Interaction of the α-Toxin of Staphylococcus aureus with the Liposome Membrane*

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When the liposome membrane is exposed to the α-toxin of Staphylococcus aureus, fluorescence of the tryptophan residue(s) of the toxin molecule increases concomitantly with the degree of toxin-hexamer formation (Ikigai, H., and Nakae, T. (1985) Biochem. Biophys. Res. Commun. 130, 175–181). In the present study, the toxin-membrane interaction was distinguished from the hexamer formation by the fluorescence energy transfer from the tryptophan residue(s) of the toxin molecule to the dansylated phosphatidylethanolamine in phosphatidylcholine liposome. Measurement of these two parameters yielded the following results. (i) The effect of the toxin concentration and phospholipid concentration on these two parameters showed first order kinetics. (ii) The effect of liposome size on the energy transfer and the fluorescence increment of the tryptophan residue(s) was only detectable in small liposomes. (iii) Under moderately acidic or basic conditions, the fluorescence energy transfer always preceded the fluorescence increment of the tryptophan residue(s). (iv) The fluorescence increment at 336 nm at temperatures below 20 °C showed a latent period, whereas the fluorescence energy transfer did not. These results were thought to indicate that when α-toxin damages the target membrane, the molecule interacts with the membrane first, and then undergoes oligomerization within the membrane.

Staphylococcus aureus produces several exotoxins (1, 2). One of the predominantly produced type being the α-toxin which is assumed to play a major role in the pathogenicity of this organism. The effect of α-toxin in staphylococcal infection is to damage the host cell by spontaneous assembly of a transmembrane channel formed from the toxin-hexamer. We have recently reported that the toxin molecule changes its secondary structure concomitantly with the monomer-to-hexamer conversion of the toxin-hexamer in the presence of sodium deoxycholate or the membrane bilayers as demonstrated by a change in the circular dichroism spectrum of the toxin molecule and its fluorescence increment at 336 nm (3). However, it is not certain whether the conformational change of the toxin molecule is related to the assembly of the toxin-hexamer or with toxin-membrane (or toxin-sodium deoxycholate) interaction. We have therefore studied the kinetics of the conformational change and the toxin-hexamer formation in the presence of PC'-liposome by measuring the fluorescence at 336 nm and by quantifying the monomer and the hexamer using an HPLC system, respectively. Interaction between the toxin and the membrane was visualized by recording the fluorescence energy transfer from the tryptophan residue(s) of the toxin molecule to dansyl-PE in the liposome membrane. The result suggested that the fluorescence increment of the tryptophan residue(s) represented the oligomerization of the toxin, and this was preceded by the toxin-membrane interaction.

A study of the assembly of the α-toxin-hexamer is of interest from the following view points. (i) Study of the cytotoxic mechanism of this toxin would help gain an understanding of the pathogenesis of staphylococcal infections; (ii) elucidation of the mechanism of transformation of the water-soluble protein into the intrinsic membrane protein would help to clarify the biogenesis of the channel-forming protein aggregates, e.g., the porin trimers (4), the complement aggregate (5), and the perforin aggregate of the T-lymphocyte (6); (iii) study of the toxin-membrane interaction would help validate the membrane trigger theory of membrane biogenesis, as hypothesized by Wickner (7).

**EXPERIMENTAL PROCEDURES**

**Materials**—Staphylococcal α-toxin was purified as described previously (3). Egg yolk PC and egg PE were purchased from Sigma and Serdy Research Laboratories (London, Canada), respectively. MEGA-9 was from Dojindo Laboratories (Kumamoto). Danaryl chloride was from Wako Pure Chemicals (Tokyo). All other chemicals were of the highest purity available.

**Preparation of Liposome**—Phospholipids were dried under a stream of nitrogen gas and kept in an evacuated desiccator for more than 2 h as described earlier (8). Four different techniques were used to prepare the liposome. (i) Phospholipids (2.5 × 10⁻⁶ mol) were suspended in 0.4 ml of Buffer A and subjected to sonic oscillation at 23 °C for 5 min as described earlier (8) using a Branson Sonifier 200 (Branson Sonic Power, Banbury) equipped with a microtip. (ii) Phospholipids (20 × 10⁻⁶ mol) in 10 ml of Buffer A) were subjected to sonic oscillation in a cup-type sonic oscillator (Kubota, Tokyo) in the presence of nitrogen gas at 180 watts for 180 min at 21 °C. (iii) Phospholipids (2.7 × 10⁻⁶ mol) were suspended in 0.4 ml of 202 mM octyl glucoside and dialyzed against a large excess of Buffer A for 20 h. (iv) Phospholipids (20 × 10⁻⁶ mol) were suspended in 1.5 ml of Buffer A and passed through a French pressure cell (cross-sectional area, 1.8 cm²; Ohake, Tokyo) at 290 kg/cm² once.

**Quantitative Determination of α-Toxin Hexamer**—The α-toxin monomer in 63 μl of Buffer A (0.256 mg of protein) was mixed with PC-liposome (2.82 × 10⁻⁶ mol of phosphorus equivalent) in 1.377 ml of Buffer A, and the mixture was incubated at 10, 23, or 35 °C for an appropriate time. The sample (100 μl) was mixed with 2% MEGA-9.

*The abbreviations used are: PC, phosphatidylcholine; HPLC, high performance liquid chromatography; PE, phosphatidylethanolamine; MEGA-9, nonanoyl-N-methylglucamide; danaryl, 5-dimethylaminonaphthalene-1-sulfonyl; MBS, 4-morpholinethanesulfonic acid; Heps, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Buffer A, 10 mM Heps, 50 mM NaCl, 3 mM NaNO₃, pH 7.3.

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in Buffer A in order to dissolve the liposome membrane. Confirmation was made that the α-toxin-monomer had not aggregated into the hexamer or that the hexamer had not dissociated into the monomer in the presence of 2% MEGA-9. One hundred microliters of the solubilized sample was injected into a 0.75-in. column (TSKgel G3000SW, Toyo Soda, Tokyo) equilibrated with Buffer A containing 30 mM MEGA-9. The column was eluted with the same solution using an HPLC system (635A, Hitachi, Tokyo) equipped with a UV monitor (635B, Hitachi) at a flow rate of 0.4 ml/min.

Preparation of Dansyl-PE—Dansyl-PE was synthesized by the procedure described by Waggoner and Stryer (9). Briefly, egg PE in chloroform (20 mg/143 µl) was mixed with 7.9 mg of dansyl chloride (powder) and 43 µl of 99% triethylamine, and the mixture was kept at 23 °C for 3 h. The mixture was passed through a silica gel column (α-10 ml glass syringe) equilibrated with chloroform/methanol (15:1, v/v), and the column was eluted with the same solvent. The purity of dansyl-PE was confirmed by thin layer chromatography with chloroform/methanol/acetic acid (60:20:3).

Fluorescence Measurement—Fluorescence was measured using a fluorescence spectrophotometer (Hitachi) with excitation and emission band slits at 5 and 10 nm, respectively. Fluorescence of the tryptophan residue(s) of α-toxin was measured at 336 nm (excitation wavelength, 284 nm) after incubation of α-toxin and PC-liposome or the liposome containing 99 mol% PC and 1 mol% PE. Fluorescence energy transfer from the tryptophan residue(s) of the toxin molecule to dansyl-PE was recorded at 512 nm (excitation wavelength, 284 nm) after incubating the toxin and the liposome containing 99 mol% PC and 1 mol% dansyl-PE.

Calculation of Fluorescence Increment of the Toxin—Fluorescence increment (Finc (%)) at 336 nm was calculated using the equation

\[ F_{\text{inc}}(\%) = \left( \frac{F_{\text{inc,t}} - F_{\text{inc,0}}}{F_{\text{inc,0}}} \right) \times 100 \]

where \( F_{\text{inc,0}} \) and \( F_{\text{inc,t}} \) are fluorescence at 336 nm of α-toxin in the presence of PC-liposome or with without 1 mol% PE at time 0 and \( t \) min, respectively. The fluorescence increment (Finc (%)) at 512 nm of dansyl-PE by excitation at 284 nm was expressed as

\[ F_{\text{inc}}(\%) = \left( \frac{F_{\text{inc,t}} - F_{\text{inc,0}}}{F_{\text{inc,0}}} \right) \times 100 \]

where \( F_{\text{inc,0}} \) and \( F_{\text{inc,t}} \) are the fluorescence at 512 nm with incubation of α-toxin and PC-liposome containing 1 mol% dansyl-PE at time 0 and \( t \) min, respectively.

RESULTS

Kinetic Study of the Toxin Oligomerization and the Fluorescence Increment—The α-toxin-monomer and the liposomes made from egg yolk PC were mixed and incubated at various temperatures, and the monomer and hexamer were quantified by separating them by gel filtration through an HPLC column after incubation for the appropriate times. The amount of the toxin monomer decreased with a concomitant increase in that of the hexamer and this monomer-to-hexamer conversion was assumed that the fluorescence increment was a measure of the monomer-to-hexamer transfer between the molecules had occurred (Fig. 1). This energy transfer was undetectable in the presence of a surfactant, 2% MEGA-9 or 1% cholesterol (octaethylene glycol dodecyl ether) (data not shown). The fluorescence increment at 512 nm could not have been due to that occurring at 336 nm as a consequence of hexamer formation, since the increase at 512 nm occurred without any increment at 336 nm (see below).

Conditions Affecting the Toxin-Membrane Interaction and Oligomerization—We tested the effect of toxin concentration on the fluorescence energy transfer and the fluorescence emission from the tryptophan residue(s) of the toxin molecule. The amount of toxin was increased from 2.5 µM to 25 µM under a fixed concentration (0.25 mM) of the target liposome formed using procedure (i) as described under “Experimental Procedures.” The results, shown in Fig. 2, revealed that the rate constant of both the energy transfer and the fluorescence of the tryptophan residue(s) of the toxin molecule appeared to be steady over the toxin concentrations tested (Fig. 2).

![Fig. 1. Fluorescence spectra of the tryptophan residue(s) of the toxin and the fluorescence energy transfer. The α-toxin (0.142 mg/57 µl of Buffer A) was mixed with 4 x 10^-2 mol/l/743 µl of PC-liposome containing 1 mol% PE (solid line) or 1 mol% dansyl-PE (dotted line), made using procedure (i) as described under “Experimental Procedures.” Fluorescence spectra were recorded 5 min after mixing by exiting at 284 nm under controlled temperature. An arrow indicates (dotted line) the fluorescence increment due to the energy transfer.](image-url)

![Fig. 2. Effect of toxin concentration on the toxin-membrane interaction and the hexamer formation. Liposome (0.25 mM phosphorus, made as described in the legend to Fig. 1) was mixed with various concentrations of the α-toxin, and fluorescence at 512 nm or 336 nm (excited at 284 nm) was recorded at 23 °C for up to 30 min. Plots show the rate constants versus toxin concentrations. C, fluorescence at 512 nm; O, fluorescence at 336 nm.](image-url)
This result suggested that the toxin-membrane interaction follows first order kinetics. In the next experiment, we tested the effect of liposome concentration on toxin-membrane interaction and -hexamer formation. The amount of liposome formed by procedure (i) as described under "Experimental Procedures" was changed from 0.1 mM to 2.5 mM per 5 μM α-toxin. The results showed that the rate constants of both the energy transfer and fluorescence at 336 nm appeared to be nearly constant within the range of the liposome concentrations tested (Fig. 3). It was not possible to reduce the amount of liposome less than 0.1 mM per 5 μM α-toxin, since this level of the liposome was the limit of the assay sensitivity. We therefore decided to use a liposome/α-toxin ratio of around 500 in subsequent experiments. Membrane damage under these liposome/toxin ratios was confirmed by determining the release of the intravesicular carboxyfluorescein (data not shown, see Ref. 16 for details). However, the extent of fluorescence quenching and/or the release of carboxyfluorescein was not significantly large under these conditions.

Another important experiment which had to be done was to test the effect of liposome size on toxin-membrane interaction and -hexamer formation. Liposomes were made as described under "Experimental Procedures" by sonic oscillation for 5 min (liposomes-5), by sonic oscillation for 180 min (liposomes-180), by dissolving phospholipids in octyl glucoside followed by dialysis (liposomes-Og), or by passing through a French pressure cell (liposomes-Fp). These liposome preparations were then subjected to gel filtration using a Sepharose CL-4B column, and their elution profiles had the following characteristics (Fig. 4A). (i) Liposomes-Og eluted as a single peak at 13–14 ml (void volume) suggesting the presence of large-sized liposomes and a homogeneous population, as reported earlier (10). (ii) Liposomes-180 were eluted at 27–28 ml as a single peak, suggesting that the liposomes were fairly small and uniform in size. (iii) Liposomes-Fp showed two peaks at the void volume and at 25 ml (see Fig. 4A). (iii) Liposomes-5 were eluted as two distinct peaks at 15 ml and 26 ml. Although the liposome size was not measured, the gel filtration profiles clearly showed that different sizes of liposomes were prepared by these techniques. Liposomes-Fp were separated, and the liposomes eluted in the void volume and at 25 ml were collected. Using these small and large liposomes, the fluorescence energy transfer was measured. The results, shown in Fig. 4B, indicated that the energy transfer between the toxin and liposomes-Fp eluted at 25 ml occurred efficiently, while no energy transfer was detectable when liposomes-Fp collected in the void volume were used. The small amount of energy transfer observed in the large liposomes was not due to the turbidity of the suspension, since the liposome suspension was negligibly turbid and no fluorescence at 512 nm was detectable at a high gain setting of photometer. Fluorescence of the tryptophan residue(s) of the toxin was increased when liposomes-Fp from fraction 25 were used, but it was undetectable when liposomes-Fp from the void volume was used (Fig. 4B). These results suggested that α-toxin does not interact at least with the PC-liposome of large size. In
order to confirm the above observations, similar experiments were carried out using liposomes-180 and liposomes-Og as above. The results showed that the fluorescent probe on liposomes-180, the smallest size of liposome practically preparable, was excellent in accepting the fluorescence energy from the tryptophan residue of the toxin and for the fluorescence increment of the tryptophan residue. On the other hand, the large type of liposome, liposomes-Og, showed neither fluorescence energy transfer nor fluorescence increment at 336 nm. Thus, it became evident that α-toxin only attacks relatively small PC-liposomes. However, for the sake of convenience, we used liposomes-5 which contain both intermediate and small liposomes, for the experiments described below. For this reason, it is possible that only a limited size range of liposomes was involved in the reaction.

There is a question of whether the hemolysis or membrane damage caused by the α-toxin is a consequence of membrane fusion triggered by the toxin. We tested this possibility by determining dequenching of rhodamine-PE fluorescence by the fusion of liposomes containing rhodamine-PE with liposomes without (11). The result showed that the α-toxin does not cause the dequenching of rhodamine-PE, although polyethylene glycol 4000 under identical conditions caused an increase in fluorescence at 590 nm (excitation at 560 nm, data not shown).

**Conditions Distinguishing the Toxin-Membrane Interaction and the Oligomerization of the Toxin**—Using the above-described assay techniques, the effect of pH on the energy transfer and fluorescence increment of the toxin molecule was monitored. When the toxin and the liposome were incubated with dansyl-PE or PE at pH 4.8, the energy transfer began immediately after mixing and gradually saturated, but the fluorescence at 336 nm was not detectable until about 3 min of incubation, after which it increased a little (Fig. 5A). The energy transfer and fluorescence at 336 nm reached 50% and 6% (ratio, 8.3), respectively, at 30 min. When a similar experiment was carried out at pH 8.2, the energy transfer occurred immediately after mixing and fluorescence at 336 nm rose after 1 min, extent of the fluorescence increments at 512 and 336 nm reaching 23% and 8% (ratio, 2.8), respectively, at 30 min (Fig. 5C). Fluorescence levels at 512 nm and 336 nm rose soon after mixing when the experiment was carried out at pH 6.2, and the extent of the fluorescence increments appeared to be 30% and 16%, respectively (Fig. 5B, ratio, 1.8). No time-lag was observed at neutral pH. It is noteworthy that both the energy transfer and the fluorescence increment at 336 nm in the presence of 2% MEGA-9 or 1% C12E8 were not influenced by a simple change of pH. These results indicated that the toxin-membrane interaction and -hexamer formation occurred most efficiently at acidic pH and neutral pH, respectively. These results suggested that the oligomerization of the toxin and the toxin-membrane interaction are distinguishable phenomena.

Similarly, the effect of incubation temperature on the fluorescence energy transfer and fluorescence increment at 336 nm were examined. The results, shown in Fig. 6, clearly indicated that the energy transfer increased from 29 to 53% as the incubation temperature was raised from 14 to 41°C, at 30 min of incubation. Fluorescence increments at 336 nm appeared to be 16, 22, and 25% at 14, 25, and 40°C, respectively. Since the ratios of the fluorescence between 512 nm and 336 nm at various temperatures appeared to be constantly around 2, the results suggest that the toxin-membrane interaction is the rate-limiting factor in the assembly of the hexamer. Although the result does not rule out a possibility that the liposome somehow causes formation of the water-soluble hexamer before the toxin being integrated into the membrane. On the basis of these results, the activation energies for the toxin-membrane interaction and -hexamer formation were calculated from the Arrhenius plots (Fig. 7), and the values appeared to be 6.9 and 6.7 kcal/mol, respectively. Although this result does not necessarily mean that the activation energies for both reactions are identical, when the data on the effect of pH are taken into consideration, the possibility remains that the reaction which precedes the other is the rate-limiting one.

**DISCUSSION**

When the α-toxin of *S. aureus* causes cytolysis of the host cell, the water-soluble toxin monomer becomes assembled into a transmembrane toxin hexamer, forming a water-filled channel or pore which penetrates the membrane (12-15). We have reported recently that the toxin molecule changes its conformation concomitantly with monomer-to-hexamer conversion...
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FIG. 7. Arrhenius plots of the hexamer formation, the fluorescence increment of the tryptophan residue(s), and the fluorescence energy transfer upon incubation of the $\alpha$-toxin with PC-liposome. Rate constants were calculated by log-$t$ time plot for the hexamer formation, the fluorescence increment of the tryptophan residue(s), and the energy transfer. Symbols: $\Delta$ and $\mathbf{B}$, the hexamer formation and the $\alpha$-toxin with PC-liposome; $\mathbf{O}$, the fluorescence increment at 336 nm upon incubation of the $\alpha$-toxin with PC-liposome containing 1 mol% PE; $\mathbf{C}$, the fluorescence (512 nm) energy transfer upon incubation of the $\alpha$-toxin with PC-liposome containing 1 mol% dansyl-PE.

Fig. 6. Effect of temperature on the fluorescence energy transfer and fluorescence of the tryptophan residue(s) in the presence of PC-liposome. Fluorescence was measured as described in the legend to Fig. 5, and the results were plotted against incubation time. The fluorescence increment was calculated as described under "Experimental Procedures." Symbols are the same as in the legends to Fig. 5. A, 14°C; B, 25°C; C, 40°C.

(3). The basic questions which need to be answered are: (i) whether the conformational change of the toxin molecule occurs as the toxin is exposed to the hydrophobic environment of the membrane or sodium deoxycholate, (ii) whether this conformational change occurs as a consequence of the oligomerization, (iii) whether the hexamer is assembled in the aqueous phase or in the membrane. In other words, whether the toxin-monomer becomes attached to the membrane before hexamer assembly. In the present work, we tested the above possibilities. Possibility (i) seems unlikely, since the conformational change was undetectable when the toxin monomer was exposed to the nonionic surfactants such as C$_{12}$E$_8$ and MEGA-$\nu$. Possibility (ii) seems more likely than (i), since the quantitatively determined oligomer formation and the fluorescence increment of the toxin molecule always paralleled one another. Moreover, the activation energy for both reactions appeared to be indistinguishable. Possibility (iii) was partially clarified by the present work, in that the toxin-membrane interaction and the oligomerization of the toxin could be distinguished (Figs. 5 and 6). Also, the fluorescence energy transfer between the toxin molecule and the membrane preceded the fluorescence increment of the toxin under acidic or basic conditions and at low temperature, and their reversed order was never seen under any conditions (Figs. 5 and 6). On the basis of these results, the preliminary process of membrane damage caused by $\alpha$-toxin of $S. aureus$ can be visualized. The toxin-monomer in the aqueous phase first interacts with the membrane and probably enters the membrane domain (see Ref. 16 for more information). Then, the monomer in the membrane become aggregated into the hexamer, resulting in a conformational change of the protein. However, the data do not exclude an alternative possibility that the liposomes somehow cause the accumulation of the water-soluble hexamer or the formation of the water-soluble hexamer transiently before being inserted into membrane.

One interesting observation made was that $\alpha$-toxin interacted with only small-sized PC-liposomes (Fig. 4). We interpret this observation as signifying that the curvature of the liposome membrane and exposure of the hydrophobic surface of the membrane is essential for interaction with the toxin. The fact that sodium deoxycholate micelles interact and induce oligomerization of the toxin, but that micelles of C$_{12}$E$_8$, MEGA-$\nu$, and Triton X-100 do not, supports the above interpretation. In the case of the large PC-liposome, the surface packing of PC molecules should be tighter than on the small PC-liposome because of the larger surface area. Under these circumstances, it is possible that the toxin might not be able to become incorporated into the membrane. However, the situation could be different in liposomes of different phospholipid composition and in biological membranes.

The activation energies for energy transfer, hexamer formation, and the change in fluorescence at 336 nm appeared to be indistinguishable, 6.5–6.7 kcal/mol. These results could be interpreted in two ways. One is that the activation energies for the assembly of the hexamer is very low compared with that for the toxin-membrane interaction. Alternatively, the activation energy required for both steps are the same, and hence, these two are indistinguishable. From the present data, we consider the former to be more likely than the latter, since
the oligomerization of the toxin seems to be preceded by toxin-membrane interaction (Fig. 5). The activation energy for α-toxin-induced hemolysis has been reported to be 2.2 kcal/mol, which is only 30% of the figure obtained in this study. This discrepancy may be due to differences in the target membrane. A similar observation has been made in the C9-aggregate formation in sheep erythrocyte ghosts and PC-liposome for which lower and higher activation energies, respectively, were calculated (17). If it is so, it would be of great interest to know the membrane factor(s) which influence the efficiency of toxin-hexamer assembly. This aspect will be dealt with in an accompanying paper (16).

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