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Assembly and channel-forming activity of a naturally-occurring nicked molecule of *Staphylococcus aureus* α-toxin

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From the culture supernatant of *Staphylococcus aureus* Wood 46, we obtained a naturally-occurring nicked molecule of staphylococcal α-toxin. The nicked α-toxin consisted of non-covalently-linked 8-kDa and 25-kDa polypeptides, which were derived, respectively, from the N-terminal and the C-terminal part of the toxin nicked at the peptide bond between Glu-71 and Gly-72. The nicked toxin, as well as native α-toxin, bound to and oligomerized in the liposome membranes composed of choline-containing phospholipids (i.e., phosphatidylcholine and sphingomyelin) and cholesterol, and formed membrane channel in the liposome membranes. However, the channel-forming activity of the nicked toxin, assessed as a toxin-induced carboxyfluorescein leakage from the liposomes, was approx. 20-fold lower than that of native α-toxin. Channel-forming activity of the nicked toxin as well as native toxin was inhibited by divalent cations including Zn^{2+}, Cd^{2+}, Ca^{2+} and Mg^{2+}, and degree of the inhibitory effect of the divalent cations was in the following order: $Zn^{2+} > Cd^{2+} > Ca^{2+} > Mg^{2+}$. Thus, although the cleavage of α-toxin at the position between Glu-71 and Gly-72 drastically reduced channel-forming activity of the toxin, the nicked toxin retained the ability to oligomerize in phospholipid-cholesterol membranes and the characteristics of channel-forming activity in terms of the specificity for phospholipids and the susceptibility to divalent cations.

Introduction

Staphylococcal α-toxin is a water-soluble 33-kDa polypeptide endowed with hemolytic, dermonecrotic, and lethal properties for laboratory animals. It is secreted by most clinical isolates of *Staphylococcus aureus*, and is considered to be a virulence factor of the bacterium [1,2]. The toxin has been shown to be membrane-damaging to artificial membranes [3–6] and to a variety of mammalian cells including erythrocytes of different sources [7,8], human platelets [9], human monocytes [10], pig pulmonary endothelial cells [11], rabbit alveolar macrophages [12] and mouse adrenocortical Y1 cells [13].

Fuessle et al. proposed the concept of α-toxin as a channel-forming protein on the basis of the finding that the 12 S ring structure of α-toxin formed on erythrocytes and liposomes was composed of hexameric toxin, which appeared to be a transmembrane channel [14]. Formation of the toxin hexamer was confirmed by different experimental techniques [15,16], and channel activity of the hexamer was characterized by several groups [17–19]. The functional role of the toxin hexamer in hemolysis caused by low concentrations of α-toxin was further studied by Bhakdi and his co-workers [20,21] and Hildebrandt et al. [22]. The channel-forming activity of the toxin is now thought to underlie the multiple biological actions of the toxin [2,23].

While purifying staphylococcal α-toxin from the culture supernatant of *Staphylococcus aureus* Wood 46, we unexpectedly encountered a naturally-occurring nicked molecule of the toxin on DEAE-Sephacel column chromatography. We isolated the nicked toxin and characterized it in terms of its assembly and channel-forming activity by using phospholipid-cholesterol liposomes.

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Abbreviations: PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CF, carboxyfluorescein; TBS, Tris-buffered saline.

Materials and Methods

Chemicals

Carboxyfluorescein (CF) was purchased from Eastman Kodak (Rochester, NY), and purified as described

by Weinstein et al. [24]. Egg yolk phosphatidylcholine (PC) was kindly supplied from Nippon Oil and Fats (Tokyo). Egg yolk phosphatidylglycerol, bovine brain phosphatidylserine, bovine brain cardiolipin, bovine brain sphingomyelin, soybean phosphatidylinositol and cholesterol were purchased from Sigma (St. Louis, MO). Cholesterol was recrystallized twice from methanol before use.

Staphylococcal α-toxin

Staphylococcal α-toxin was isolated from the culture supernatant of *Staphylococcus aureus* Wood 46 as described previously [5], and stored at −80°C. Before use, the toxin preparation was chromatographed on a column of Sephadex G-75, to eliminate hexamer of the toxin spontaneously formed in the toxin stock.

Isolation of a naturally-occurring nicked molecule of staphylococcal α-toxin

Staphylococcus aureus Wood 46 was grown in Tryptic Soy Broth (Difco, Detroit). The culture supernatant obtained was fractionated with 70% saturated ammonium sulfate. Resultant precipitate was dissolved in 50 mM Tris-HCl buffer (pH 8.5) and dialyzed against the same buffer. The dialysate was chromatographed on a column (2.1 × 56 cm) of CM-Sepharose CL-6B (Pharmacia, Uppsala) that had been equilibrated with the same buffer. Flow-through hemolytic fractions were pooled, dialyzed against 50 mM Tris-HCl buffer (pH 8.5) and applied onto a column (2.1 × 45 cm) of DEAE-Sephacel (Pharmacia, Uppsala) equilibrated with the same buffer. Two peaks of absorbance at 280 nm were obtained in the unadsorbed fractions. The first peak fraction, which contained α-toxin, exhibited a strong hemolytic activity, whereas the second peak fraction was hemolytically weak (Fig. 1A). The second peak fraction was rechromatographed on another DEAE-Sephacel column (2.1 × 45 cm) to eliminate trace amounts of α-toxin (Fig. 1B).

N-terminal amino acid sequence of fragments of the nicked α-toxin

The purified weakly-hemolytic fraction was electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [25], and electroblotted onto ProBlott™ membrane (Applied Biosystem). Protein bands on the membrane were stained with 0.1% Coomassie brilliant blue R250, cutted out, and subjected to sequence analysis using a Protein Sequencer 473A (Applied Biosystem) [26].

Hemolytic activity

In a U-bottomed 96-well microplate (Nunc, Roskilde), 50 μl of serial dilutions of test samples were mixed with 50 μl of 2% (v/v) rabbit erythrocyte suspension in 10 mM Tris-HCl buffer (pH 7.2) containing 0.85% NaCl (Tris-buffered saline, TBS), and incubated at 37°C for indicated time periods (30–180 min). After the incubation, the microplate was centrifuged at 200 × *g* for 10 min, to remove unlyzed erythrocytes. 50 μl of the supernatants were transferred to another microplate, and subjected to measurement of absorbance at 550 nm with a reference at 630 nm by use of a microplate reader MTP-32 (Corona Electric, Katsuda, Japan). Hundred percent of hemolysis was defined as the maximal value of absorbance at 550 nm obtained with > 2 μg/ml of native α-toxin.

Multilamellar liposomes

A mixture of phospholipid (1 μmol) and cholesterol (1 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 further dried for 30–60 min under vacuum, and dispersed by vortexing at 50–60°C in 0.1 ml of either 0.1 M carboxyfluorescein (CF) or TBS. Multilamellar liposomes formed were collected by centrifugation at 23 000 × *g* for 20 min, and washed three times by centrifugation to remove unentrapped CF.

Toxin-induced CF release from liposomes

In a 96-well U-bottomed microplate, 25 μl of serial dilutions of test samples were mixed with 25 μl of CF-loaded liposome and incubated at 37°C for 30–120 min. Fluorescence intensity was measured with a microplate fluorometer MTP-32 (Corona, Katsuda, Japan) with an exitation at 490 nm and an emission at 530 nm. 100 percent of CF release was defined as the fluorescence intensity that was gained upon exposure of liposomes to 0.5% Triton X-100 at 37°C for 30–120 min. Concentrations of liposomes were adjusted on the basis of the fluorescence intensity obtained upon 100% CF release.

Assembly of nicked α-toxin in liposome membrane

A mixture of the nicked toxin (30 μg) and egg yolk PC-cholesterol liposome (0.1 μmol of lipids) in TBS (1 ml) was incubated at 37°C for 1 h. Liposome-bound toxin was collected by centrifugation at 23 000 × *g* for 20 min, washed two times by centrifugation, and solubilized in 1% SDS at 25°C for 1 h. The solubilized toxin was subjected to SDS-PAGE on a 13% polyacrylamide gel essentially as described by Laemmli [25]. However, boiling of the solubilized toxin in the presence of 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol was omitted to avoid dissociation of oligomer molecules [14,27]. The gel was stained with Coomassie brilliant blue R250 as described by Fairbanks et al. [28]. For comparison, native α-toxin (30 μg) was also used instead of the nicked toxin in the same assay.

Miscellaneous

Protein determination was performed essentially as described by Bradford [29], using bovine serum albumin as a standard. Phosphorus was determined according to the method of Gerlach and Deuticke [30]. Cholesterol was determined with an assay kit from Nissui Phamaceuticals (Tokyo).

Results

A naturally-occurring nicked molecule of staphylococcal α-toxin obtained from the culture supernatant of Staphylococcus aureus Wood 46

When we attempted to purify staphylococcal α-toxin from the culture supernatant of *Staphylococcus aureus* Wood 46 according to the method of Ikigai and Nakae [16], we found two hemolytic peaks, a hemolytically high peak of α-toxin and a weakly-hemolytic peak of unknown substance(s), in the unadsorbed fractions of a DEAE-Sephacel column chromatography (Fig. 1A). Since the weakly-hemolytic fraction exhibited the same antigenic properties as α-toxin (data not shown), it appeared to contain polypeptide fragment(s) of α-toxin. We rechromatographed the fraction on another DEAE-Sephacel column to eliminate trace amounts of coexsisting α-toxin (Fig. 1B). In a representative experiment, we obtained approximately 8 mg protein in the rechromatographed weakly-hemolytic fraction from 5 litres of the culture supernatant. To check purity of the rechromatographed weakly-hemolytic fraction, a portion of the fraction was withdrawn and subjected to a high performance gel permeation chromatography with Asahipack GS510 column (Asahi, Tokyo). As shown in Fig. 1C, the fraction gave a single peak of absorbance at 280 nm, indicating that the fraction was apparently homogeneous in molecular size. In addition, the weakly-hemolytic fraction was eluted at an elution volume similar to that of native α-toxin (data not shown).

To study antigenic properties of the weakly-hemolytic fraction, immunodiffusion test was performed by using rabbit antiserum raised to α-toxin. As shown in Fig. 2, the weakly-hemolytic fraction reacted with the anti serum and formed a precipitin line, which was fused with the precipitin lines formed between native α-toxin and the same serum. On SDS-PAGE using a 15% polyacrylamide gel, however, the weakly-hemolytic fraction gave two different polypeptides with apparent M_r of 8000 and 25 000 regardless of boiling in the presence of 2% SDS and 5% 2-mercaptoethanol (Fig. 3A). Thus, although the weakly-hemolytic fraction had the same antigenic properties as native α-toxin, it was composed of 8-kDa and 25-kDa polypeptides instead of a single 33-kDa polypeptide of native α-toxin. Taken together with the evidence that the weakly-hemolytic fraction behaved as a homogeneous preparation in terms of molecular size (Fig. 1C), we presumed that the fraction contained a nicked molecule of α-toxin.

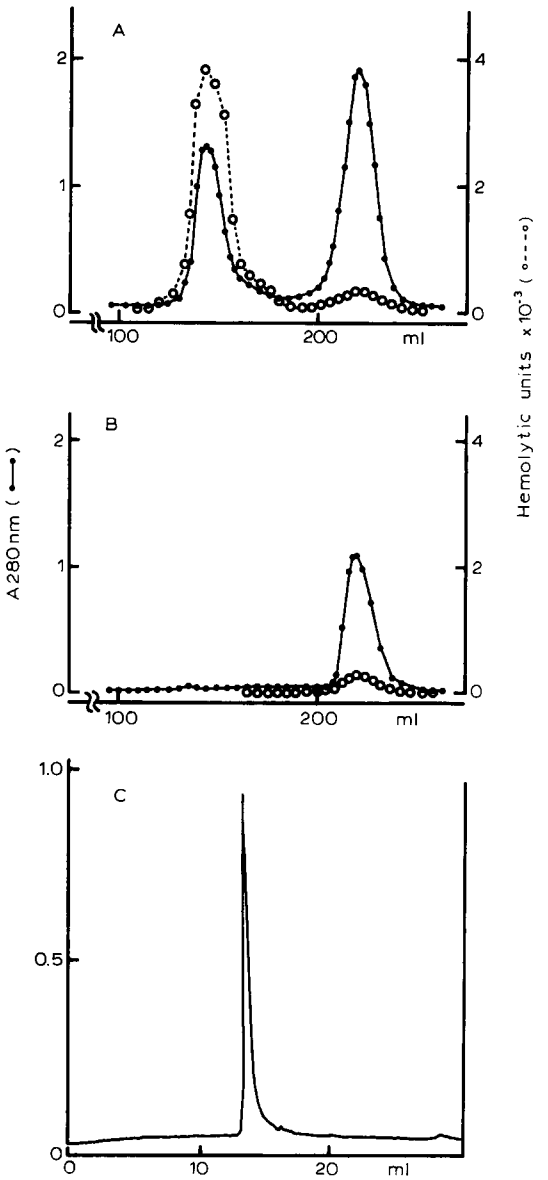

Fig. 1. Isolation of weakly-hemolytic fraction from the culture supernatant of *Staphylococcus aureus* Wood 46. Hemolytic fractions eluted from CM-Sepharose CL-6B column was subjected to DEAE-Sephacel column chromatography (A). The weakly-hemolytic fractions, i.e., the latter peak of absorbance at 280 nm in the unadsorbed fractions, were collected and rechromatographed on another DEAE-Sephacel column (B). Weakly-hemolytic fractions eluted in the flow through of the DEAE-Sephacel column chromatography were collected. A portion of the combined fraction was withdrawn and subjected to a high performance liquid chromatography with Asahipack GS 510 (Fig. 1C). Absorbance at 280 nm (●——●) and hemolytic activity (○——○) of the fractions obtained from representative chromatography were illustrated.

We analyzed N-terminal amino acid sequences of the 8-kDa and the 25-kDa polypeptides by using a gas phase protein sequencer (Fig. 3B), and compared the data with the amino acid sequence of α-toxin, which

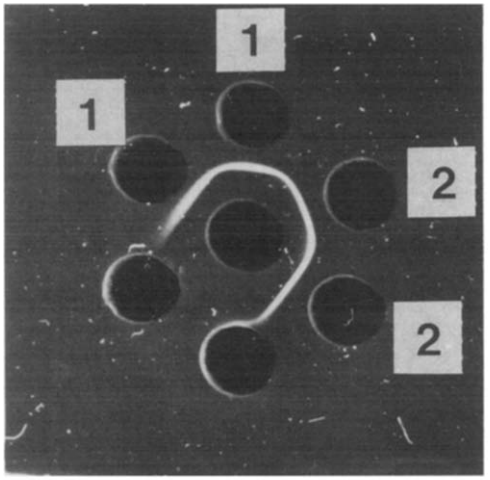

Fig. 2. Antigenic properties of the weakly-hemolytic fraction. Using rabbit antiserum raised to α-toxin, immunodiffusion test was performed in 1% agarose gel for the weakly-hemolytic fraction and native α-toxin. The antiserum was added to the central well. Well 1: the weakly-hemolytic fraction obtained from the second DEAE-Sephacel column chromatography. Well 2: native α-toxin.

was deduced from the DNA sequence reported by Gray and Kehoe [31]. The sequence of N-terminal 20 amino acids of the 8-kDa polypeptide was determined to be Ala-Asp-Ser-Asp-Ile-Asn-Ile-Lys-Thr-Gly-Thr-Thr-Asp-Ile-Gly-Ser-Asn-Thr-Thr-Val, indicating that

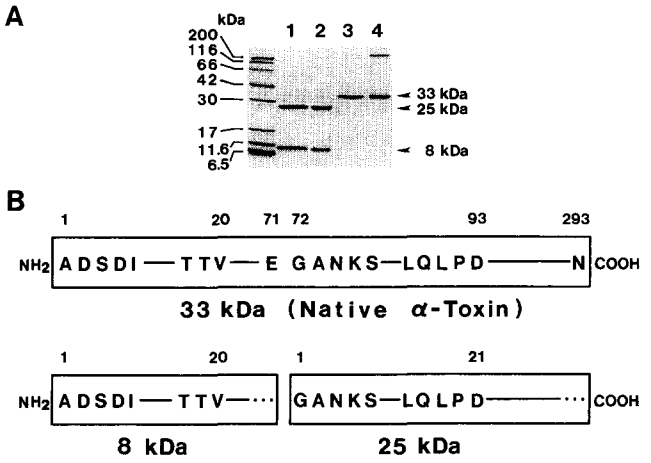

Fig. 3. SDS-polyacrylamide gel electrophoresis of the weakly-hemolytic fraction (A) and amino acid sequences of the polypeptides obtained from the electrophoresis (B). (A) A portion of the weakly-hemolytic fraction was electrophoresed on a 15% acrylamide gel with (Lane 1) or without (Lane 2) boiling in the presence of 2% SDS and 5% 2-mercaptoethanol For comparison, native α-toxin was electrophoresed on the same gel with (Lane 3) or without (Lane 4) the boiling. (B) N-terminal amino acid sequences of the 8 kDa and 25 kDa polypeptides, determined by a protein sequencer as described in Materials and Methods, are partially illustrated. The amino acid sequence of α-toxin, which was deduced from the DNA sequence reported by Gray and Kehoe [23], is also partially illustrated. Standard single letter codes for amino acids are used in the illustrations. Corresponding three letter codes are: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

the sequence coincided with the N-terminal amino acid sequence of α-toxin that was deduced from the DNA sequence (Fig. 3B). N-terminal amino acid sequence of the 25-kDa polypeptide was determined to be Gly-Ala-Asn-Lsy-Ser-Gly-Leu-Ala-Try-Pro-Ser-Ala-Phe-Lys-Val-Gln-Leu-Gln-Leu-Pro-Asp. The data indicated that the 25-kDa polypeptide was derived from the C-terminal part of the α-toxin cleaved at the position between Glu-71 and Gly-72 (Fig. 3B). Although we have not analyzed C-termini of the fragments, their apparent molecular weights were in good agreement with the calculated molecular weights for the N-terminal and C-terminal fragments of the toxin nicked at the peptide bond between Glu-71 and Gly-72 (i.e., 7900 and 25 282, respectively). Densitometric study of the gels stained with Coomassie brilliant blue R-250 indicated that molar ratio of the 25-kDa fragment to the 8-kDa fragment was approximately 1 : 1 in the nicked toxin (data not shown).

Channel-forming activity and assembly of the nicked molecule of α-toxin

We assayed hemolytic activity of the nicked α-toxin, and compared it with that of native toxin on the basis of protein concentration. The nicked or native toxin of different concentrations was incubated with 1% rabbit erythrocytes at 37°C for various time periods between 30 and 180 min. Under the conditions, concentrations of toxin for inducing 50% hemolysis were determined to be 5.2–4.4 μg/ml for the nicked toxin and 0.24–0.088 μg/ml for native toxin, respectively. Thus, the nicked toxin was much less active hemolytically than native toxin (i.e., there was apparently 20–50-fold difference in the hemolytic activity between native and the nicked toxin).

By use of multilamellar liposomes of various compositions, we showed previously (i) that α-toxin specifically binds to the membranes composed of choline-containing phospholipids, phosphatidylcholine and sphingomyelin [5], and (ii) that the toxin assembles into hexamer and exhibits transmembrane channel activity on/in the liposome membranes when the membranes were fluidized by the inclusion of >20 mol% cholesterol [6,27]. Based on these data, we studied whether or not the nicked toxin induces membrane damage in phospholipid-cholesterol membranes, and whether or not it has the same specificity for phospholipids in the channel-forming activity. We used multilamellar liposomes composed of various phospholipids and cholesterol in a molar ratio of 1 : 1, and assessed channel-forming activity of the nicked toxin as leakage of internal carboxyfluorescein (CF) from the liposomes upon exposure to the toxin for 30 min at 37°C. The nicked toxin induced 50% CF release from egg yolk PC-cholesterol liposome at 100 μg/ml (Fig. 4), whereas native α-toxin induced 50% CF leakage from the same

liposome at 5 μg/ml (Fig. 4). These results indicated that the nicked toxin was approx. 20-fold less active in the channel-forming activity than the native toxin (Fig. 4). Similar results were obtained when the incubation period was extended to 2 h (i.e., concentrations for inducing 50% CF leakage were 78 and 3.5 μg/ml for the nicked and the native toxin, respectively). Concerning the phospholipid specificity of the nicked toxin, the nicked toxin induced CF leakage only from PC-cholesterol and sphingomyelin-cholesterol liposomes (Fig. 4). Thus, the nicked toxin exhibited the same phospholipid specificity as native toxin, although it was 20-fold less active in the channel-forming activity than native α-toxin.

We also examined the ability of the nicked toxin to oligomerize in membrane. The nicked toxin was incubated with egg yolk PC-cholesterol liposome at 37°C for 1 h. Liposome-bound nicked toxin was collected by centrifugation, solubilized in 1% SDS, and subjected to SDS-PAGE without boiling in the presence of 2% SDS and 5% 2-mercaptoethanol. As shown in Fig. 5, a substantial amount of the nicked toxin solubilized from the liposome was mobilized as a band of approx. 200-kDa, indicating that the nicked toxin assembled into oligomer in the membrane (Lanes 1 and 2). For comparison, oligomerization of native α-toxin was also assayed under the same conditions (Fig. 5, Lanes 3 and 4). These results indicated that oligomerization of the nicked toxin occurred in the membrane at a level comparable with that of the native α-toxin (Fig. 5).

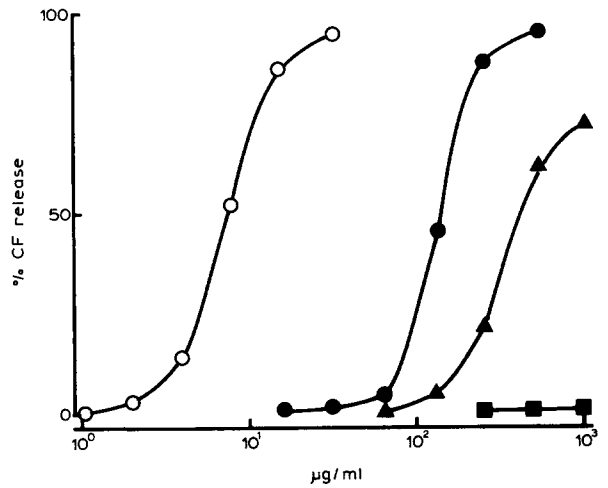

Fig. 4. Channel-forming action of the nicked α-toxin on phospholipid-cholesterol liposomes. Carboxyfluorescein (CF)-loaded liposomes composed of egg yolk PC (●), bovine brain sphingomyelin (▲), bovine brain phosphatidylserine (■), egg yolk phosphatidylglycerol (■), soybean phosphatidylinositol (■) or bovine cardial muscle cardiolipin (■) and cholesterol in a molar ratio of 1:1 were exposed to various doses of the nicked toxin. CF release from the liposomes was assayed as described in Materials and Methods. For comparison, CF release from egg yolk PC-cholesterol liposome upon exposure to native α-toxin was also illustrated (○). Representative data from three independent experiments were illustrated.

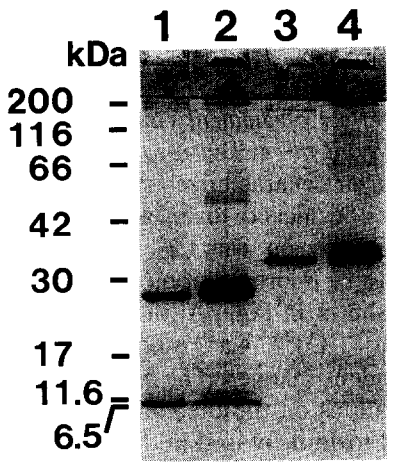

Fig. 5. Oligomer formation of the nicked α-toxin in liposome membrane. The nicked toxin or native toxin was incubated with egg yolk PC-cholesterol liposome at 37°C for 1 h. Liposome-bound toxin was collected, solubilized in 1% SDS and subjected to SDS-PAGE without boiling prior to electrophoresis, as described in Materials and Methods. Lane 1: the nicked toxin used; Lane 2: liposome-bound nicked toxin; Lane 3: native toxin used; Lane 4: liposome-bound native toxin.

Incidentally, oligomer of the nicked toxin was not dissociated to monomers during the SDS-PAGE (Fig. 5, Lane 2). Thus, the oligomer of the nicked toxin as

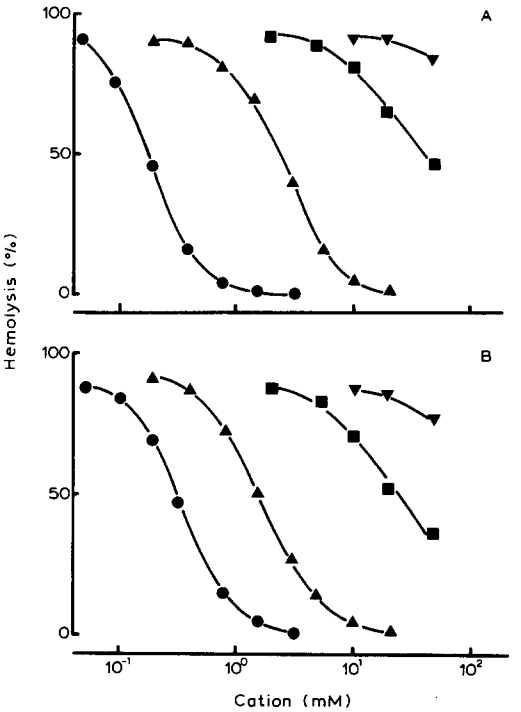

Fig. 6. Inhibitory effect of divalent cations on the channel-forming activity of the nicked α-toxin In the presence of Zn^{2+} (●), Cd^{2+} (▲), Ca^{2+} (■) or Mg^{2+} (▼), CF-loaded, egg yolk PC-cholesterol liposome was incubated at 37°C for 30 min with a given amount of the nicked toxin (A) or native toxin (B) that induced 90% CF release when the divalent cations were absent. CF release from the liposome was measured as described in Materials and Methods. Mean values obtained from three independent experiments were plotted.

well as the native toxin was resistant to the treatment with 1% SDS.

Inhibitory effect of divalent cations on the channel-forming activity of the nicked α-toxin

To further characterize the membrane channel formed by the nicked toxin, we studied the effect of divalent cations on the CF leakage from egg yolk PC-cholesterol liposome upon exposure to the nicked toxin. As shown in Fig. 6A, the CF leakage was inhibited by Zn^{2+}, Cd^{2+}, Ca^{2+} and Mg^{2+} in a concentration-dependent manner. The inhibitory effect of these divalent cations on the the channel-forming activity of the nicked toxin increased in the order of Mg^{2+}, Ca^{2+}, Cd^{2+} and Zn^{2+} (Fig. 6A). Similar results were obtained with native α-toxin (Fig. 6B). These results indicated that both the nicked toxin and the native toxin formed membrane channel with similar, if not identical, characteristics in terms of the susceptibility to divalent cations. The inhibitory effect of divalent cations was not due to displacement of the nicked toxin and native toxin from the membrane by the cations, since the nicked toxin as well as native toxin remained to be associated with the liposome in the presence of Zn^{2+} or Cd^{2+} (unpublished observation).

Discussion

In this study, we isolated and characterized a naturally-occurring nicked α-toxin from the culture supernatant of *Staphylococcus aureus* Wood 46. The nicked toxin comprised noncovalently-linked two polypeptides, the 8-kDa and the 25-kDa polypeptides, which were derived, respectively, from the N-terminal part and the C-terminal part of the α-toxin nicked at the peptide bond between Glu-71 and Gly-72. These two fragments behaved together on the high-performance gel permeation column chromatography (Fig. 1C), and on a native polyacrylamide gel electrophoresis (data not shown). However, the fragments were separated on the SDS-PAGE regardless of boiling in the presence of SDS and 2-mercaptoethanol (Fig. 3A). They were also partially separated on the high-performance gel permeation column chromatography when 10% isopropanol was present in the buffer (data not shown). Taken together, it may be a hydrophobic interaction that linked the two polypeptides in the nicked toxin.

Since *Staphylococcus aureus* V8 proteinase has substrate specificity for the peptide bond Glu-X [32], we studied preliminarily whether or not the proteinase hydrolyzes the peptide bond between Glu-71 and Gly-72 in spontaneously-formed hexameric toxin (which was obtained from the toxin stock by Sephadex G-75 column chromatography). We treated the hexameric toxin with the proteinase, separated proteolytic products on SDS-PAGE, and analyzed N-terminal sequences of the products. Our data indicated that the proteinase preferentially hydrolyzed the peptide bond between Glu-71 and Gly-72 of the spontaneously-formed hexamer of α-toxin. Thus, the peptide bond between Glu-71 and Gly-72 in the spontaneously-formed hexameric α-toxin was accessible by staphylococcal V8 proteinase, suggesting that the position between Glu-71 and Gly-72 is exposed on the external surface of the hexameric toxin, and that the site is not directly included in the channel-forming domain of the toxin.

Regarding the channel formation in phospholipid-cholesterol membranes, the nicked α-toxin exhibited essentially the same specificity for phospholipids and the same susceptibility to divalent cations as native toxin (Figs. 4 and 6). It was also shown that the nicked toxin assembled into oligomer (perhaps hexamer) in the PC-cholesterol membrane as efficiently as native toxin (Fig. 5). Thus, the nicked toxin retained abilities for channel formation and oligomerization in membrane, suggesting that tertiary structure of the nicked toxin and/or its oligomer does not largely differ from that of native α-toxin and/or its hexamer. Furthermore, since the membrane channel of the nicked toxin was inhibited by Zn^{2+}, Ca^{2+} and Mg^{2+} in the same manner as previously reported for native toxin [6,17], it may have rather intact cation-binding site(s), which is postulated to be located at the mouth of membrane channel.

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