Influence of Membrane Fluidity on the Assembly of *Staphylococcus aureus* α-Toxin, a Channel-forming Protein, in Liposome Membrane*

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By use of multilamellar phosphatidylcholine (PC) liposomes of different acyl composition and cholesterol content as model membranes, we studied whether or not membrane fluidity affects the assembly process of *Staphylococcus aureus* α-toxin. Under conditions using fluid and solid membranes, we assayed accessibility (or hemolytic activity) of liposome-bound α-toxin to rabbit erythrocytes added, hexamerization of membrane-bound toxin using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonde-naturating conditions, and susceptibility of liposome-bound toxin to trypsin digestion. Our data indicated 1) that α-toxin bound to PC membrane as a hemolytically active monomer (or reversibly bound state); 2) that when the membrane was fluidized either by phase transition of PC or by inclusion of cholesterol over 20 mol %, the hemolytically active monomer of the toxin was irreversibly converted to nonhemolytic monomer (and/or unstable oligomer) in a first-order kinetics with a τ of about 1 min, and thereafter hexamerization of the toxin gradually proceeded in the following 60–90 min; 3) that α-toxin might have different topology and/or conformation in PC membrane, depending on the presence or absence of cholesterol in the PC membrane; and 4) that coexistence of unsaturated acyl chain-containing PC and cholesterol was a prerequisite for efficient hexamerization of α-toxin in membrane. Thus, increase in membrane fluidity promoted the assembly process of *S. aureus* α-toxin.

Staphylococcal α-toxin is a water-soluble 33-kDa polypeptide secreted by *Staphylococcus aureus*. It is hemolytic, demonecrotic, and lethal for experimental animals and is considered to be an important virulence factor of the bacterium. The toxin has also been shown to cause membrane damage to a variety of cultured mammalian cells and artificial membranes (1–5). Of the multiple biological activities, toxin-induced hemolysis has been the one most intensively studied, and it is thought to involve the following steps: 1) binding of the native monomeric toxin to the cell membrane, 2) oligomerization of the monomers on/in the membrane to form a transmembrane channel consisting of hexameric toxin, and 3) leakage of small ions and molecules, leading to colloid osmotic lysis of erythrocytes (4, 5). Functional membrane damage by α-toxin in other types of mammalian cells, including mouse adrenocortical Y1 tumor cells (6), rabbit alveolar macrophages (7), and human platelets (8), may also be caused by the formation of a transmembrane channel in a similar mechanism.

The concept of α-toxin as a channel-forming protein was proposed by Fuessele et al. (9) based on the finding that the 12 S ring structure of α-toxin formed on liposomes and erythrocytes was composed of hexameric toxin, which appeared to form a transmembrane channel (4, 9, 10). The functional role of the toxin hexamer in hemolysis caused by a low concentration of α-toxin was established by Bhakdi et al. (11, 12). Formation of the toxin hexamer was confirmed by using different experimental techniques (9, 13, 14), and channel activity of the hexamer was characterized by several groups (15–18). However, little knowledge has been obtained so far on the physicochemical properties of membrane that affect the assembly of α-toxin in membrane.

By use of multilamellar liposomes of various compositions, we showed previously that α-toxin specifically interacts with the choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin (19). The idea of the choline-containing phospholipids as the binder molecules for α-toxin is consistent with the asymmetrical preferential distribution of these lipids in the outer leaflet of the cytoplasmic membrane of erythrocytes. Our study also showed that cholesterol was not essential for the initial binding of α-toxin to lipid bilayer, but inclusion of cholesterol into the liposome membrane enhanced release of carboxyfluorescein (internal marker) from the liposome upon exposure to α-toxin (19). Therefore, we considered that cholesterol fluidized the membrane and thereby facilitated the post-binding steps of the toxin-membrane interaction as a structural component (19).

In this paper we studied the influence of membrane fluidity on the post-binding steps of membrane channel formation of α-toxin by using multilamellar PC liposomes of different acyl chain composition and cholesterol content as model membranes.

MATERIALS AND METHODS

Lipids—Egg yolk PC, soybean phosphatidylcholine, dilauroyl-L-α-phosphatidylcholine (DLPC), dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), distearoyl-L-α-phosphatidylcholine (DSPC), and dioleoyl-L-α-phosphatidylcholine.

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line (DOPC) were kindly supplied by Nippon Oil and Fats Co., Ltd. (Tokyo), β-Oleoyl-γ-palmitoyl-L-α-phosphatidylcholine (OPPC), β-palmitoyl-γ-oleoyl-L-α-phosphatidylcholine (POPC), and cholesterol were purchased from Sigma. Cholesterol was recrystallized twice from methanol before use. The purity of all lipids used was checked by thin layer chromatography on silica gel plates (Merck, Darmstadt, Federal Republic of Germany).

Staphylococcal α-Toxin—α-Toxin was purified from the culture supernatant of S. aureus Wood 46 as described previously (19) and stored at -80 °C. Before use, the toxin preparation was chromatographed on a column of Sephacryl G-75 to eliminate the toxin hexamer spontaneously generated.

Liposomes—Multilamellar liposomes were prepared as described previously (19). A mixture of PC (2 μmol) and cholesterol (0-2 μmol) in chloroform was evaporated under reduced pressure to form a lipid film on the wall of a conical-bottomed flask. The lipid film was dried for 30-60 min in an evacuated desicator, and it was dispersed in 1 ml of phosphate-buffered saline (PBS) by vortexing at 45-50 °C or above the temperature of gel-liquid crystalline phase transition (Tₘ) of the PC. Multilamellar liposomes were collected by centrifugation of the lipid dispersion at 22,000 × g for 15 min at 4 °C. Mean diameters of the liposomes, determined by Microtrac SPA (Leeds and Northrup, St. Petersburg, FL), were in the range between 5.70 and 6.74 μm.

Accessibility of the Liposome-bound α-Toxin to Rabbit Erythrocyte—Rabbit erythrocytes were employed as an indicator to assess hemolytic activity of liposome-bound α-toxin, since they are the most sensitive cells to the toxin. α-Toxin (5-50 μg; 7.6-11 nmol) and liposome (1.4 μmol of lipids) in 1 ml of PBS were incubated for 30 min at a given temperature. The toxin-liposome complexes formed were collected by centrifugation at 22,000 × g for 15 min at 0-4 °C and centrifuged twice to remove unbound toxin. The toxin-liposome complexes were suspended in 100 μl of ice-cold PBS and divided into three portions for determinations of hemolytic activity and protein amount of the membrane-bound toxin and for analysis of the hexamer formation on SDS-PAGE (see below).

For the assay of hemolytic activity, the suspension of the toxin-liposome complex was serially diluted 2-fold with ice-cold PBS for five hours. After 50 μl of the diluted suspension was mixed with 50 μl of 1% suspension of rabbit erythrocytes in a 96-well microplate and allowed to stand for 30 min at 37 °C. After the incubation, the microplate was centrifuged at 2000 rpm for 10 min at room temperature. Absorbance at 550 nm of the supernatant was measured with a reference at 630 nm in MTP96 Microplate Reader (Corona Electric Co., Ltd., Katuda, Japan). 100% hemolysis was defined as the maximum value at 550 nm obtained with native α-toxin of >2 μg/ml.

For determination of the amount of protein of membrane-bound toxin, the toxin-liposome complexes were solubilized at 37 °C in PBS containing 1% Triton X-100 for 30 min unless otherwise stated. All samples of the solubilized sample were subjected to protein determination, essentially according to Lowry's method (20) using a bovine serum albumin solution containing 1% Triton X-100 as standard. The white precipitate, if formed, was removed by the centrifugation at 10,000 rpm for 15 min.

Assessment of Hexamerization of α-Toxin on SDS-PAGE under Nondenaturating Conditions—Toxin-liposome complexes obtained as described above were collected by centrifugation at 4 °C, solubilized in ice-cold PBS containing 1% Triton X-100 or 1% octyl-β-glucoside, and subjected to SDS-PAGE. SDS-PAGE was performed on a 10% polyacrylamide slab gel, essentially according to Laemmli’s method (21). However, boiling of the solubilized toxin in the presence of 2% SDS and 5% 2-mercaptoethanol was omitted to avoid dissociation of the toxin hexamer formed. Toxin hexamer is considered to be resistant to treatment with 1% Triton X-100 and 1% SDS at room temperature as described by others (9, 13, 14). No further hexamerization was induced by the incubation of α-toxin in PBS containing 1% Triton X-100 at least for 90 min at 37 °C (Fig. 2).

To analyze the hexamerization of α-toxin in the erythrocyte membrane, native α-toxin (45 μg in 100 μl) was incubated with the erythrocyte membrane (66 μg of protein in 10 μl) for 30 min at 0 or 37 °C. After α-toxin was collected by centrifugation, the supernatant was solubilized in 1% Triton X-100, and subjected to the SDS-PAGE system as described above.

The gel was stained with Coomassie Brilliant Blue R250 as described by Fairbanks et al. (22) and subjected to densitometry for determination of percent of hexamer formation. The percent of hexamer formation was defined as follows: (area of hexamer/area of monomer and hexamer) × 100.

Susceptibility of Liposome-bound Toxin to Trypsin Treatment—A mixture of α-toxin (150 μg) and liposome (0.4 μmol of phosphate) in 0.5 ml of PBS was incubated for 1 h at 10 °C and centrifuged twice at 22,000 × g for 20 min at 0-4 °C to collect liposome-bound toxin. The liposome-bound toxin was suspended in 200 μl of PBS and divided into two portions. The divided portion of the liposome-bound toxin received either trypsin (0.5 mg/ml; Sigma Type XI; Sigma) or PBS and was then incubated for 30 min at 10 °C. After addition of soybean trypsin inhibitor (0.5 mg/ml; Sigma), the mixture was centrifuged at 22,000 × g for 20 min at 0-4 °C. The trypsin-treated liposome-bound toxin thus obtained was boiled in the presence of 2% SDS and 5% 2-mercaptoethanol for 5 min and subjected to SDS-PAGE on a 15% polyacrylamide gel as described by Laemmli (21).

Miscellaneous—Erythrocyte membrane was prepared essentially as described by Dodge et al. (23). Determination of phosphorus was performed according to the method of Gerlach and Deuticke (24). Cholesterol was determined enzymatically by use of an assay kit of Nissui Pharmaceuticals Co., Ltd. (Tokyo).

RESULTS

Dissociation of Membrane-bound α-Toxin to Hemolytic and Nonhemolytic State—By use of multilamellar liposomes composed of synthetic PC with saturated acyl chains of different chain lengths, we assayed the accessibility (or hemolytic activity) of liposome-bound toxin to rabbit erythrocytes added. Above or below the phase transition temperature from gel to liquid crystalline state (Tₘ) of the PC, the α-toxin was bound to each of the liposomes composed of DLPC, of which Tₘ is 0 °C (25); DMPC, of which Tₘ is 23 °C (25, 26); or DSPC, of which Tₘ is 58 °C (26). The hemolytic activity of the liposome-bound toxin to the rabbit erythrocytes added was assayed at 37 °C and compared with that of native toxin on the basis of protein amount.

When α-toxin was bound to DMPC liposome at 0 °C (i.e. below Tₘ of DMPC), the liposome-bound toxin retained 73% of the hemolytic activity in comparison with native toxin on the protein basis (Fig. 1B and Table I). By contrast, α-toxin lost ≥90% hemolytic activity when bound to the same liposome at 37 °C (i.e. above Tₘ of DMPC; Fig. 1B and Table I). Furthermore, ≥90% hemolytic activity of α-toxin was lost when bound to DLPC liposome at 0 °C as well as at 37 °C (i.e. at or above Tₘ of DLPC, Fig. 1A), whereas a major
portions (63-96%) of the liposome-bound toxin remained hemolytically active after the contact with DSPC liposome at 37 °C, as well as 0 °C (i.e., below T_m of DSPC; Fig. 1C and Table I). Under these conditions, no significant difference was observed in the overall binding of the toxin between different liposomes and between different temperatures (Table I), and toxin-free liposome prepared from any PC used evoked no significant hemolysis (data not shown). These results indicated that membrane-bound α-toxin comprised two distinguishable states, hemolytically active and nonhemolytic states. The data also indicated that liposome-bound toxin remained hemolytically active when bound to the liposome below T_m of PC, but it became nonhemolytic upon contact with the liposome at or above T_m. Thus, membrane fluidity was suggested to affect the post-binding step(s) of the interaction between α-toxin and membrane.

To investigate the correlation between the accessibility of membrane-bound α-toxin to erythrocytes added and hexamerization of the toxin in membrane, we analyzed hexamer formation in DLPC and DSPC membranes above or below T_m of the PC. Hexamer formation was assayed on SDS-PAGE after solubilizing the liposome-bound toxin in 1% Triton X-100. Boiling of the solubilized toxin prior to electrophoresis was omitted to avoid disassembly of hexamer. As shown in Fig. 2, almost all (>95%) of the toxin solubilized from DSPC liposome was recovered as a monomer of 33 kDa. By contrast, around 30% of the toxin solubilized from DLPC liposome was mobilized as hexamer molecules of about 200 kDa (Fig. 2). These results suggested that hexamerization of α-toxin occurred more efficiently in the fluid DLPC membrane than in the solid DSPC membrane and that the membrane-bound hemolytically active state of the toxin predominately comprised monomer (and/or unstable oligomers, which might be dissociated to monomer during the Triton X-100 treatment and SDS-PAGE), whereas the nonhemolytic state of the membrane-bound toxin contained a substantial amount of stable hexamer. Trace amounts of hexamer seen in the lane of native toxin (Fig. 2) are probably derived from the toxin preparation, because the hexamer disappeared when boiled in the presence of 2% SDS and 5% 2-mercaptoethanol prior to electrophoresis (data not shown). In addition, treatment of native toxin with 1% Triton X-100 induced no further hexamerization (Fig. 2).

To study topology and/or conformation of α-toxin on/in fluid and solid membranes, we assayed the susceptibility of liposome-bound toxin to trypsin digestion. α-Toxin was bound to DLPC and DSPC liposomes for 1 h at 10 °C and exposed to trypsin (0.5 mg/ml) for 30 min at 10 °C. The treatment and SDS-PAGE), whereas the nonhemolytic state of the membrane-bound toxin contained a substantial amount of stable hexamer. Trace amounts of hexamer seen in the lane of native toxin (Fig. 2) are probably derived from the toxin preparation, because the hexamer disappeared when boiled in the presence of 2% SDS and 5% 2-mercaptoethanol prior to electrophoresis (data not shown). In addition, treatment of native toxin with 1% Triton X-100 induced no further hexamerization (Fig. 2).

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### Table I

| Liposome & Concentration | Binding & Concentration |
|-------------------------|-------------------------|
| DLPC MLV 0 °C 6.6 | 6.3 | 7.0 | 6% (5%) |
| DLPC MLV 37 °C 5.2 | 5.8 | 0.59 | 73% (3%) |
| DSPC MLV 0 °C 5.8 | 4.8 | 0.45 | 96% (63%) |

a MLV, multilamellar vesicle.

b Native α-toxin used for the experiments induced 50% hemolysis at 0.43 μg/ml. The percent residual hemolytic activity of the liposome-bound toxin was calculated as 100 x (the concentration of the native toxin required for inducing 50% hemolysis/the concentration of the liposome-bound toxin required for inducing 50% hemolysis).
the SDS-PAGE as intact toxin of 33 kDa and partially digested toxin of 32 kDa after the trypsin digestion (Fig. 3A). Thus, a substantial amount of the membrane-bound toxin was sequestered from the trypsin digestion in fluid DLPC membrane, and the amount (20–30%) of the sequestered toxin in the DLPC membrane apparently coincided with the percent hexamerization (20–30%) in the same membrane (Fig. 2). The 32-kDa toxin fragment derived from a cleavage between positions 8 and 9 in the native toxin, and it could be generated upon incubation above or below T,m of PC. α-Toxin that had been bound to DMPC liposome at 0 °C was further incubated at 37 °C (data not shown). These results indicated that hexamerization of α-toxin occurred in the fluid DMPC membrane, and it proceeded at a much lower rate, compared with the reduction of hemolytic activity of the membrane-bound toxin (Figs. 4 and 5).

Cholesterol Effect on the Hemolytic State of Liposome-bound α-Toxin—Since it is generally accepted that cholesterol fluidizes PC membrane at concentrations over 20 mol % (27), we studied the cholesterol effect on the hemolytic state of membrane-bound α-toxin by using liposome composed of DMPC and cholesterol in a molar ratio of 1:1. α-Toxin lost approximately 90% of the hemolytic activity when incubated with the DMPC-cholesterol liposome for 30 min at 0 °C (Table II). Similar results were obtained when the toxin was associated at 0 °C with liposome composed of dipalmitoylphosphatidylcholine (DPPC, T,m = 41 °C) (25, 26) and cholesterol in a molar ratio of 1:1 (Table II). Thus, inclusion of cholesterol into membrane facilitated formation of the nonhemolytic state of membrane-bound toxin even below the T,m of the PC used.

For further study of the cholesterol effect on the hemolytic state of membrane-bound α-toxin, we prepared DPPC liposomes with various cholesterol contents between 0 and 50 mol % and examined hemolytic activity of α-toxin that had been bound to each of these liposomes at 0 °C. As shown in Fig. 6, the hemolytic activity of liposome-bound toxin was markedly decreased at 0 °C when membrane contained cholesterol over 20 mol %, suggesting that the membrane-fluidizing effect of

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Footnote:

1 T. Tomita, M. Watanabe, and T. Yasuda, unpublished data.
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TABLE II

| MLV* | Concentration | 0°C | 37°C |
|------|---------------|-----|------|
|      | µg/ml         |     |      |
| DMPC MLV | 0.68 (68%) | 10 (4.6%) |
| DPMC-Chol MLV | 4.2 (11%) | 12 (3.8%) |
| DPPC MLV | 0.65 (71%) | ND* |
| DPPC-Chol MLV | 3.6 (13%) | ND |
| DOPC MLV | 4.7 (9.8%) | 17 (2.7%) |
| DOPC-Chol MLV | 9.8 (4.7%) | 48 (0.96%) |
| Egg PC MLV | 5.8 (7.9%) | 17 (2.7%) |
| Egg PC-Chol MLV | 10 (4.6%) | 45 (1.0%) |

*a The percent residual hemolytic activity was calculated as described in the legend of Table I. The native-α-toxin used in the experiments induced 50% hemolysis at 0.46 µg/ml.
*b MLV, multilamellar vesicle.
*c Chol, cholesterol.
*d ND, not done.

Fig. 6. Effect of cholesterol content on the hemolytic activity of liposome-bound α-toxin. α-Toxin was incubated for 30 min at 0°C with the liposomes composed of DMPC and cholesterol in various molar ratios. Concentration of each liposome-bound toxin required for inducing 50% hemolysis was determined as described under “Materials and Methods.” Percent residual hemolytic activity was calculated as 100 × (concentration of native toxin required for 50% hemolysis/concentration of each liposome-bound toxin for 50% hemolysis) and plotted for each of the liposomes with various cholesterol contents.

Cholesterol promoted the formation of nonhemolytic toxin in membrane.

Cholesterol Effect on the Hexamerization of α-Toxin in Membrane—α-Toxin was bound to DMPC and DMPC-cholesterol liposome at 0°C, solubilized with 1% Triton X-100, and subjected to the SDS-PAGE system described above. As shown in Fig. 7A, 6 and 12% of the liposome-bound toxin were hexamerized in DMPC and DMPC-cholesterol membrane, respectively. Similar results were obtained with DPPC and DPPC-cholesterol liposome (data not shown). Taken together with the data that the toxin preparation used contained hexamer at approximately 3% (Fig. 2), these results indicated that hexamerization of α-toxin occurred severalfold more efficiently in the PC-cholesterol membranes than in the membranes composed of PC alone. However, it should be noted that the percent of hexamerization of the toxin in the DMPC-cholesterol membrane was much lower, compared with that in the erythrocyte membrane (Fig. 7A and D).

Since natural PC extracted from cell membrane contains unsaturated fatty acids, we assayed hexamerization of α-toxin in DOPC and DOPC-cholesterol membrane. As shown in Fig. 7, A and B, hexamerization of α-toxin occurred more efficiently in DOPC membrane than in DMPC membrane, suggesting that unsaturated acyl residue(s) of PC facilitated hexamerization of α-toxin more efficiently than saturated fatty acyl residue(s). More importantly, hexamerization of the toxin was induced in DOPC-cholesterol membrane to a high level (>70%) comparable with that in erythrocyte membrane (Fig. 7, B and D). Similar results were obtained with egg yolk PC and soybean PC instead of DOPC (data not shown). Thus, both unsaturated acyl chain-carrying PC and cholesterol were required for efficient hexamerization of α-toxin in membrane.

We also studied whether or not the position of unsaturated acyl chain in PC molecule affected hexamerization of α-toxin in membrane. To answer this question, we prepared liposomes composed of OPPC, Tω + = −7.9 °C (28) or DOPC, Tω + = −0.8 °C (28) and cholesterol in a molar ratio of 1:1. As shown in Fig. 7C, hexamer formation of the toxin occurred efficiently in both OPPC-cholesterol and POPC-cholesterol membrane at a high level similar to that in DOPC-cholesterol membrane. These results indicated that a single unsaturated acyl residue at any position was adequate to induce efficient hexamerization of α-toxin in the cholesterol-containing membrane.

Cholesterol Effect on the Susceptibility of Membrane-bound Toxin to Trypsin Digestion—To study cholesterol effect on the topology and/or conformation of membrane-bound α-toxin, we assessed trypsin accessibility of liposome-bound toxin by use of DMPC and DOPC liposomes containing 0 or 50 mol% cholesterol. α-Toxin was bound to each of the liposomes for 1 h at 10°C and exposed to trypsin (0.5 mg/ml) for 30 min at 10°C. The trypsin-treated liposome-bound toxin was collected by centrifugation, boiled for 5 min in the presence of 2% SDS and 5% 2-mercaptoethanol, and electrophoresed on a 15% polyacrylamide gel.

As shown in Fig. 8 (lanes 1 and 2), almost all the toxin bound to DMPC liposome was digested by the trypsin treatment, indicating that α-toxin was accessible by trypsin when associated with DMPC membrane below Tω, of the PC. In contrast, 15–25% of the toxin bound to the DOPC-cholesterol liposome was recovered mainly as a 32-kDa fragment of the toxin after the trypsin treatment (Fig. 8, lanes 3 and 4). These results indicated that cholesterol facilitated sequestration of membrane-bound toxin from trypsin digestion even below Tω, of the PC. It should also be noted that almost all toxin was recovered as a 32-kDa fragment (i.e. a cleaved product of α-toxin at the position between 8 and 9) from the cholesterol-containing membrane. When α-toxin was bound to the DOPC liposome, 25–36% of the membrane-bound toxin was recovered as intact toxin plus the 32-kDa fragment after the trypsin treatment (Fig. 8, lanes 5 and 6), indicating that sequestration of membrane-bound toxin from the trypsin digestion occurred when toxin was associated with fluid membrane. In addition, similar amounts of the intact toxin and the 32-kDa fragment remained after the trypsin digestion of the membrane-bound toxin (Fig. 8, lane 6). From the DOPC-cholesterol liposome, 45–60% of the membrane-bound toxin was recovered mainly as the 32-kDa fragment after the trypsin treatment (Fig. 8, lane 8), and only a trace amount of intact toxin was observed in the lane.

Thus, the presence of cholesterol in membrane facilitated sequestration of membrane-bound α-toxin from the trypsin digestion, perhaps by its membrane-fluidizing effect. It was also suggested that topology and/or conformation of the sequestered toxin in membrane was slightly different, depending on the presence or absence of cholesterol in membrane. In addition, percent sequestration of the membrane-bound toxin from the trypsin treatment was apparently in good correlation with percent hexamerization of the toxin in the PC-cholesterol membrane (Figs. 7 and 8).
Fig. 7. Hexamerization of α-toxin in various PC-cholesterol membranes and erythrocyte membrane. α-Toxin was incubated for 30 min at 0 (open bar) or 37 °C (dotted bar) with liposome or human erythrocyte membrane. Membrane-bound toxin was collected by centrifugation, solubilized in the ice-cold PBS containing 1% Triton X-100, and subjected to SDS-PAGE under non-denaturing conditions as described under “Materials and Methods.” A, DMPC and DMPC-cholesterol liposome; B, DOPC and DOPC-cholesterol liposome; and C, the liposome composed of OPPC or POPC and cholesterol were used as artificial membranes, in which cholesterol was contained at 50 mol %. As a natural membrane, human erythrocyte membrane was employed (D). Mean values of percent hexamerization obtained from three independent experiments are illustrated in the upper panels. Chol, cholesterol.

Fig. 8. Susceptibility to trypsin digestion of α-toxin bound to various PC and PC-cholesterol liposomes. α-Toxin was incubated with each of DMPC, DMPC-cholesterol, DOPC, and DOPC-cholesterol liposomes for 1 h at 10 °C. Liposome-bound toxin was collected by centrifugation and exposed to trypsin (0.5 mg/ml) or PBS for 30 min at 10 °C. The trypsin-treated liposome-bound toxin was boiled and subjected to SDS-PAGE as described under “Materials and Methods.” Chol, cholesterol.

DISCUSSION

By using fluid and solid PC liposomes as model membranes, we showed that an increase in membrane fluidity promotes the assembly process of S. aureus α-toxin. Our data indicated 1) that the membrane-bound state of α-toxin was experimentally dissociated to the hemolytically active and hemolytically inactive states; 2) that when membrane was fluidized either by phase transition of PC or by inclusion of cholesterol over 20 mol %, the hemolytically active monomer of the toxin assembled into stable hexamer via non-hemolytic monomer (and/or unstable oligomer); 3) that α-toxin may have a different topology and/or conformation in PC membrane, depending on the presence or absence of cholesterol in membrane; and 4) that coexistence of unsaturated acyl chain-carrying PC and cholesterol is a prerequisite for efficient hexamerization of α-toxin in membrane.

Several lines of evidence supported the interpretation that the membrane-bound hemolytically active state of α-toxin corresponded to membrane-bound monomeric toxin. 1) A major portion (>90%) of the hemolytically active toxin solubilized from solid membranes was mobilized as monomer of 33 kDa on the SDS-PAGE (Figs. 2, 5, and 7). 2) The membrane-bound hemolytically active toxin was accessible by trypsin (Figs. 3 and 8). As shown in Fig. 3B and in the previous works (9, 14), monomer toxin is sensitive to trypsin digestion, whereas hexamer toxin is resistant to the protease. 3) α-Toxin associated with solid membrane was so highly active hemolytically as the native monomeric toxin on the basis of protein amount (i.e. the membrane-bound toxin and the native toxin induced 50% hemolysis at the concentrations of 0.45–0.65 and 0.37–0.43 μg/ml, respectively) (Tables I and II). In contrast, the hexamer (spontaneously generated in the toxin stock) exhibited no hemolytic activity at a concentration as high as 100 μg/ml. 4) Our preliminary data indicated that a significant amount of hemolytic activity of α-toxin was recovered from solid DPPC membrane, but not from fluid DLPC membrane; 1–3% of the hemolytic activity of α-toxin which had been bound to DPPC liposome at 10 °C was recovered in the supernatant when incubated at 10 °C in the presence of 0.1% bovine serum albumin and then centrifuged at 22,000 × g for 20 min at 0–4 °C. By contrast, less than 0.1% of hemolytic activity of the membrane-bound toxin was recovered from DLPC liposome under the same conditions. This evidence suggested that the membrane-bound hemolytically active state of α-toxin contained, at least in part, reversibly bound toxin, which was dissociable from the membrane probably as monomer molecule and able to lyse the rabbit erythrocytes added. Blomqvist and Thelestam (29) also demonstrated the reversibly bound state of Staphylococcal α-toxin with mouse adrenocortical Y1 cells; they recovered substantial amounts of hemolytically active toxin from the adherent Y1 cells, which had been exposed to the toxin at 0 °C and thereafter rinsed twice.

Kinetic studies on the hemolytic activity and the hexamer formation of α-toxin in fluid membrane showed that the appearance of stable hexamer was preceded by the formation of the nonhemolytic state of the toxin (Figs. 4 and 5). Major portions (70–80%) of the nonhemolytic toxin associated with fluidized DLPC and DMPC membranes were trypsin-digestible (Figs. 3 and 8) and behaved as monomeric molecules on the SDS-PAGE (Figs. 2 and 7). These results suggested the existence of the nonhemolytic but monomeric state of α-toxin in membrane. However, the experimental methods employed did not eliminate the possibility of concomitant formation of unstable oligomeric states, which were in an equilibrium with the monomeric state in membrane and dissociated to monomeric toxin.
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Membrane during the solubilization and/or electrophoresis of membrane-bound toxin. First-order kinetics of the reduction in the hemolytic activity of α-toxin in fluid membrane also favored the formation of nonhemolytic but monomeric state at least in a short-time range (Fig. 4). Thus, circumstantial evidence supported the existence of the nonhemolytic but monomeric state of α-toxin in fluid membrane. To obtain more information on the molecular identities of the membrane-bound α-toxin, a study involving use of cross-linking reagents is now in progress. Fluorescence-labeled toxin might also be a useful probe for analysis of structural aspects of α-toxin in fluid and solid membranes.

The assembly process of α-toxin in membrane was evidently promoted by the inclusion of cholesterol into PC membranes as well as by the phase transition of PC membranes from gel to liquid crystalline state. These phenomena can be interpreted as the effect of membrane fluidity. However, as far as the trypsin accessibility of the membrane-bound toxin was concerned, trypsin digestion of the toxin took place differently between the PC-cholesterol membranes and the membranes composed of PC alone. Although significant amounts of intact toxin were recovered when the toxin was associated with fluid PC liposome and exposed to trypsin, almost all toxin was recovered as a 32-kDa fragment (i.e. a cleaved product of α-toxin at the position between 8 and 9) from the cholesterol-containing membranes. These results suggested that the N terminus of the toxin was exposed at the surface of the cholesterol-containing membranes, whereas it was partially sequestered from the trypsin digestion on/in the PC membranes. Thus, cholesterol might have a different effect on the membrane-bound state of α-toxin at least in terms of topology and/or conformation of the toxin in membrane.

It should also be noted that coexistence of unsaturated acyl chain in PC membrane was required for enhancing hexamerization of the toxin to a high level comparable with that in the isolated erythrocyte membrane. Since the membranes contained 50 mol% cholesterol, it is not likely that the unsaturated acyl chain of PC drastically fluidized the cholesterol-containing membranes. A possible explanation would be as follows. An unsaturated acyl residue confers on the cholesterol-containing membrane a more fluid microenvironment (which cannot be well characterized by methods such as differential thermal calorimetry), and thereby it makes the membrane less tightly packed, permitting lateral and/or rotational motion of α-toxin molecules in the membrane. Incidentally, α-toxin induced leakage of internal carboxyfluorescein from various PC-cholesterol liposomes at 25 °C in the following order: DOPC -> DLPC -> DMPC -> DPPC -> DSPC-cholesterol, i.e. efficiency of the formation of membrane channel by α-toxin in the cholesterol-containing membranes is apparently in a good correlation with the Tm of PC.

By measuring fluorescence energy transfer from tryptophan residue of α-toxin to dansylated phosphatidylethanolamine in PC liposomes, Ikigai and Nakae (30) reported that Staphylococcal α-toxin interacts most efficiently with small unilamellar liposomes, but it does not bind to the large liposomes (perhaps multilamellar liposomes) that were eluted in the void fraction on Sepharose CL-4B column chromatography. To monitor assembly of α-toxin in the membrane of small unilamellar liposomes, Ikigai and Nakae (31) measured the fluorescence increment at 336 nm, which may be conferred by the tryptophan residues of the adjacent toxin molecules (31). They concluded that the presence of unsaturated fatty acid(s) in phospholipid is a prerequisite for the assembly of α-toxin and that α-toxin does not assemble in the membrane composed of DPPC or DMPC at any temperatures between 15 and 45 °C (i.e. membrane fluidity does not affect the assembly process of the toxin). The discrepancy between our data presented in this paper and the results of Ikigai and Nakae (30, 31) may be due to the difference in the liposome preparations used and in the methods for measuring binding and hexamer formation of α-toxin. In order to control the thermal behavior of PC membranes more precisely, we employed large multilamellar liposomes whose curvatures were in the size comparable with that of the erythrocyte. Because of the small curvature, however, small unilamellar liposome does not exhibit thermotropic response well, and it may expose the hydrophobic surface.

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