

## Localization and Environment of Tryptophans in Soluble and Membrane-Bound States of a Pore-Forming Toxin from *Staphylococcus aureus*

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**ABSTRACT** The location and environment of tryptophans in the soluble and membrane-bound forms of *Staphylococcus aureus*  $\alpha$ -toxin were monitored using intrinsic tryptophan fluorescence. Fluorescence quenching of the toxin monomer in solution indicated varying degrees of tryptophan burial within the protein interior. *N*-Bromosuccinimide readily abolished 80% of the fluorescence in solution. The residual fluorescence of the modified toxin showed a blue-shifted emission maximum, a longer fluorescence lifetime as compared to the unmodified and membrane-bound  $\alpha$ -toxin, and a 5- to 6-nm red edge excitation shift, all indicating a restricted tryptophan environment and deeply buried tryptophans. In the membrane-bound form, the fluorescence of  $\alpha$ -toxin was quenched by iodide, indicating a conformational change leading to exposure of some tryptophans. A shorter average lifetime of tryptophans in the membrane-bound  $\alpha$ -toxin as compared to the native toxin supported the conclusions based on iodide quenching of the membrane-bound toxin. Fluorescence quenching of membrane-bound  $\alpha$ -toxin using brominated and spin-labeled fatty acids showed no quenching of fluorescence using brominated lipids. However, significant quenching was observed using 5- and 12-doxyl stearic acids. An average depth calculation using the parallax method indicated that the doxyl-quenchable tryptophans are located at an average depth of 10 Å from the center of the bilayer close to the membrane interface. This was found to be in striking agreement with the recently described structure of the membrane-bound form of  $\alpha$ -toxin.

### INTRODUCTION

$\alpha$ -Toxin is a soluble hemolytic protein exotoxin secreted by *Staphylococcus aureus* that is thought to be a major factor contributing to the pathogenicity of *S. aureus*. The amino acid sequence of  $\alpha$ -toxin has been deduced from its gene sequence (Kehoe et al., 1983; Gray and Kehoe, 1984). The toxin is composed of 293 amino acids and corresponds to a calculated molecular weight of 33,400, which is in fair agreement with the SDS-PAGE-based determinations.  $\alpha$ -Toxin damages membranes by the formation of nonspecific oligomeric pores in the target membranes, which lead to cell lysis (Fussle et al., 1981; Bhakdi et al., 1981). These pores are large in size, and diameters of 1–2 nm have been reported (Fussle et al., 1981; Menestrina, 1986). An insight into the mechanism of membrane insertion and channel formation by *S. aureus*  $\alpha$ -toxin and the amino acids involved in channel formation have been emerging from the extensive investigations on  $\alpha$ -toxin, based on mutational (Walker et al., 1993, 1994; Panchal and Bayley, 1995), biochemical (Tobkes et al., 1985; Walker et al., 1995), and biophysical approaches (Ward et al., 1994; Valeva et al.,

1996), culminating in the model confirmed by the recently solved x-ray structure of the detergent-solubilized heptamer (Song et al., 1996). The developments in the understanding of the transformation of this water-soluble toxin monomer to a channel-forming membrane-bound oligomer has been described in excellent reviews (Bhakdi and Tranum-Jensen, 1991; Thelestam and Blomqvist, 1988; Bhakdi et al., 1996; Gouaux, 1998).

According to the recently described structure of the heptameric form of  $\alpha$ -toxin (Song et al., 1996), the transmembrane pore complex is composed of three regions, the cap, the stem, and the rim domains. The large protrusions in electron microscopic images of the toxin (Ward and Leonard, 1992) have been identified as the cap and portions of the rim domain. The stem domain, which defines the 28-Å wide transmembrane channel, is described as a 14-strand antiparallel  $\beta$ -barrel composed of two 65-Å-long  $\beta$ -strands contributed by each monomer. The rim domains protrude from the underside of the heptamer and are in close proximity to the bilayer. However, the structure of the soluble form is yet unknown, and the formation of the heptameric pore requires structural transition from a water-soluble monomeric form into the oligomeric form and is believed to be catalyzed by binding to an unidentified receptor on the membrane or by binding to the membrane surface. Comparative studies of the soluble and membrane-bound forms therefore should give important information on the structural transitions that result in the functional heptameric pore.

Fluorescence spectroscopy is one of the spectroscopic techniques that provides structural information (although at a lower resolution) about structural and dynamic changes in proteins and is very useful in studying the interaction of

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soluble proteins with membranes. Aqueous soluble quenchers like iodide and acrylamide have been used to provide information on the gross location of tryptophan residues in the complex three-dimensional structure of soluble and membrane-bound proteins (Eftink, 1991). On the other hand, membrane-associated quenchers such as bromine atoms (East and Lee, 1982; Markello et al., 1985) or nitroxide (London and Feigenson, 1981; Blatt et al., 1984), groups covalently linked to fatty acids or phospholipids derived from these fatty acids, have been effectively used to evaluate involvement of tryptophan-containing regions of membrane-interacting proteins. They have also been used to determine the location of tryptophan residues of membrane-bound proteins in the bilayer (Meers, 1990; Jiang et al., 1991; Chattopadhyay and McNamee, 1991; Chung et al., 1992) and to follow the insertion of soluble proteins in membranes (Gonzalez-Manas et al., 1992). Another useful method to study the environment and organization of tryptophans is red edge excitation shift (REES) (Demchenko, 1988; Mukherjee and Chattopadhyay, 1994, 1995), which is a shift in the wavelength of emission maxima toward a higher wavelength caused by a shift in the excitation wavelength toward the red edge of the absorption band. This effect is observed when a polar fluorophore is present in a motionally restricted environment and arises from the slow rates of solvent relaxation around the excited state of the fluorophore caused by motional restriction on the solvent molecule in the immediate vicinity of the fluorophore.

In this study we have utilized the intrinsic fluorescence of tryptophan to evaluate structural changes in  $\alpha$ -toxin on transition from a water-soluble native form to a membrane-bound oligomeric form. The gene sequence of  $\alpha$ -toxin predicts 8 tryptophan residues in the toxin at positions 80, 167, 179, 187, 260, 265, 274, and 286 (Kehoe et al., 1983, Gray and Kehoe, 1984). Based on the recent structure of the detergent-solubilized heptamer, 6 tryptophans at positions 80, 179, 187, 260, 265, and 274 appear to be present in the rim domain, whereas Trp-167 and Trp-286 seem to be present in the cap domain (Song et al., 1996). Besides tryptophans, the rim domain contains several tyrosine residues and is thus rich in aromatic residues. We have studied tryptophan fluorescence quenching using the aqueous soluble quenchers, and spin-labeled and brominated membrane-bound quenchers were used to evaluate tryptophan-containing regions of  $\alpha$ -toxin in its soluble and membrane-bound forms. Quenching studies using aqueous soluble quenchers indicated that the tryptophan residues of the soluble  $\alpha$ -toxin were deeply buried within the protein tertiary structure. *N*-Bromosuccinimide (NBS) modification, time-resolved fluorescence measurements, and REES confirmed that some of the tryptophan residues were very deeply buried within the monomeric toxin structure. The fluorescence of membrane-bound  $\alpha$ -toxin, on the other hand, was quenched by iodide, indicating exposed tryptophan residues. This was also indicated by a shorter average fluorescence lifetime of the tryptophan residues of the membrane-bound toxin in comparison with the native toxin. Although brominated

membrane probes failed to quench the tryptophan fluorescence of the membrane-bound toxin, there was significant quenching by spin-labeled probes. Depth calculation using the parallax method (Chattopadhyay and London, 1987) suggested a location of tryptophans at an average depth of 10 Å from the center of the bilayer, indicating that the most of the tryptophan residues are located at the membrane-water interface. Based on the x-ray structure, Song et al. (1996) suggested that some of the residues in the rim domain might have some contact with the membrane hydrophobic environment. This is supported by studies of fluorescence changes on binding of acrylodan-labeled single cysteine mutant at position 266 (Valeva et al., 1996) and studies of the spectroscopic analysis of conformational changes in  $\alpha$ -toxin associated with membrane binding and insertion (Vecsey-Semjen et al., 1997).

It is being increasingly recognized that tryptophan residues of membrane-bound proteins are preferentially located at the membrane-water interface (Weiss and Schulz, 1992; Deisenhofer et al., 1995; Ostermeier et al., 1996; Grigorieff et al., 1996). Our studies thus confirm that the membrane-bound oligomeric pore formed by *S. aureus*  $\alpha$ -toxin is another example corroborating the preference of tryptophan residues to reside in the membrane-water interface.

## EXPERIMENTAL PROCEDURES

Reagents of commercial grade and highest purity were used. Spectral grade water obtained using Milli-Q Plus from Millipore Corporation, Bedford, MA, was used in all experiments. Potassium iodide was bought from Loba Chemicals and acrylamide was from SRL, Bombay, India. NBS was purified by recrystallization from water. 9,10-dibromostearic acid was prepared by addition of bromine to oleic acid in  $\text{CCl}_4$  at 0°C according to the procedure of Nevenzel and Howton (1957). The product after chromatography on silica gel appeared as a single spot. The product was further characterized by IR, NMR, and mass spectroscopy. Bis-9,10-dibromostearoyl phosphatidylcholine (9,10-BrPC) was prepared from 9,10-dibromostearic acid according to the procedure of Regen et al. (1983) and characterized by NMR. 5- and 12-Doxyl stearic acids were from Molecular Probes (Eugene, OR). Asolectin (Sigma Chemical Company, St. Louis, MO) was further purified by the procedure of Kagawa and Racker (1971) and stored at  $-20^\circ\text{C}$  as a 0.25 M stock solution in chloroform as determined by phosphate assay (Ames and Dubin, 1960). All lipid concentrations expressed are based on phosphate assay. Samples of lyophilized  $\alpha$ -toxin were a kind gift from Dr. S. Bhakdi (Institute of Medical Microbiology, University of Mainz, Augustusplatz, Mainz, Germany). Concentration of  $\alpha$ -toxin was estimated from absorbance at 280 nm using an  $A_{280}$  of  $1.1 \text{ mg}^{-1} \text{ ml}^{-1}$  (Harshman et al., 1988). All experiments were done at  $23^\circ\text{C}$ , using 10 mM Tris containing 100 mM NaCl at pH 7.0 (referred to as standard buffer) unless otherwise specified.

## Steady-state fluorescence studies

Steady-state fluorescence measurements were done with a Shimadzu RF-540 or Hitachi F-4010 spectrofluorometer using a quartz cuvette of 1-cm path length. For quenching experiments, the excitation wavelength was set at 295 nm with a slit width of 5 nm, and the emission range was set between 300 and 500 nm, with the slit width kept at 10 nm. Steady-state fluorescence quenching was carried out by measuring the fluorescence intensities at the emission maxima as a function of the quencher concentration or as a function of time. Increasing concentrations of the quencher were added from a concentrated stock solution of the quencher in water.

Fluorescence intensities were corrected for dilution. For acrylamide quenching studies fluorescence measurements were further corrected for the attenuation of the excitation light intensities due to the added acrylamide (Parker, 1968), which has a molar extinction coefficient of  $\sim 0.23$  at 295 nm, by multiplying the measured fluorescence by the factor as given,

$$\text{Factor} = \text{antilog}[(A_{\text{ex}} + A_{\text{em}})/2]$$

where  $A$  is the absorbance of a given concentration of acrylamide at the excitation and emission wavelengths. The fluorescence quenching data was analyzed by the Stern-Volmer equation (Stern and Volmer, 1919)

$$(F_0/F) - 1 = K_{\text{sv}}[Q]$$

where  $F_0$  is the initial fluorescence,  $F$  is the fluorescence at the quencher concentration  $[Q]$ , and  $K_{\text{sv}}$  is Stern-Volmer's constant. A plot of  $(F_0/F) - 1$  versus  $[Q]$  gives rise to a linear plot which passes through the origin. However, if all the tryptophans are not accessible to the quencher, the Stern-Volmer plot deviates from linearity and the quenching process can be described by a modified equation by Lehrer (Eftink, 1991):

$$F_0/(F_0 - F) = 1/K_{\text{sv}} \cdot [Q] + 1/f_a$$

where  $(F_0 - F)$  refers to the change in fluorescence intensity on addition of the quencher and  $f_a$  refers to the fraction of tryptophans accessible to the quencher.

For REES studies, the fluorescence emission spectra of the samples were scanned at excitation wavelengths varying from 280 to 310 nm using a bandwidth of 5 nm for excitation and emission. For each sample the corresponding blank spectra were recorded and subtracted from the sample spectra.

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer operated in the time-correlated single-photon counting mode. The machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas ( $15 \pm 1$  inch of Hg vacuum) and runs at 22–25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox as the scatterer. To optimize the signal-to-noise ratio, 5000 photons were collected in the peak channel. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. The sample was excited at 297 nm with a slit of 2 nm, and the fluorescence intensity decay at 340 nm was measured using a slit of 4 nm. The data were collected and stored in a multichannel analyzer and were routinely transferred to an IBM PC/AT computer for analysis. Intensity decay curves were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i)$$

where  $\alpha_i$  is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime  $\tau_i$ . The decay parameters were recovered using a nonlinear least-square iterative fitting procedure based on the Marquardt algorithm (Bevington, 1969). The program includes statistical and plotting subroutine packages (O'Connor and Phillips, 1984). The goodness of the fit of a given set of observed data and the chosen function was evaluated by the reduced  $\chi^2$  ratio, the weighted residuals (Lampert et al., 1983), the autocorrelation function of the weighted residuals (Grinvald and Steinberg, 1974), the runs test (Hamburg, 1985), and the Durbin-Watson parameters (Durbin and Watson, 1950). A fit was considered to be acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation of approximately 0 with a  $\chi^2$  value not more than 1.2. Mean (average) lifetimes  $\langle \tau \rangle$  for the biexponential decays of the fluorescence were calculated from the decay times and pre-exponential factors using the equation

(Lakowicz, 1983)

$$\langle \tau \rangle = \frac{(\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2)}{(\alpha_1 \tau_1 + \alpha_2 \tau_2)}$$

## Liposome preparation

Small unilamellar vesicles were prepared from aolectin or bis-9,10-dibromostearoyl phosphatidylcholine. 5  $\mu\text{mol}$  of lipid was dried on the walls of a test tube. The thin film was dried under vacuum for 8 h. The film was hydrated with 1 ml of standard buffer, which was then vortexed. The suspension was sonicated for 12 min twice using a Branson B-30 sonicator fitted with a microtip. The resulting clear solution was then spun at 15,000 rpm ( $15,850 \times g$ ) to pellet any titanium particles shed from the microtip during sonication.

## N-Bromosuccinimide modification of $\alpha$ -toxin

$\alpha$ -Toxin (24  $\mu\text{g}/\text{ml}$ ) was subjected to NBS modification (Spande and Witkop, 1967; Lundblad, 1995) using 5-, 10-, 20-, 40-, 50-, and 100-fold molar excess of NBS over the toxin, using a 0.24 mM stock solution of NBS in water. The fluorescence intensity at 332 nm was monitored as a function of time after excitation at 280 nm. In each case the fluorescence intensity of the same amount of  $\alpha$ -toxin without NBS was used as a control.

## RESULTS

### Fluorescence studies of the soluble monomeric $\alpha$ -toxin

#### *Fluorescence quenching of $\alpha$ -toxin by aqueous soluble quenchers*

The emission maxima of the native toxin and denatured toxin in 8 M urea were observed at 333 and 352 nm, respectively, indicating that the tryptophans were buried in the nonpolar interior of the toxin. The quenching of  $\alpha$ -toxin in solution by aqueous quenchers like iodide, acrylamide, and trichloroethanol permitted useful conclusion to be drawn about the degree of tryptophan exposure. Although iodide is known to quench only the tryptophan residues accessible to the solvent, i.e., residues in a polar environment, acrylamide and trichloroethanol penetrate the protein interior and can also quench tryptophan residues buried in the protein (Eftink, 1991).

The Stern-Volmer plots for the quenching of tryptophan fluorescence of the native toxin and the toxin denatured in 8 M urea by iodide are shown in Fig. 1. In the case of iodide quenching of the native toxin, only 8.6% of the fluorescence was quenched at the highest quencher concentration and more than 90% of the fluorescence was unquenched. The effective  $K_{\text{sv}}$  expressed in terms of  $(F_0/F) - 1/[Q]$  at the highest concentration of iodide was  $0.38 \text{ M}^{-1}$ . The Lehrer analysis gave an accessibility factor ( $f_a$ ) value of 0.14, indicating that most of the tryptophan residues of the toxin are inaccessible to iodide and hence must be deeply buried in the protein interior. In the case of the toxin denatured in 8 M urea,  $\sim 60\%$  of the initial fluorescence was quenched by iodide, and the Stern-Volmer plot shows a negative deviation from linearity. The analysis of the data using the

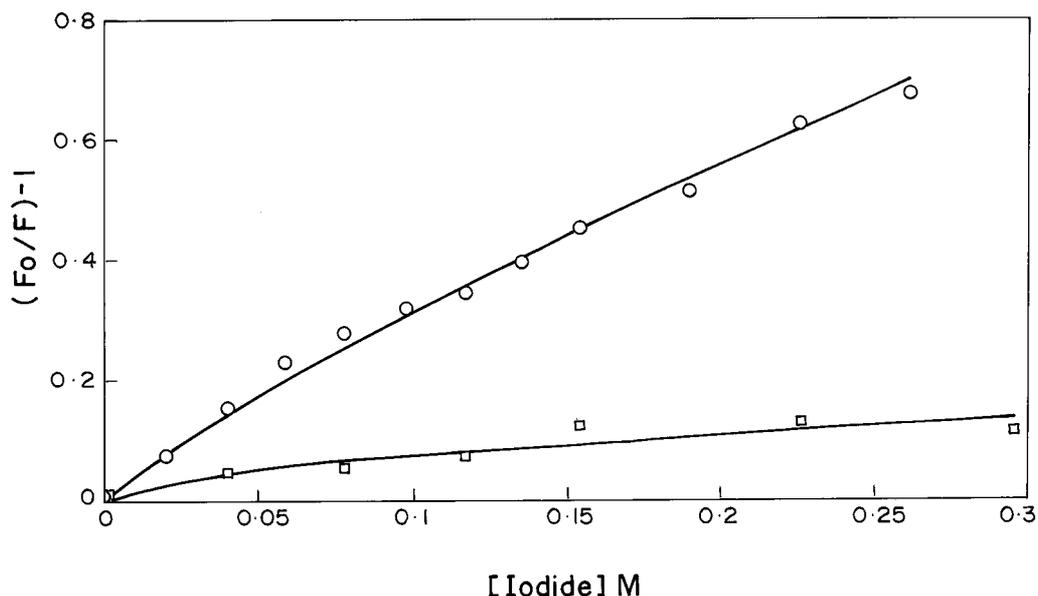


FIGURE 1 Stern-Volmer plots for iodide quenching of native ( $\square$ ) and denatured ( $\circ$ )  $\alpha$ -toxin. Increasing aliquots from a 4 M stock solution of KI containing 200  $\mu$ M sodium thiosulfate to prevent oxidation of  $I^-$  to  $I_2$  or  $I_3^-$  was added to 19  $\mu$ g/ml of  $\alpha$ -toxin in standard buffer. The intensity of fluorescence at the emission maximum (332 nm for native  $\alpha$ -toxin or 352 nm for denatured  $\alpha$ -toxin) was measured after excitation at 295 nm. The emission bandwidth was kept at 10 nm, whereas the excitation bandwidth was 5 nm.  $F_0$  is the fluorescence intensity in the absence of the quencher, whereas  $F$  is the intensity in the presence of the quencher.

Lehrer equation gave  $f_a$  value of 0.63 for the iodide-accessible fraction of  $\alpha$ -toxin in 8 M urea, showing that only 63% is accessible to quenching by iodide. These results indicated that some of the tryptophan residues are still buried even on denaturation of the toxin in 8 M urea. All of these clearly point out to the fact that the tryptophan residues in the native toxin must be deeply buried in the toxin interior.

In order to study the buried tryptophan residues in  $\alpha$ -toxin, we used acrylamide and trichloroethanol as neutral quenchers, which can penetrate the protein interior and quench buried tryptophans (Fig. 2). Using acrylamide it was observed that only 16% of the tryptophans in  $\alpha$ -toxin were quenched at the highest quencher concentration. Thus nearly 84% of the tryptophan residues are refractive to

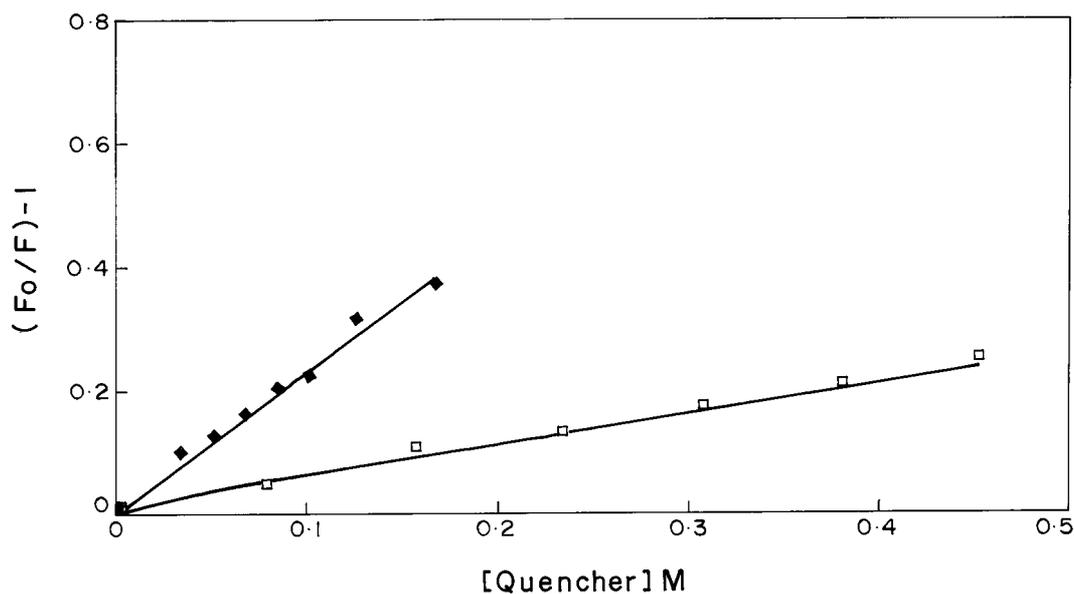


FIGURE 2 Stern-Volmer plots for quenching of native  $\alpha$ -toxin by acrylamide ( $\square$ ) and TCE ( $\blacklozenge$ ). Increasing aliquots from an 8 M stock solution of acrylamide in water or neat TCE (10.2 M) were added to 19  $\mu$ g/ml of  $\alpha$ -toxin in standard buffer. The intensity of fluorescence emission at 332 nm (slit = 10 nm) was measured after excitation at 295 nm (slit = 5 nm).  $F_0$  is the fluorescence intensity in the absence of the quencher, whereas  $F$  is the intensity in the presence of the quencher.

quenching by acrylamide. The quenching is nevertheless greater than that by iodide. The fluorescence quenching fitted into a linear Stern-Volmer plot with  $K_{SV} = 0.58 \text{ M}^{-1}$  is shown in Fig. 2. This is very low in comparison to the Stern-Volmer quenching constant for a fully exposed tryptophan as in the case of *N*-acetyl-L-tryptophanamide in water ( $K_{SV} = 17.5 \text{ M}^{-1}$ ) (Eftink and Ghiron, 1976), indicating the buried nature of tryptophans in  $\alpha$ -toxin. However, it is higher than the quenching constant for acrylamide quenching of aldolase ( $K_{SV} = 0.2 \text{ M}^{-1}$ ) at pH 5.5 (Eftink and Ghiron, 1976). The low  $K_{SV}$  value signifies that the tryptophan residues must be buried. Trichloroethanol (TCE) is another neutral quencher, which is less polar than acrylamide and has been shown to be more effective as a hydrophilic quencher than acrylamide in some cases (Eftink et al., 1977). TCE was indeed found to quench the tryptophan residues of  $\alpha$ -toxin much more than acrylamide. Nearly 30% of the fluorescence was quenched at the highest quencher concentration used. The Stern-Volmer plot was linear with a  $K_{SV}$  value of  $2.3 \text{ M}^{-1}$  and is also shown in Fig. 2. The emission maximum of the tryptophans not quenched by TCE was observed at 329 nm, which is blue-shifted with respect to the native toxin. This clearly indicated tryptophan heterogeneity, i.e., some of the tryptophan residues were more superficially located, whereas others are buried within the tertiary structure of the toxin.

### ***N*-Bromosuccinimide modification of $\alpha$ -toxin in solution**

Fluorescence quenching studies of  $\alpha$ -toxin in solution using iodide, acrylamide, and trichloroethanol showed that the tryptophans of the toxin were deeply buried. We hence decided to further probe accessibility of the tryptophans to chemical modification using NBS (Spande and Witkop, 1967; Lundblad, 1995). The toxin was modified using various molar ratios of NBS to toxin, and the reduction in

fluorescence emission at 333 nm was monitored after excitation at 295 nm. Fig. 3 shows the plot of the residual toxin fluorescence versus NBS/toxin ratio, and the inset shows a decrease in the fluorescence of toxin caused by oxidation of tryptophans by NBS at various NBS/toxin molar ratios as a function of time. The modification was rapid as seen from the fact that the fluorescence dropped to its limiting value in about 2.5 min. At an NBS/toxin molar ratio of 5, ~22% of the fluorescence is abolished. Further increase in the NBS/toxin molar ratio leads to modification of more tryptophan residues. However, even at 100 times molar excess of NBS, ~25% of the fluorescence was still observable, indicating that some of the tryptophan residues are inaccessible to chemical modification. It was also observed that the residual fluorescence had an emission maximum at 328 nm and was 4–5 nm blue-shifted, indicating that the residual fluorescence is probably due to some tryptophans, which were in a very hydrophobic environment.

### **Fluorescence studies of membrane-bound $\alpha$ -toxin**

#### *Iodide quenching of membrane-bound $\alpha$ -toxin*

Iodide was found to effectively quench the tryptophans of  $\alpha$ -toxin in the membrane-bound form, quite unlike the soluble form, which showed minimal quenching by iodide. Almost 50% of the fluorescence was quenched at the highest quencher concentration. The Stern-Volmer plot showed a negative deviation from linearity. Analysis of the data according to the modified Stern-Volmer equation indicated a fractional accessibility of 0.78. The Stern-Volmer constant for the accessible fraction was calculated to be  $2.96 \text{ M}^{-1}$ , while the effective  $K_{sv}$  in terms of  $(F_0/F) - 1/[Q]$  at the highest quencher concentration was  $2.7 \text{ M}^{-1}$ . The Stern-Volmer plot of the quenching data is shown in Fig. 4, while the analysis according to the Lehrer equation is shown in the

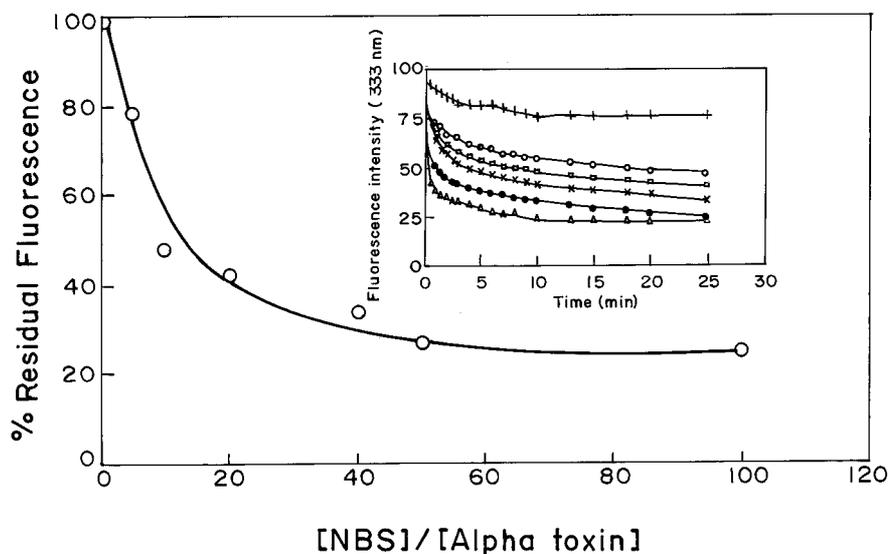
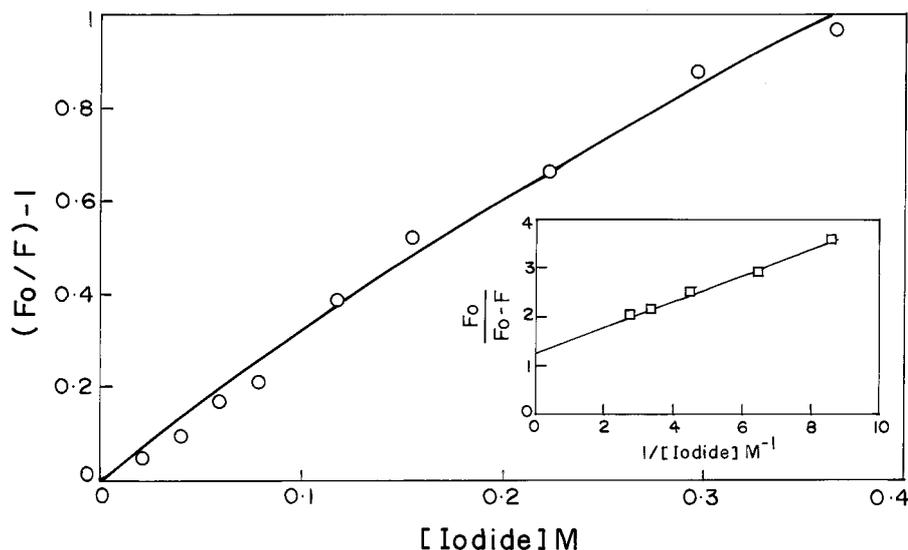


FIGURE 3 NBS modification of  $\alpha$ -toxin.  $\alpha$ -Toxin ( $24 \mu\text{g/ml}$ ) was modified with a 5- (+), 10- (○), 20- (□), 40- (×), 50- (●), and 100- (△) fold molar excess of freshly prepared 10 mM stock solution of *N*-bromosuccinimide in water. The decrease in emission intensity at 332 nm (slit = 10 nm) was monitored as a function of time after excitation at 280 nm (slit = 5 nm). A plot of the percent residual fluorescence (○) at equilibrium as a function of molar ratio of NBS/ $\alpha$ -toxin is shown. The inset shows the time course of modification of tryptophans of  $\alpha$ -toxin by NBS.

FIGURE 4 Stern-Volmer plot for fluorescence quenching of membrane-bound  $\alpha$ -toxin by iodide.  $\alpha$ -Toxin ( $40 \mu\text{g/ml}$ ) was added to asolectin vesicles ( $300 \text{ nmol/ml}$ ) and incubated for 1 h. The lipid/toxin molar ratio was 250:1. The membrane-toxin complex was titrated with increasing aliquots from a 4 M stock solution of KI containing  $200 \mu\text{M}$  sodium thiosulfate to prevent oxidation of  $\text{I}^-$  to  $\text{I}_2$  or  $\text{I}_3^-$ . The intensity of fluorescence at 332 nm was measured after excitation at 295 nm. The emission bandwidth was kept at 10 nm, whereas the excitation bandwidth was 5 nm.  $F_0$  is the fluorescence intensity in the absence of the quencher, whereas  $F$  is the intensity in the presence of the quencher. The inset shows a Lehrer plot for the iodide quenching data.



inset. These results suggest that the buried tryptophan residues of  $\alpha$ -toxin in the soluble form are exposed in the membrane-bound form.

#### Quenching of tryptophan fluorescence of $\alpha$ -toxin by membrane-bound quenchers, bis-9,10-dibromostearoyl phosphatidylcholine, 9,10-dibromostearic acid, and doxyl stearic acids

The quenching of tryptophan fluorescence of membrane-bound proteins or peptides by brominated lipids have been used for evaluating the exposure of tryptophans to lipids on interaction of a protein or a peptide with lipids (East and Lee, 1982; Berkhout et al., 1987; Bolen and Holloway, 1990; Gonzalez-Manas et al., 1992, 1993). We therefore studied the insertion of  $\alpha$ -toxin into lipid vesicles using quenching of tryptophan fluorescence of  $\alpha$ -toxin using vesicles made of 9,10-BrPC. We also studied the quenching of membrane bound  $\alpha$ -toxin with 9,10-dibromostearic acid, 5-doxyl stearic acid, and 12-doxyl stearic acid.

Membrane pore-forming activity of the  $\alpha$ -toxin preparation in vesicles prepared with these reporter lipid molecules was confirmed by checking the release of encapsulated calcein from asolectin vesicles and 9,10-BrPC vesicles. We decided to follow the quenching of  $\alpha$ -toxin fluorescence on its interaction with 9,10-BrPC vesicles under these conditions. However, we observed that there was no appreciable quenching of the toxin fluorescence, which remained almost the same. This is illustrated in Fig. 6, where the value of  $F_0/F$  does not show any appreciable change. The quenching of tryptophan fluorescence should result in an increase in the value of  $F_0/F$ , which saturates with time. As a positive control we checked the quenching of  $\alpha$ -lactalbumin fluorescence, which has been one of the proteins used as a model for studying membrane-protein interactions in our laboratory (Lala et al., 1995). This is shown in the inset in Fig. 5.

Quenching of the membrane-bound  $\alpha$ -toxin using 9,10-dibromostearic acid showed similar results. No decrease in the fluorescence intensity of the  $\alpha$ -toxin bound to asolectin vesicles were observed (Fig. 6). Although brominated quenchers cause minimum perturbation to the membrane, spin labels can quench virtually any fluorophore including tryptophans and hence may be more useful than brominated probes. The radius of quenching for brominated probes is 8–9 Å (Bolen and Holloway, 1990), whereas spin-labeled probes quench over a range of 11–12 Å (London and Feigenson, 1981; Chattopadhyay and London, 1987). We therefore studied the fluorescence quenching of membrane-bound  $\alpha$ -toxin by 5- and 12-doxyl stearic acids. It was indeed encouraging to find that both 5- and 12-doxyl stearic acids effectively quenched the fluorescence of membrane-bound  $\alpha$ -toxin. This is shown in Fig. 6. Quenching of the membrane-bound toxin by 5-doxyl stearic acid was more than that by 12-doxyl stearic acid, indicating that the tryptophan residues were closer to the membrane-water interface. About 37% of the fluorescence was quenched by 5-doxyl stearic acid, whereas 12-doxyl stearic acid quenched 25% of the fluorescence. The Stern-Volmer plot showed a negative deviation from linearity, indicating fractional accessibility to the quencher. An analysis using the Lehrer equation gave an  $f_a$  value of 0.5 in the case of 5-doxyl stearic acid, indicating that 50% of the tryptophan residues of the membrane-bound  $\alpha$ -toxin are accessible to quenching by 5-doxyl stearic acid. A similar analysis in the case of 12-doxyl stearic acid gave an  $f_a$  value of 0.39, indicating 39% accessible tryptophans. An average depth calculation based on the parallax method (Chattopadhyay and London, 1987) suggested that the tryptophan residues are located at a distance of 10 Å from the center of the bilayer. The observation that brominated quenchers fail to quench the fluorescence of membrane-bound  $\alpha$ -toxin, while doxyl probes effectively quench, also leads to the conclusion that tryptophan residues are not directly in contact with

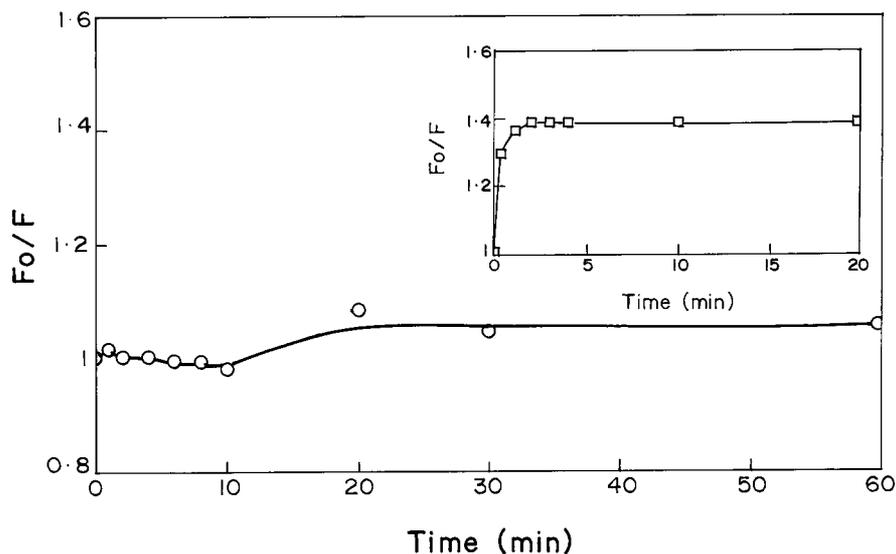


FIGURE 5 Fluorescence quenching of  $\alpha$ -toxin in 9,10-BrPC vesicles.  $\alpha$ -Toxin (20  $\mu\text{g/ml}$ ) was added to vesicles made of 9,10-BrPC (122 nmol/ml). The lipid/toxin molar ratio was 200:1. The binding of  $\alpha$ -lactalbumin to 9,10-BrPC vesicles was taken as a positive control.  $\alpha$ -Lactalbumin (30  $\mu\text{g/ml}$ ) was added to 9,10-BrPC vesicles (416 nmol/ml) in 10 mM citrate/phosphate buffer containing 100 mM NaCl at pH 3.8 (lipid/protein = 200:1). The fluorescence intensity of  $\alpha$ -toxin or  $\alpha$ -lactalbumin added to asolectin vesicles under the same conditions at a given time was taken as  $F_0$ , whereas  $F$  was taken as the intensity of  $\alpha$ -toxin or  $\alpha$ -lactalbumin added to 9,10-BrPC vesicles at the corresponding time. All fluorescence intensities were corrected for vesicle scatter by subtracting the fluorescence value of the vesicles at 332 nm. The inset shows the quenching of  $\alpha$ -lactalbumin on binding to 9,10-BrPC vesicles

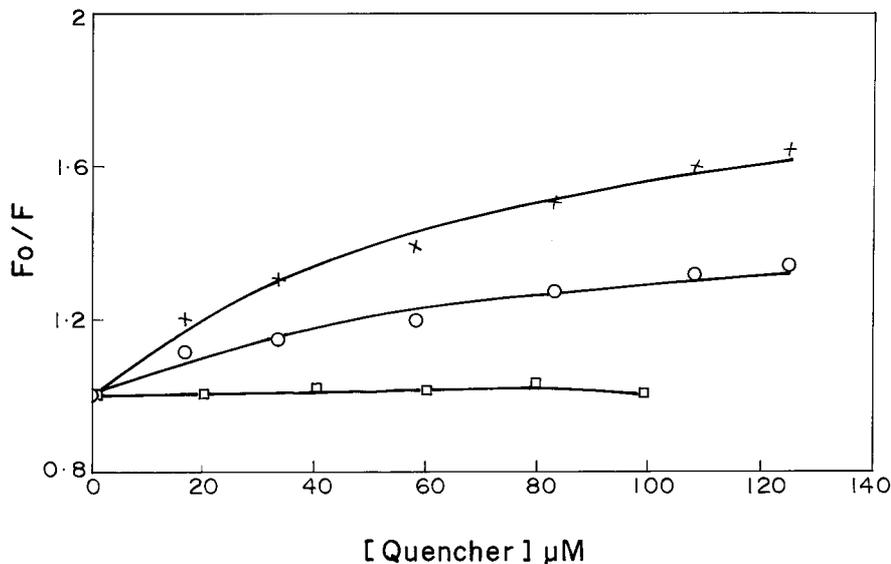
the lipid hydrocarbon phase of the bilayer. Addition of iodide to the doxyl-quenched samples could not further quench the fluorescence of the membrane-bound  $\alpha$ -toxin (data not shown). This observation, along with the fact that iodide, a quencher that barely penetrates the membrane, effectively quenched the fluorescence of membrane-bound  $\alpha$ -toxin, leads to the conclusion that both the quenchers are sensing the same set of tryptophan residues and hence most of tryptophan residues in the membrane-bound  $\alpha$ -toxin oligomer must be located near the membrane-water interface.

This was indeed found to be true based on the recent x-ray structure of the  $\alpha$ -toxin pore (Song et al., 1996).

#### Fluorescence lifetime measurements

The steady-state fluorescence studies of soluble and membrane-bound  $\alpha$ -toxin showed that the tryptophan residues are buried in the native form, whereas it gets exposed in the membrane-bound form of the toxin. We sought to confirm

FIGURE 6 Fluorescence quenching of membrane-bound  $\alpha$ -toxin by 9,10-dibromostearic acid, 5-doxyl stearic acid, and 12-doxyl stearic acid. Asolectin vesicle-bound  $\alpha$ -toxin was prepared as described in the legend to Fig. 4. The membrane-toxin complex was titrated with increasing aliquots from a 10 mM stock solution of 9,10-dibromostearic acid ( $\square$ ), 5-doxyl stearic acid ( $\times$ ), or 12-doxyl stearic acid ( $\circ$ ). The samples were incubated for 20 min after each addition. At the end of 20 min, the intensity of fluorescence at 332 nm was measured after excitation at 295 nm. The emission bandwidth was kept at 10 nm, whereas the excitation bandwidth was 5 nm.  $F_0$  is the fluorescence intensity in absence of the quencher, whereas  $F$  is the intensity in presence of the quencher.



this by fluorescence lifetime measurements of the native, NBS-modified, and membrane-bound  $\alpha$ -toxin. Table 1 shows the lifetimes of tryptophan residues of  $\alpha$ -toxin in solution after NBS modification and after membrane binding. In all the cases the fluorescence decay fitted into a biexponential function, with a major component having a short lifetime and a minor component having a relatively longer lifetime. The mean lifetime for the native toxin was found to be 1.79 ns. In the membrane-bound form the tryptophans are found to have a shorter lifetime of 1.35 ns and hence a faster rate of relaxation. This indicates that there is a change in the microenvironment around the tryptophan residues. Changes in fluorescence lifetime, in general, can be attributed to a number of factors. However, change in polarity of the environment is known to reduce the lifetime of tryptophans (Kirby and Steiner, 1970). Since our results from iodide quenching experiments also indicate that the tryptophan residues in the membrane-bound toxin are more exposed to the aqueous environment than in its soluble form, the decrease in tryptophan lifetime could arise from a more polar environment experienced by the tryptophan residues in the membrane-bound toxin complex. This conclusion is strengthened by comparing the second order bimolecular quenching constants ( $k_q$ ) obtained from the effective  $K_{sv}$  and the measured unquenched lifetime. The  $k_q$  for quenching of  $\alpha$ -toxin by iodide increases from  $0.378 \text{ M}^{-1} \text{ ns}^{-1}$ , measured in solution, to  $1.48 \text{ M}^{-1} \text{ ns}^{-1}$ , measured in the membrane-bound form, reflecting the increase in aqueous exposure of the average tryptophan environment.

The residual fluorescence of NBS-modified toxin was seen to have an emission maximum of 328 nm, blue-shifted with respect to the unmodified toxin, indicating that it was very deeply buried and in a restricted environment. Lifetime measurement of the NBS-modified  $\alpha$ -toxin supported this conclusion, as the mean lifetime was seen to be much longer (2.05 ns) as compared to the native form.

### Red edge excitation shift studies of $\alpha$ -toxin

REES is a powerful tool to gain information about the environment around tryptophan residues of a protein in solution and to monitor structural changes during transformation from a soluble form to a membrane-bound form (Demchenko, 1988; Mukherjee and Chattopadhyay, 1994, 1995). We used REES to get additional information about soluble and membrane-bound  $\alpha$ -toxin. Both native and membrane-bound  $\alpha$ -toxin showed a 2-nm REES (Fig. 7), indicating that on average, tryptophan residues were not in

a motionally restricted environment. It also suggests that there was no drastic change in the average environment around the tryptophan residues on membrane binding especially in terms of solvent reorientation dynamics. The results are shown in Fig. 7.

NBS modification of the native toxin showed that even a 100-fold molar excess of NBS could not abolish the fluorescence of the toxin. The residual fluorescence showed an emission maximum at 328 nm and was blue-shifted with respect to the unmodified toxin, which had an emission maximum of 332 nm. This argued for the fact that some of the tryptophan residues must be in a highly hydrophobic environment and must be facing motional restriction from its surroundings. This was confirmed by REES of NBS-modified toxin, which gave a REES of 5–6 nm. This result is also shown in Fig. 7.

## DISCUSSION

Structural aspects of the mechanism of pore formation in membranes by pore-forming toxins can be understood only by the identification of regions of the toxin interacting with the membrane. Song et al. (1996) have recently described the structure of the detergent-solubilized heptameric form of *S. aureus*  $\alpha$ -toxin. However, the structure of the water-soluble monomer is not yet known. The  $\alpha$ -toxin monomer has been described in terms of a two-domain model by Tobkes et al. (1985). The molecule is thought to be composed of separately folded N-terminal and C-terminal domains connected by a glycine-rich region described as the hinge region. On the basis of the heptamer structure, the protomer core has been described as a  $\beta$  sandwich formed by separately folded N- and C-terminal domains, connected by the loop region, which forms the stem domain of the heptameric complex (Song et al., 1996; Gouaux, 1998). The toxin is thought to bind to the membrane surface or an unidentified receptor on the surface as a monomer and to oligomerize into a non-lytic heptameric prepore complex. The prepore oligomeric complex is converted to the lytic oligomer by insertion of the glycine-rich loop (residues 110–148) from each protomer, which organizes into a 14-stranded  $\beta$ -barrel in the membrane hydrophobic core.

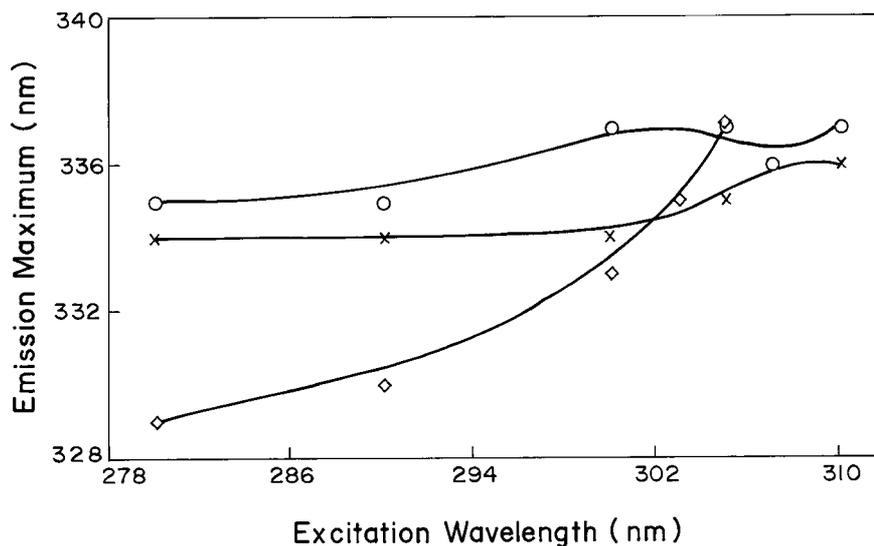
The protein must go through several structural changes before reaching its functional pore-forming state. The steps we can visualize are 1) binding of monomer to the membrane, probably accompanied by a mild denaturation of the monomer at the interface, resulting in a conformational change and formation of a molten globule state of the

**TABLE 1** Fluorescence lifetimes of tryptophans in soluble and membrane-bound  $\alpha$ -toxin

Sample	$\alpha_1$	$\tau_1$	$\alpha_2$	$\tau_2$	$\chi^2$	$\langle\tau\rangle$
Native $\alpha$ -toxin	0.95	0.71	0.05	4.82	1.19	1.79
NBS-modified $\alpha$ -toxin	0.90	0.59	0.1	4.00	1.12	2.05
Membrane-bound $\alpha$ -toxin	0.96	0.56	0.04	4.00	1.03	1.35

Excitation at 297 nm, emission monitored at 340 nm.

FIGURE 7 Red edge excitation shift in fluorescence of native, NBS-modified, and membrane-bound  $\alpha$ -toxin. The fluorescence emission spectra of native ( $\times$ ), membrane-bound ( $\circ$ ), and NBS-modified ( $\diamond$ )  $\alpha$ -toxin were scanned at various excitation wavelengths ranging from 280 to 310 nm. A bandwidth of 5 nm was used for emission and excitation. The fluorescence emission spectra, for excitation at 305–310, were an average of three scans recorded with a high photomultiplier gain. For each sample the blank spectra were recorded and subtracted from the sample spectrum. An  $\alpha$ -toxin concentration of 40  $\mu\text{g/ml}$  was used for native and membrane-bound  $\alpha$ -toxin. For studying REES of NBS-modified  $\alpha$ -toxin, a concentration of 132  $\mu\text{g/ml}$  was used. Asolecitin vesicle-bound  $\alpha$ -toxin was prepared as described in the legend to Fig. 4.



monomer; 2) formation of heptamer by lateral diffusion in the plane of the membrane (interfacial region) and stabilization of the heptamer by formation of intermonomeric contacts; and 3) a second conformational change, resulting in the spontaneous insertion of the loop into the membrane and formation of the membrane active heptameric pore.

Our studies reveal several features of the monomer structure and structural changes occurring in the transition from soluble to a membrane-bound form. In the monomer, the tryptophan residues were buried within a hydrophobic environment, as indicated by an emission maximum of 332 nm, as well as quenching studies using iodide, acrylamide, and TCE. NBS modification and REES of the NBS-modified toxin supported the conclusion. Upon binding to membranes the structure goes through a conformational change as indicated by the exposure of tryptophan residues (which indicates change in the tertiary structure of the toxin) as suggested by iodide quenching and the shift in the lifetime. Iodide quenching of the membrane-bound  $\alpha$ -toxin indicated that about 80% of the tryptophan residues were exposed and quenched by iodide. The fluorescence lifetime of the membrane-bound  $\alpha$ -toxin also supported this conclusion, as the mean lifetime of the tryptophan residues of the membrane-bound toxin was shorter (1.35 ns) as compared to that of the native toxin (1.79 ns).

Fluorescence quenching by brominated and spin-labeled fatty acids indicated that in the membrane-bound state the tryptophans were not exposed to the lipid hydrocarbon core. One of us (Chattopadhyay and London, 1987) has previously described an elegant method to determine the depth of a fluorophore in a membrane by comparing the quenching by two membrane-bound quenchers, with the quencher group at different depths on the fatty acyl chain. Using this method we calculated the average depth of the membrane-quenchable tryptophans to be located at a distance of about 10 Å from the center of the bilayer. This means that these must lie close to the membrane-water interface. Interestingly, both native and membrane-bound toxins did not show

much REES, indicating that on average the tryptophan residues do not encounter motional restriction. As mentioned earlier, most of the tryptophan residues are localized in the rim domain of the pore complex described by Song et al. (1996). It is thought that some regions of the rim domain may dip into the membrane (Song et al., 1996; Vecsey-Semjen et al., 1997). Our measurement of the depth of Trp residues confirms this belief.

The membrane-bound form of  $\alpha$ -toxin thus appears to be another example of a protein in which aromatic residues seem to be sequestered at the membrane boundary. Other examples are bacterial porins (Weiss and Schulz, 1992), the bacterial photosynthetic reaction center (Deisenhofer et al., 1995), cytochrome *c* oxidase (Ostermeier et al., 1996), and bacteriorhodopsin (Grigorieff et al., 1996). Tryptophan is a unique amino acid in that it has the largest nonpolar surface area and is a polar amino acid due to the presence of indole N-H, which gives it the ability to form an H-bond near the interfacial region of the membrane. This duality in chemical structure helps it to float in the interfacial region. Kachel et al. (1995) have analyzed the depths intrinsically favored by tryptophan and tyrosine by studying the location of membrane associating Trp and Tyr analogues using the parallax analysis of fluorescence quenching. These were found to be located at the same depths as Trp and Tyr in membrane proteins. The amphipathic nature of Trp and Tyr residues has been implicated in its interfacial partitioning and acting as anchors or floats for membrane protein inserted into the membrane. This gives stability to the vectorial nature of membrane proteins (Chattopadhyay et al., 1997).

For hydrophilic channel-forming proteins such as  $\alpha$ -toxin, this property of aromatic residues should be one of the factors stabilizing the intermediate membrane-bound monomer. Besides aromatic residues, the crevice between the stem and the rim domain of  $\alpha$ -toxin is also rich in basic amino acids. This could provide the basis of the initial electrostatic interaction of the monomer with the membrane surface, besides participating in interactions with the phos-

pholipid headgroups. The anchoring of the protein also could be giving the necessary orientation for the formation of this prepore complex, reducing the collisional requirement from three dimensions to two dimensions and subsequent stabilization of the prepore complex by electrostatic, hydrophobic, and hydrophilic interactions. It is striking to note that some of the bacterial pore-forming toxins whose structures are known, like aerolysin and perfringolysin, are rich in tryptophan residues and have domains rich in tryptophan. These are aerolysin from *Aeromonas hydrophila*, which has tryptophan-rich domain 2 (Parker et al., 1994), and the perfringolysin O from *Clostridium perfringens*, which has tryptophans concentrated in domain 4 (Rossjohn et al., 1997). More interestingly, these have been implicated in binding to its membrane receptors. It is therefore tempting to speculate that these tryptophans may be playing a similar role in the process of the transformation of these family of toxins from a water-soluble to a membrane-bound form.

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