presence of Br-DOPG and KI is in a hydrophobic environment. This is also what one would expect if the remaining fluorescent fraction was due to the limited quenching efficiency of bromines.

Tryptophan Fluorescence upon Insertion of α-Toxin into Non-brominated DOPG Vesicles—The preceding analysis of the accessibility of tryptophan residues to soluble and hydrophobic,
membrane-bound, quenchers indicated that at least 60% of the residues were accessible to quenching by bromines. Here we have investigated whether these residues were also accessible to KI. To perform this experiment we analyzed the effect of KI on the tryptophan fluorescence of the α-toxin channel formed in non-brominated DOPG vesicles.

These experiments first showed that membrane insertion of α-toxin led to an increase in fluorescence intensity as well as a 5 nm red shift in $\lambda_{\text{max}}$ (Fig. 3A) indicating that a fraction of the tryptophan residues became exposed to a hydrophilic environment upon membrane binding and insertion. The effect of KI was then analyzed as indicated previously; vesicles containing the α-toxin channel were sedimented by centrifugation and resuspended in a buffer containing either 150 mM KI or 150 mM NaCl. As shown in Fig. 3B, only 20 ± 3% ($n = 3$) of the fluorescence was quenched by KI, a percentage that is essentially identical to the one previously obtained when using Br-DOPG vesicles (Fig. 2). This observation implies that the large fraction of tryptophans that were quenched by bromine in the previous experiments were not accessible to KI.

The present observation that the fluorescence increased upon membrane insertion indicates that the quenching by Br-DOPG is in fact more pronounced than initially suggested by Fig. 2A. For proper evaluation of the percentage of quenching, the tryptophan spectrum of α-toxin inserted into Br-DOPG vesicles should be compared with that of α-toxin inserted into non-brominated DOPG vesicles and not to that of α-toxin in solution. Taking this correction into account the percentages of inhibition by Br-DOPG as well as by KI were recalculated and are shown in Table I. It appears that at least 6 of the 8 tryptophan residues in α-toxin were accessible to bromine quenching (taking into account that the quenching efficiency of bromines is of about 80%).

Uncoupling of Binding and Insertion—In order to study the conformation of α-toxin when it is bound to the membrane prior to insertion, we have tested whether we could establish conditions where most of the toxin would be trapped as a membrane-bound intermediate. We have previously shown that the channel-forming rate of α-toxin could be modulated by changing the pH or the amount of negatively charged lipids in the vesicles (5). Here we have analyzed the effect of brominated vesicles composed of Br-DOPG/Br-DOPC (50:50) on the intrinsic fluorescence of α-toxin at various pH values in the hope of finding conditions where binding would still occur but not insertion. As shown in Fig. 4B, the effect of Br-DOPG/Br-DOPC vesicles on the intrinsic fluorescence varied significantly from one pH to another. Under the same conditions, we have analyzed the α-toxin-induced chloride efflux.

At pH 4.25, the intrinsic fluorescence was readily quenched by Br-DOPG/Br-DOPC in agreement with the fact that channels readily form (5, Fig. 4).

At pH 4.5, the changes in fluorescence observed upon addition of Br-DOPG/Br-DOPC vesicles were particularly interesting because kinetic intermediates in the channel formation process could be discriminated. The fluorescence first increased and then decreased (Fig. 4B). To check whether the decrease was indeed due to bromine quenching, the same experiment was performed with vesicles containing non-brominated DOPG/DOPC (50:50). As shown in the inset of Fig. 4B, the decrease was not observed indicating that the toxin first bound to the vesicles and probably oligomerized, thereby leading to an increase in fluorescence. A subpopulation of toxin then formed channels in the vesicles, whereby their tryptophan fluorescence became quenched by bromines. Under the same experimental conditions, chloride was rapidly released from the vesicles. The apparent discrepancy between the observation that most α-toxin molecules do not insert into the membrane and the observation that chloride is rapidly released is due to the difference in sensitivity between the two methods used. As mentioned previously, less than two channels are necessary to release all the chloride from one vesicle within milliseconds (5).

In the present experiments, in order to be able to follow quenching of tryptophan fluorescence, the equivalent of 80 heptamers of α-toxin were added per liposome. Therefore, even if only 2% of the α-toxin molecules inserted into the membrane and formed a channel, chloride efflux would be observed, whereas the overall tryptophan fluorescence would not be significantly quenched by bromine. We have previously shown that at lower protein to vesicles ratios (2 heptamers per vesicle) chloride is not released at pH 4.5 (5).
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It is surprising that such a minute increase in pH (4.25–4.5) led to such a dramatic drop in the membrane insertion rate. We have previously described a similarly steep pH dependence for the rate of α-toxin channel formation (5). We have shown that the change in channel formation rate correlated with the appearance of a molten globule folding intermediate at the membrane surface (5, 23, 31). Aspartic or glutamic acids, which have a pk_c around 4 (pk_c = 4.4), are possible contributors to this pH effect. Walker and Bayley (30) have systematically mutated all charged residues to cysteine in single amino acid mutants and studied the effect on binding, oligomerization, and channel formation in erythrocyte membranes. Only three mutations led to a decrease in hemolytic activity without affecting binding and oligomerization, two of which were aspartic acids Asp-24 and Asp-152, the latter being located immediately downstream from the central loop. Therefore protonation of Asp-152 may promote insertion of the central loop.

At pH 5, addition of Br-DOPG/Br-DOPC vesicles led only to an increase in fluorescence suggesting that in contrast to what was observed at pH 4.5, most of the toxin underwent a conformational change upon binding and oligomerization but was trapped at that stage. A minor population, however, did insert and make channels as witnessed by a chloride efflux (Fig. 4A). Maximal chloride release was not observed suggesting that 1% or less of the toxin present in these experiments formed channels. The intermediate conformation reached by α-toxin when bound to Br-DOPG/Br-DOPC vesicles at pH 5 will be studied in more detail below.

Finally at pH 7, no change in fluorescence could be observed upon addition of vesicles. Even though the amount of toxin bound to the vesicles was lower at pH 7 than at pH 5 (24 ± 3 instead of 69 ± 2%), the fact that no increase in fluorescence was observed suggests that binding alone is not sufficient to induce the conformational change that leads to an increase in tryptophan fluorescence and that there is an additional pH effect.

Characterization of α-Toxin Bound to DOPG/DOPC Vesicles—The conformation of membrane-bound but not inserted α-toxin, i.e., α-toxin bound to DOPG/DOPC vesicles at pH 5, was studied in more detail. When adding the vesicles to α-toxin about 70% of the protein was bound to the membranes (Fig. 5A). Binding was extremely tight since it resisted high salt and high pH treatments. SDS-PAGE analysis of the membrane-associated toxin showed that most of the toxin was in an oligomeric form (Fig. 7) and therefore corresponded in majority to the non-lytic pre-pore complex (4, 9, 12).

As illustrated in Fig. 5A, binding led to an increase in fluorescence and a red shift in the maximum emission wavelength as previously observed by Ikigai and Nakae (32, 33). These changes in the tryptophan emission spectrum are likely due to a pH-induced conformational change at the membrane surface and to oligomerization.

The accessibility of tryptophan residues to quenchers was analyzed. As shown in Fig. 5A, addition of Br-DOPG/Br-DOPC vesicles did not lead to a decrease but an increase in fluorescence intensity. The same increase was observed upon addition of non-brominated DOPG/DOPC vesicles (data not shown). Therefore, the tryptophan residues seemed to be inaccessible to quenching by Br-DOPG/Br-DOPC indicating that they must be located at a distance from the plane of the membrane (see “Discussion”).

We therefore investigated whether tryptophans were accessible to the soluble quencher KI. α-Toxin was incubated with Br-DOPG/Br-DOPC vesicles for 60 min to allow binding, and the toxin bound to the liposomes was sedimented by centrifugation and resuspended in buffer (20 mM MES, pH 5) containing either 150 mM KI or 150 mM NaCl. As shown in Fig. 5B and Table I, addition of KI led to 78 ± 2% quenching of the tryptophan fluorescence. The accessibility of the tryptophans to solvent is also illustrated by the fact that λ_max was 335.5 nm in the absence of KI, corresponding to a 5 nm red shift when compared with the soluble toxin. In contrast in the presence of KI, λ_max was 329 nm indicating that KI had quenched the solvent-exposed tryptophans and that the KI-inaccessible residues were in a very hydrophobic environment probably in the protein interior or at a monomer/monomer interface. These observations indicate that most tryptophans in the membrane-bound α-toxin were accessible to the soluble quencher in contrast to both the soluble state (KI had no significant effect on the fluorescence of α-toxin in solution at pH 5) and the membrane-inserted state. This confirms that the toxin undergoes a first major conformational change upon binding/oligomerization and a second upon insertion/channel formation.

Near UV CD Analysis of α-Toxin in Its Various Forms—The changes in tertiary structure undergone by α-toxin upon oligomerization, membrane binding, and insertion were analyzed by near UV CD. As shown Fig. 6A, α-toxin at pH 5 has the
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![Graph A](image1)

**Fig. 5.** Effect Br-DOPG/Br-DOPC and KI on the tryptophan fluorescence of α-toxin. A, the tryptophan fluorescence spectra were measured for α-toxin (180 nM) in 150 mM NaCl, 20 mM MES, pH 5 as well as after 60 min incubation with Br-DOPG/Br-DOPC vesicles (0.24 mM of lipid)(●). After pelleting the vesicles by centrifugation, the spectrum of the supernatant (▲) was measured. B, the pellet was resuspended in 20 mM MES, pH 5, containing either 150 mM KI (■) or 150 mM NaCl (○), and the spectra were measured.

The ellipticity measured for α-toxin in the presence of DOPG/ DOPC vesicles at pH 5 was more pronounced in the 295–325 nm range indicating a reorganization of the tertiary structure. The spectrum of the heptamer was similar to the one previously obtained by Ikigai and Nakae (32) for oligomers prepared in deoxycholate. The ellipticity measured for α-toxin in the presence of DOPG/ DOPC vesicles at pH 5 was more pronounced in the 295–325 nm range when compared with that of the heptamer in solution, whereas the signal was somewhat lower in the 280–290 nm range. The signal was mainly due to the bound toxin since the signal was somewhat lower in the 280–290 nm range when compared with that of the heptamer in solution. The changes in structure when compared with both the monomer or the oligomer in solution (Fig. 6A).

Unexpectedly, α-toxin inserted into DOPG vesicles (pH 4.75) had a very weak near UV CD signal (Fig. 6A). The positive ellipticity observed for the oligomer in the 250–280 nm range gave rise to a broad negative peak, whereas the well defined peak around 294 nm completely collapsed. The overall weakening of the near UV CD signal indicated that most of the aromatic residues in the toxin were no longer in an asymmetric environment and became mobile. A similar collapse of the near UV CD spectrum was observed upon membrane binding of the pore-forming toxin colicin A (34).

As shown in the inset of Fig. 6A, the near UV signal of the α-toxin channel increased again upon addition of the non-ionic detergent Triton X-100. All peaks were, however, shifted to lower wavelengths (spectrum was taken 5 min after addition of 0.07% detergent) when compared with the peaks of the spectrum of the heptamer in solution. This observation might explain why Nakae and Ikigai (32) measured a well defined near UV CD spectrum for oligomeric α-toxin that had been purified from detergent-solubilized erythrocytes after treatment with the toxin. These results also indicate that the aromatic residues in the heptamer have a different conformation in the presence of a lipid bilayer than in detergent.

The changes in secondary structure occurring upon oligomerization, membrane binding, and insertion were also analyzed by far UV CD spectroscopy. As shown in Fig. 6B, the far UV CD spectrum of α-toxin was not significantly affected by heptamerization and membrane binding. The spectra of monomeric α-toxin, heptameric α-toxin in solution, or α-toxin bound to DOPG/DOPC membrane all showed a minimum at 215 nm as previously observed (5, 6, 32). A significant change in spectrum, however, occurred upon membrane insertion. The signal became more intense suggesting a slight increase in the amount of β-sheet, and the minimum was shifted to 217.5 nm. Proteins containing β-barrels such as porins also show a minimum ellipticity at 217 nm (35, 36). Therefore this change in far UV CD spectrum could be due to folding of the central loops into a β-barrel upon membrane insertion.

**Protease Sensitivity of α-Toxin**—In order to further characterize the changes in conformation undergone by α-toxin upon membrane binding and insertion, we have analyzed the cleavage pattern of α-toxin after limited proteolysis with Pronase. Treatment of monomeric α-toxin in solution at pH 5 or 4.75 led to a major band of approximately 17 kDa (Fig. 7). This pattern is characteristic of α-toxin cleavage in the central loop which leads to two fragments of similar size (6, 8, 37). Binding of α-toxin to DOPG/DOPC vesicles (pH 5) rendered the protein insensitive to Pronase treatment in agreement with previous work showing that binding leads to inaccessibility of the central loop to proteases (3, 4, 6).

Interestingly Pronase treatment of the α-toxin channel in DOPG vesicles (pH 4.75) led to a completely different, very reproducible, cleavage pattern confirming the fact that a conformational change occurred when the bound toxin penetrated into the membrane. A number of cleavage sites, which remain to be determined, became accessible to the protease giving rise to eight main fragments with apparent molecular masses of 28, 25, 20, 16 kDa and 4 bands in the 10–12 kDa range. Given the sizes of the peptides, cleavage must have occurred all along the linear sequence of the protein thereby suggesting that the conformational change that occurred upon insertion was not restricted to a small region but involved the entire protein.

**DISCUSSION**

Channel formation by staphylococcal α-toxin is a complex multi-step process. In order to analyze the configuration of the toxin at each step, we have established conditions where the toxin accumulated either at the stage of the membrane-bound pre-pore complex or continued to form the transmembrane channel. We report here that under certain conditions α-toxin will accumulate at the membrane surface in the pre-pore state without the need of introducing mutations that affect channel-forming properties. In the pre-pore state, the tryptophan residues were shown to be at a distance from the plane of the membrane and accessible to the soluble quencher KI, whereas they were inaccessible to KI in the soluble state. These obser-
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vations suggest that binding led to a rearrangement of the tryptophan side chains but that they did not directly interact with the membrane at this stage (Fig. 8). Upon channel formation, a second conformational change occurred. Most tryptophan residues reached a hydrophobic environment where they were accessible to quenching by bromide atoms attached to the middle of the acyl chain of the lipids suggesting that the C-terminal half of the protein, which contains 7 out of the 8 tryptophans in the protein, came in close contact with the lipid bilayer. Most aromatic residues in the channel were rather mobile since they no longer gave rise to a CD signal in the near UV. Since it is unlikely that the C-terminal half of the protein actually penetrates deeply into the membrane (see “Discussion” below), we speculate that the tryptophan side chains dip into the membrane in order to anchor the large extramembranous part of the protein to the lipid membrane in a manner similar to what has been described for other membrane proteins such as porins (Fig. 8).

Membrane Binding and Oligomerization—We have shown that at pH 5, α-toxin will readily bind to DOPG/DOPC vesicles but will not insert into the membrane. At the protein concentration used in this study, most of the membrane-bound toxin was in the heptameric form as indicated by SDS-PAGE analysis under non-boiling conditions (Fig. 7). The conformational change occurring upon binding and oligomerization of the toxin led to (i) a relative resistance to Pronase treatment, (ii) a change in near UV CD spectrum, (iii) an increase in tryptophan fluorescence, (iv) a red shift in maximum emission wavelength ($\lambda_{\text{max}}$), (v) accessibility of 78% of the tryptophan residues to the soluble quencher KI but (vi) total inaccessibility to quenching by brominated lipids. The first observation indicates that upon binding/oligomerization the central loop becomes hidden in agreement with previous observations (3, 4, 6). The second and third observations show that a rearrangement of the aromatic residues occurs but that the tertiary structure remains rigid since the ellipticity in the near UV, although modified, was not dramatically reduced. The red shift in $\lambda_{\text{max}}$ suggests that a fraction of the tryptophan residues moves to a more hydrophilic environment, closer to the surface of the protein as also illustrated by the fact that most tryptophan residues are accessible to KI. The tryptophan residues, however, remain at a distance from the membrane plane as they are not accessible to quenching by bromide atoms situated at the mid-point of the acyl chains. It is not clear what triggers the conformational changes at the membrane surface. It may be the lower pH at the

![Figure 6](image_url)

**Fig. 6.** Effect of oligomerization, membrane binding, and membrane insertion on the near (A) and far (B) CD spectra of α-toxin. ▲, α-toxin monomer in solution at pH 5; △, α-toxin heptamer in solution at pH 5; ●, α-toxin bound to DOPG/DOPC vesicles at pH 5; ○, α-toxin channel in DOPG vesicles at pH 4.75. Inset, the sample of the α-toxin channel in DOPG vesicles at pH 4.75 (○) was treated for 5 min with 0.07% Triton X-100, the spectrum was measured again (■) and compared with that of the α-toxin heptamer (△). △ and ○ are the same as in the main figure.

![Figure 7](image_url)

**Fig. 7.** Effect of pH and membrane interaction on the Pronase cleavage pattern of α-toxin. α-Toxin was treated with Pronase (1:50 protease/toxin ratio, w/w) under different conditions for 30 min (37°C). Samples were analyzed by SDS-PAGE. All samples were boiled unless specified. Lane 1, untreated α-toxin monomer at pH 5; lane 2, α-toxin monomer treated with Pronase at pH 5; lane 3, α-toxin bound to DOPG/DOPC vesicles, unboiled sample; lane 4, α-toxin bound to DOPG/DOPC vesicles; lane 5, α-toxin bound to DOPG/DOPC vesicles treated with Pronase; lane 6, α-toxin inserted into DOPG vesicles, unboiled sample; lane 7, α-toxin inserted into DOPG vesicles; lane 8, α-toxin inserted into DOPG vesicles treated with Pronase.

interface as previously mentioned (5) and/or the lower dielectric constant (38).

Insertion and Channel Formation—We have shown that under conditions where α-toxin forms channels, the fluorescence of the tryptophan residues could be quenched by bromines situated at the mid-point of the acyl chains. This event, which we have termed insertion, could not be uncoupled from channel formation and had the same energy requirement suggesting that these two measurements correspond to the same event. Insertion leads to a major conformational change involving all part of the molecule as illustrated by the appearance of Pronase cleavage sites along the entire primary sequence (Fig. 7).

The observation that the fluorescence of tryptophans in the α-toxin channel can be quenched by bromide atoms indicates that these residues come in close proximity of the hydrophobic core of the bilayer. In a detailed study on the quenching of tryptophan fluorescence by brominated phospholipids, Bolen and Holloway (22) have shown that quenching does not necessarily require contact between the chromophore and the bromide atom, even though the quenching is efficient only at a very short range. They have estimated that the quenching
Conformational Changes of α-Toxin upon Membrane Interaction

FIG. 8. Working model for the various steps that lead to channel formation by α-toxin. This model is consistent with earlier findings in the literature and the present data. In the soluble form, α-toxin (i) is a monomer formed of at least two structurally independent domains separated by a protease-sensitive, glycine-rich, central loop. Tryptophan residues (some of which are symbolized by black hexagons) are in a hydrophobic environment and inaccessible to the soluble quencher KI. Upon membrane binding and subsequent oligomerization (ii), the toxin undergoes a conformational change whereby most tryptophan residues become accessible to KI but are still inaccessible to quenching by bromines attached at positions 9 and 10 of the lipid acyl chains. At this stage the central loop is no longer accessible to proteases. Finally upon membrane insertion (iii), the central loop might fold into a β-hairpin and insert into the lipid bilayer to form, in conjunction with the hairpins from the other monomers, a 14-stranded β-barrel. In the membrane-inserted conformation, most of the tryptophan residues would dip into the head group-acyl chain boundary region. The gray circles represent the region around the bromide atoms where significant quenching can occur.

efficiency is 50% when the tryptophan-bromine pair is separated by a distance of 9 Å. In the light of these observations and considering that in the present study the bromines were situated approximately 7 Å deep in the hydrophobic core of the bilayer (39), bromines would not only quench the fluorescence proportion of the aminated approximately 7 Å deep in the hydrophobic core of the protein do not penetrate into the bilayer. The current view is that only 15 amino acids in the central loop penetrate deeply into the membrane upon channel formation (16), although it has not been explicitly shown that other parts of the protein do not penetrate into the bilayer. It seems unlikely, however, that most tryptophans that are scattered along the entire protein (Trp-80, Trp-167, Trp-179, Trp-187, Trp-260, Trp-265, Trp-274, Trp-285) would all penetrate into the membrane. We rather believe that they dip into the head group-acyl chain boundary region where they would be accessible to quenching by bromines at positions 9–10 of the acyl chain. Other aromatics might also lie at the interface such as the three tyrosine and one phenylalanine residues that surround the glycine-rich loop (Tyr-112, Tyr-118, Phe-120, Tyr-148). These aromatic residues could play a role in anchoring the extramembranous part of the toxin to the lipid bilayer. Such a role for aromatic residues has been suggested for certain toxins (41) as well as for a number of membrane proteins based on the amphipathic nature of these side chains and on the observation that aromatic residues show a clear preference for the membrane interface region. The most striking example is that of bacterial porins in which two rings of aromatic residues surround the porin trimer at the inner and outer border between the polar and non-polar parts of the membrane (42, 43). Sequestration of aromatics at the membrane boundary has also been found in the bacterial photoreaction center (44), cytochrome c oxidase (45), and bacteriorhodopsin (46) although to a lesser extent. A statistical analysis of 115 type I membrane proteins has also shown that tryptophan residues occur preferentially at the extracellular boundary of the hydrophobic transmembrane segments (47). Finally, it has been suggested for soluble, membrane-associated proteins such as annexins that tryptophan residues might contribute to membrane anchoring (48, 49).

The possibility that the tryptophan residues and possibly other aromatics are dipping into the membrane could partly explain the weakness of the near UV CD signal of the α-toxin channel. A drop in near UV CD signal indicates that the aromatic residues reach an isotropic environment and is often interpreted, when studying soluble proteins, as a partial unfolding of the protein. In the case of membrane proteins it is, however, not clear how such an observation should be interpreted. Penetration of aromatics into the membrane interface might be sufficient to abolish the near UV CD signal without necessarily evoking partial unfolding of the protein. In the present case, however, both a partial unfolding (also suggested by the increased protease sensitivity) and the mobile environment provided by the lipid head group region are likely to contribute to the low near UV CD signal. The increase in the far UV CD signal indicates that there is an increase in β-sheet content which could be due to folding of the central loops into a 14-stranded β-barrel.

Conclusion—Upon membrane binding and oligomerization, α-toxin undergoes a major conformational change that leads to...
rearrangement of tryptophan side chains and their accessibility to soluble quenchers (Fig. 8). Upon subsequent membrane penetration, a second change in conformation occurs that is not solely restricted to the penetration of the central loop into the membrane. Here we show that tryptophan residues scattered along the entire C-terminal half of the protein come in close contact with the membrane and possibly anchor the extramembranous part of the heptameric complex to the lipid bilayer.

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Note Added in Proof—The structure of the α-toxin channel at 1.9 Å resolution became available after this work had been accepted (50). The heptameric complex is a mushroom-shaped structure divided in three domains: the cap region, the rim regions, and the stem region. The stem region is thought to span the lipid membrane and is composed of a 14-stranded β-barrel (residues 110–148 in each promoter). Between the stem and the rim domains in each promoter is a crevice that is very rich in acidic and aromatic residues and has been suggested to constitute a lipid binding region. In particular, in agreement with the present work, this crevice contains several tryptophan residues (Trp-179, Trp-187, Trp-254, Trp-260) as well as tyrosine residues (Tyr-112, Tyr-118, and Tyr-148). Unfortunately the positions of the other five tryptophans contained in α-toxin are not mentioned. It is important to note that the structure that has been solved is that of the detergent-solubilized heptameric complex to the lipid bilayer.

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