Cobra cardiotoxins (CTXs) have previously been shown to induce membrane fusion of vesicles formed by phospholipids such as cardiolipin or sphingomyelin. CTX can also form a pore in membrane bilayers containing an anionic lipid such as phosphatidyserine or phosphatidylglycerol. Herein, we show that the interaction of CTX with negatively charged lipids causes CTX dimerization, an important intermediate for the eventual oligomerization of CTX during the CTX-induced fusion and pore formation process. The structural basis of the lipid-induced oligomerization of CTX A3, a major CTX from *Naja atra*, is then illustrated by the crystal structure of CTX A3 in complex with SDS; SDS likely mimics anionic lipids of the membrane under micelle conditions at 1.9 Å resolution. The crystal packing reveals distinct SDS-free and SDS-rich regions; in the latter two types of interconnecting CTX A3 dimers, D1 and D2, and several SDS molecules can be identified to stabilize D1 and D2 by simultaneously interacting with residues at each dimer interface. When the three CTX-SDS complexes in the asymmetric unit are overlaid, the orientation of CTX A3 monomers relative to the SDS molecules in the crystal is strikingly similar to that of the toxin with respect to model membranes as determined by NMR and Fourier transform infrared methods. These results not only illustrate how lipid-induced CTX dimer formation may be transformed into oligomers either as inverted micelles of fusion intermediates or as membrane pore of anionic lipid bilayers but also underscore a potential role for SDS in x-ray diffraction study of protein-membrane interactions in the future.

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The atomic coordinates and structure factors (code 1h0j) have been deposited in the Protein Data Bank, Research Collaborators for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: CTX, cardiotoxin; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; ATR, attenuated total reflection; FTIR spectroscopy, Fourier transform infrared spectroscopy; Rh, rhodamine; HPLC, high performance liquid chromatography; 6-CF, 6-carboxyfluorescein; ATR, attenuated total reflection.

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phatidylglycerol (PG) and phosphatidic acid (PA), induce a significant increase in the β-sheet content of CTX A3 (7, 13, 15, 16). To address these issues and to gain an insight into the mechanism of CTX-induced membrane leakage and fusion processes, we co-crystallized CTX A3 with SDS and determined its three-dimensional structure under micelle conditions. The results also provide the first high resolution molecular model of CTX A3 to understand how lipid-induced CTX dimerization may contribute to the CTX oligomerization required for the formation of fusion intermediate and membrane pores in the previously reported CTX-induced membrane related activities.

EXPERIMENTAL PROCEDURES

Materials and Purification—Rhodamine B isoiothiocyanate, fluorescently labeled dextran FD-4 and FD-70, and fluorescein isoiothiocyanate-conjugated anti-rabbit IgG were purchased from Sigma. The phospholipids of PC, phosphatidylyserine (PS), PG, and PA used in this study were obtained from Avanti Polar Lipids. Because these phospholipids contain palmitoyl-olefatty acyl chains, they are named POPC, POPS, POPG and POPA, respectively. CTX A3 and CTX A5 were purified by SP-Sephadex C-25 ion exchange chromatography followed by HPLC on a reverse phase C-18 column from crude venom of N. naja atra (snake’s education farms, Tainan, Taiwan) previously described (7).

Vesicle Preparation—Lipids were dried under vacuum overnight and then hydrated with 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl. The suspension was frozen and thawed several times and was successively extruded through a polycarbonate filter with the pore size of 0.1 µm for obtaining homogeneous large unilamellar vesicles. For the pore size determination experiments, the buffer contained 2 mg/ml FD-4 and 4 mg/ml FD-70 or fluorescein isoiothiocyanate-conjugated IgG (17). Vesicles used in fluorescence-leakage experiments were formed in the presence of 10 mM Tris (pH 7.4), 75 mM NaCl, and 50 mM 6-carboxyfluorescein (6-CF). Sepharose CL-4B column was used to remove the residual fluorescent molecules outside of the vesicles, and the lipid concentration was determined by inorganic phosphate assay as described (18).

Chemical Modification of Methionine—Chemical modification of methionine residues was performed as described (19) with a slight modification. Briefly, 1 mM CTX A3 in 100 mM phosphate buffer (pH 2.5) containing 6 M guanidine-HCl was reacted with 10 mM iodoacetamide at room temperature. The reaction was monitored by analytical reverse phase HPLC followed by mass spectrometry. Two single- and one double-alkylated product could typically be obtained from this reaction. Each product was further characterized by mass spectrometry after phase HPLC followed by mass spectrometry. Two single- and one double-alkylated product could typically be obtained from this reaction.

Vesicle Leakage—Release of vesicle contents was detected by 6-CF fluorescence intensity. Although 6-CF displays low fluorescence intensity at high concentration, its intensity increases sharply at low concentrations. Vesicles containing 6-CF were incubated at a final volume of 1 ml of buffer in a 1 × 1 cm quartz cuvette. After the addition of CTX, the fluorescence intensity was monitored as a function of time for the CTX-induced vesicle leakage process. The 6-CF leakage was calculated using the following expression: leakage (%) = ([F2 – F1]/F1) × 100, where F1 is the initial fluorescence before adding proteins, F2 is the fluorescence reading at time t, and F3 is the final fluorescence determined by adding Triton X-100 (0.02%). The excited and emitted wavelengths were 480 and 520 nm. The selectivity was defined by the ratio of intrinsic leakage factor of co-encapsulated markers.

Crystalllography—Crystals of CTX A3 in complex with SDS, belonging to P2,2,2 space group with cell parameters of a = 74.90 Å, b = 76.20 Å, and c = 47.78 Å, were grown by the hanging drop method. One µl of the protein solution (10 mg/ml) was mixed with 1 µl of reservoir solution containing 100 mM sodium acetate (pH 4.6), 20% polyethylene glycol 400, 3% glycerol, and 24 mM SDS, which is well above its critical micelle concentration (7–10 mM). Crystals were flash-frozen in liquid nitrogen followed by cryo-data collection on an X-Ray IV imaging plate mounted on a Rigaku RU 300 rotating anode and subsequent data processing using DENZO (20). The structure was solved by the molecular replacement method using the crystal structure of CTXγ (accession code ITGX) as the search model. Using AMoRe (21), the initial solution containing three molecules in the asymmetric unit had an R-factor of 40%. After manually adjusting the position of L2, which significantly differed from that of the model (ITGX), as revealed by omit map of residues 26–34, and the addition of SDS and water molecules, the R-factor dropped to about 30%. Because the L2 of each molecule in the asymmetric unit adopts a different conformation, non-crystallographic symmetry was not imposed during refinement, for which CNS (22) and XtaView (23) was used. The current model with R-factor and R-free values of 22 and 22.3% for model and 30–1.54–Å resolution (Table I) was obtained after several iterative cycles of CNS refinement. PROCHECK (24) showed that 85.6% of the residues are in the most favored region with the remaining residues in the additional favored region. Coordinates have been deposited in the Protein Data Bank under accession code 1h0j.

Fluorescence Labeling—CTX (0.2 mM) was mixed with rhodamine B isothiocyanate (0.4 mM) in the presence of 100 mM phosphate buffer (pH 7.4) containing 6 M guanidine-HCl. The reaction mixture was incubated at room temperature for 12 h, and the resulting fluorescence-conjugated CTX was purified by HPLC. Single fluorescent probe-conjugated CTX was further identified using electrospray ionization mass spectrometry (Quatro Ultima, MicorMass). For characterization of the conjugated position on CTX, the sample was dissolved in 100% trifluoroacetic acid at 40 °C for 20 min to obtain conjugated amino acids of CTX and the subsequent molecular weight verified by mass spectrometry. Concentrations of fluorescence-labeled and unlabeled CTX were determined using extinction coefficients of εmax = 105,000 M−1 cm−1 for rhodamine B-conjugated CTX A3, εmax = 4185 M−1 cm−1 for CTX A3, and εmax = 2813 M−1 cm−1 for CTX A5. For each experiment, only N-terminal fluorescence-conjugated CTXs were used.

Attenuated Total Reflection Fourier Transform Infrared (ATR FTIR)

Experiments—ATR-FTIR spectra were collected at ambient temperature using a Bomem DA 6.3 FTIR system with a liquid nitrogen-cooled MCT detector. The internal reflection element was a zinc-selenium ATR plate 5 × 5 × 2 mm, Harrick, Ossining, NY) with an incidence angle of 45°. The ATR plates were washed with alcohol and deionized water and cleaned by plasma cleaner (Harrick) for the generation of clean and dust surface. CTX (20 µg), dissolved in D2O solution in the absence and presence of lipids (40 µg), was dried on the surface of the ATR plate.
and sealed in a D,O-saturated sample holder. The spectra (200 scans) were recorded at a spectral resolution of 2 cm⁻¹ with triangular apodization. Fourier self-deconvolution was calculated, with the optimal parameter of 14⁻¹ cm for the half-width of undeconvolution band and 2.2 for the resolution enhancement factor K, as previously described (25).

**Fluorescence Homotransfer**—The steady-state fluorescence spectra for the determination of CTX oligomerization upon binding to anionic lipids were obtained on an SLM-4800 fluorescence spectrometer with excitation and emission wavelengths set at 550 and 580 nm, respectively. Fluorescence-labeled and unlabeled CTX were mixed in an appropriate molar ratio, as shown in Fig. 2, in the presence of 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl. The final concentration of both proteins was maintained at 0.1 M. Upon the addition of anionic lipids (vesicles (30 μM) to fluorescence-labeled CTX, the fluorescence intensity spontaneously decreased as a result of the fluorescence energy homotransfer (self-quenching) during the oligomerization process. The effect of self-quenching became less if the intrinsic CTX was added to dilute the fluorescence-labeled CTX (26, 27). All experiments were performed at 25 °C.

**RESULTS AND DISCUSSION**

**Anionic Lipid-induced Oligomerization and Membrane Pore Formation of CTX A3**—Although P-type CTXs are known to bind to micelles of zwitterionic lipid or membrane vesicles of sphingomyelin, their interaction with PC membranes at liquid crystalline state causes no detectable lytic effect (green line, Fig. 1B). However, introduction of acidic lipids such as PS into the model membrane leads to a significant CTX A3-induced leakage of 6-CF fluorescence probe (red and black lines, Fig. 1B). In contrast, despite stronger interaction of CTX A5 than CTX A3 with the PC membranes, there is no detectable CTX A5-induced leakage even in vesicles with 100% PS (blue line, Fig. 1B). That similar CTX-induced vesicle leakage can also be observed for vesicles formed by PG, PA, or sulfatide suggests electrostatic interactions between anionic lipids and cationic CTX A3 play a role in the CTX-induced leakage of negatively charged membranes. Concentration-dependent study of the effect of CTX A3-induced leakage further reveals a bimolecular interaction of CTX A3 might be involved since the initial leakage rate of the process depends on the square of the CTX A3 concentration (Fig. 1C and the inset).

CTX A3-induced membrane leakage may stem from formation of a toxin pore and/or its direct lytic action on membranes; both mechanisms require membrane-induced toxin oligomerization. To investigate whether the CTX A3 and CTX A5 molecules oligomerize in the presence of anionic lipid membranes, fluorescence energy transfer experiments were performed in the presence of rhodamine-labeled CTX A3 (Rh-CTX A3), or CTX A5 (Rh-CTX A5). If Rh-CTX A5 is a monomer near the membrane surface, dilution of the Rh-CTX with the intrinsic CTX molecules could cause no change in the fluorescence intensity (black dashed line in Fig. 2). Conversely, if Rh-CTX A5 exists as either dimer or oligomer upon dilution of Rh-CTX on intrinsic CTX, the efficiency of fluorescence energy transfer among Rh-CTXs would decrease. Theoretically, the quantitative effect of fluorescence energy transfer suggests a linear decrease for the CTX dimer and an even faster decrease for the CTX oligomer (Fig. 2A). Quantitative analysis of the result indicates that although CTX A3 forms oligomer (>dimer) in the presence of negatively charged lipids (green line in Fig. 2A), CTX A5 forms only dimer (Fig. 2B). This in turn suggests an interactive relationship between oligomerization and membrane leakage in the case of CTX A3.

To see whether membrane pore formation of CTX A3 indeed occurs near the anionic lipid membrane surface, we ask whether the CTX-induced leakage of the co-entrapped fluorescence probe exhibits selectivity toward molecules with different sizes. Although Triton-treated PS vesicles allow the complete leakage of the fluorescently labeled dextran probe of both

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**Fig. 1.** Sequence alignment of four CTXs and CTX A3-induced vesicle leakage of 6-CF. A, sequences of CTX A3, CTX A5 (accession code 1KXI), CTX V4 (accession code 1CTD), and CTX γ (accession code 1TGX) were aligned based on their structures. The labeled secondary structural elements derived from this work are shown above the alignment. B, the leakage of different sized molecules is plotted as a function of time after adding toxin. Black line, CTX A3 within pure PS vesicle; red line, CTX A3 within 50% PS/PC vesicle; green line, CTX A3 within pure PC vesicle; blue line, CTX A5 within pure PS vesicle. Concentrations of CTX A3 and CTX A5 were 0.16 and 10 μM, respectively. C, initial rate of 6-CF leakage as function of CTX A3 concentration. Black square, pure PS; red circle, 50% PS/PC; green triangle, pure PC. The inset shows re-plotting of C with the squared x axis of toxin concentration.

FD-70 (M₀ = 50.7 kDa) and FD-4 (M₀ = 4.4 kDa) (green line in Fig. 3A), CTX A3-treated PS vesicles retain more of FD-70. FD-70 has been suggested to be a prolate ellipsoid (28). Based on the short axis of dextran, it is estimated that the lower limit of the pore size is ~20 Å (17). The selectivity value of CTX-induced pore was determined to be ~1.8 ± 0.3, which was smaller than that of melittin-treated POPC vesicles. This result implies that the size or lifetime of the CTX-induced pore is larger or longer than that of the melittin-induced pore. Because all co-entrapped IgG remained within the CTX A3-treated vesicles (Fig. 3B), the result further suggests an upper limit for the size of the pore is ~100 Å, which corresponds to the diameter of an IgG molecule (29). Based on the aforementioned result, we conclude that the anionic lipid may induce the oli-
gomerization of CTX A3 near the membrane surface and formation of a pore with a size ranging between 20 and 100 Å. Because the interaction of CTX with other anionic lipid such as cardiopin also induces the formation of well-defined membrane particles, presumably inverted micelles, as its fusion intermediates, it is desirable to obtain a high resolution structure to understand the mechanism responsible for the CTX action on phospholipid membranes. We consequently co-crystallized CTX A3 with anionic lipid of SDS and determined its three-dimensional structure at 1.9-Å resolution.

Overall Structure of CTX A3—There are three crystallographically unrelated CTX A3 molecules in an asymmetric unit; each molecule forms a dimer with its closest neighbor from an adjacent asymmetric unit. The crystal structure of CTX A3 contains five β-sheets comprising residues 2–4 (β1), 11–13 (β2), 20–26 (β3), 35–39 (β4), and 49–54 (β5). The three functional loops are formed by residues 4–11 (L1), 26–35 (L2), and 39–49 (L3) (Fig. 1A). The Co structural overlay of the three molecules in the asymmetric unit (Fig. 4A) reveals that the most significant structural variation between monomers occurs at the tip of L2. Two monomeric (blue and magenta in Fig. 4A) L2s adopt similar conformations, with each bound to an SDS head group, whereas the L2 of the third CTX A3 molecule (green in Fig. 4A) has a conformation that closely resembles that of the molecule in solution determined by 1H NMR (yellow in Fig. 4A) (11, 30). This suggests that L2 undergoes a local and specific conformational change in the presence of SDS.

CTX A3 contains only two acidic residues, Asp-40 and Asp-57, of which the side chain of Asp-40 is exposed to the solvent, and the OD1 of Asp-57 hydrogen bond with the NH of Lys-2. The latter observation is consistent with fluorescence data generated from a study into the effect of the mutation of Asp-57 to Asn on the unfolding process of CTXs (31).

Interactions of a CTX A3 Monomer with SDS Molecules—In the current structure, 10 SDS molecules were found to interact with 3 CTX A3 monomers in the asymmetric unit. Although any of the positively charged amino acids distributed throughout the slightly curved surface of CTX A3 can potentially interact with the SDS sulfate head groups, those residing on or near the three loops are found to be involved in the CTX A3-SDS interactions (Figs. 4B and 5A). Interestingly, the toxin backbone of 2 amino acid triads, Lys-5-Leu-6—Val-7 and Thr-29—Pro-30—Lys-31, from the respective L1 and L2 are also important contributors in CTX A3-SDS interactions, as each adopts a conformation that wraps around the SDS sulfate moiety (see SDS 2 and 4 in Fig. 4B). Consistent with previous biochemical and NMR studies in aqueous solution, the mode of the CTX A3-SDS interaction observed in the crystal implies that the three loops, L1-L3, initiate the CTX A3-membrane interaction. However, the current structure provides a molecular model on how negatively charged lipids may interact with the positively charged amino acids flanking the three hydrophobic loops that enhance the CTX-membrane interactions.

Although all the three loops interact with SDS, they differ in the intensity of their interaction, with L2 providing the strongest interaction with SDS molecules and L3 the least. More importantly, the head groups of half of the total SDS population hydrogen bond with Lys-31 of L2 of the 3 CTX monomers, suggesting that Lys-31 is the most important residue for the toxin-membrane electrostatic interaction, even though it is not strictly conserved in all CTXs. In the absence of a recombinant CTX A3, it is difficult to verify whether Lys-31 has a critical role in the toxicity of CTX A3. Nevertheless, being the major component of the venom of the wild cobra implies that CTX A3 has a major role in the toxicity of the venom, thereby suggesting that Lys-31 may also be important for its specific activity.

Last, Pro-30, which is only present in P-type CTXs, enhances the CTX-membrane interaction (see below), with the Val-7—Pro-8 peptide bond of L1, similar to that of cytotoxin II of N. oxiama (12), having a trans conformation. Apart from polar interactions between CTX A3 and the SDS head groups, the
hydrophobic residues of CTX A3 make extensive interactions with the acyl moieties of SDS molecules, as discussed below. The most stable water molecule, WAT1, having the lowest B-factor (Fig. 4B), resides at the center of the ω-shaped tip of L2, where it hydrogen-bonds with the O of Thr-29 and Val-32 and the NH of Met-26. As shown previously by NMR studies and molecular dynamics studies, it plays a critical role in the stability of L2 (11). Several other stable water molecules (very low B-factors) can also be identified near the tip of three loops, most of which are involved in CTX A3-SDS interactions (Fig. 4B). Water, therefore, appears to have an important intermediary role in the CTX A3-membrane interactions (see below).

To gain an insight into the orientation on which CTX A3 initiates its interaction with the cell membrane, we overlaid the three CTX A3-SDS complexes in the asymmetric unit. This revealed that the toxin interacts with SDS in an edgewise manner (Fig. 5A). Remarkably, polarized ATR FTIR experiments of CTX A3 in the presence of the aligned negatively charged PG monolayer also indicate a similar toxin orientation (−48° ± 20°) relative to the model membrane (Fig. 5B). Because similar binding modes involving the three hydrophobic loops of CTX A3 have previously been suggested using solid state NMR (32) and solution NMR (14) studies of CTXs with zwitterionic PC membranes, our result implies that the initial peripheral binding mode of CTX A3 with negatively charged membranes probably remains similar with neutral phospholipid membranes. Interestingly, the 3 CTX A3 monomers in the asymmetric unit interact with 6, 7, and 7 SDS molecules, which is remarkably consistent with the result of the fluorescence studies (33), showing that every CTX molecule interacts with 7 singly negatively charge phospholipids. In summary, all the available spectroscopic data suggest that CTX A3, via its three loops, interacts initially with surfaces of both neutral- and negatively charged membranes in an edgewise manner. But what remains to be addressed is how such an interaction leads to the final formation of a toxin pore or inverted micelles fusion intermediates near the membrane surfaces.

**Dimers**—In the crystal, the CTX A3 molecules interact with one another and form two types of dimers, D1 and D2 (or D2′). D1 (Fig. 6A) is formed by interactions of residues residing on the three loops; it buries 105-Å² hydrophobic and 890-Å² hydrophobic interactions using a probe radius of 1.5 Å. By comparison, D2 (Fig. 6B) or D2′ (Fig. 6C), which is situated in a different SDS environment than that of D1, buries 297- and 617-Å² hydrophilic and hydrophobic interactions, respectively. The interface of D2 or D2′ is mainly contributed by the residues

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**Fig. 4.** Structural overlay of the NMR and crystal structures of CTX A3 and stereo view of the CTX A3-SDS complex. A, structural overlay of the three CTX A3 monomers (magenta, green, blue) in the asymmetric unit with that of 1H NMR one (yellow) (accession code 1I02). DINO (www.dino3d.org.) was used for drawing Figs. 4, 5, 6, A–C, 8, and 9. B, stereo ribbon diagram of a CTX A3 monomer, 6 SDS, and 4 water molecules showing direct or water-mediated interactions of CTX A3 with the SDS molecules. The SDS molecules are represented as ball-and-stick models. The 2Fo − Fc electron density map in light blue mesh was generated after omitting the six SDS molecules and contoured at 1σ.

**Fig. 5.** Orientation of CTX A3 relative to SDS and negatively charged monolayer. A, overlay of the three CTX A3 monomers and their interacting SDS molecules in the asymmetric unit. CTX A3 interacts with the head groups of the SDS molecules at an angle of ~45°. B, the orientation of CTX A3 with respect to the negatively charged monolayer is depicted in panel B. Lipid interface is on the x-y plane, and the N terminus of CTX A3 faces the bulk phase. The slightly curved CTX A3 molecule is shown to bind to the membrane surface with an angle of ~48° ± 20°, as measured between the plane of CTX A3 and that normal to the monolayer.

forming β5 (Figs. 1A, 6B, and 6C).

Because interfaces of biologically relevant dimers usually bury 1400 Å² (34, 35), it is unlikely that D1 and D2 can form in solution, as evidenced by NMR studies showing the existence of only mono- meric species (11, 30). Conversely, both dimers are likely to form when either SDS molecules or model membranes are present in the solution. As shown in Fig. 6D, six SDS molecules involving hydrophilic and hydrophobic interactions at the D1 interface bury another 1000-Å² surface area. Similarly, the participation of 6 SDS molecules at the D2 interface, which contribute another 1000-Å² surface area, results in the further stabilization of D2 (Figs. 6D). The interactions of the SDS molecules with the interfaces of D1 and D2 are reminiscent of those of detergents crystallized with membrane proteins such as bacteriorhodopsin (36), as the acyl groups of several of them reside in the grooves of the toxin dimers.

The next obvious issue is whether CTX A3 forms D1 and D2 dimers in model membranes. In the D1 dimer, the side chains of Met-24 and Met-26 of each monomer forming D1 are clustered in close proximity to each other (Figs. 6A). These residues have been implicated in CTX-membrane interactions (14) and cytotoxicity (19). The side chains of Met-26 and Met-24, respectively, bury ~50- and ~25 Å² at the dimer interface, suggestive of their important role in the D1 formation. To test whether methionines are important for the membrane-active property of CTX A3, we performed fluorescence experiments to check the effect of chemical modification on CTX A3-induced leakage of
6-CF trapped in large unilamellar vesicles. Whereas the chemical modification of Met-24 with iodoacetamide caused a ~40% reduction in 6-CF leakage compared with wild type CTX A3, that of Met-26 reduced it by 80%. The subsequent modification of both Met-24 and Met-26 diminished it by ~95% (Fig. 7A). By contrast, CTX A5, which lacks methionine residues at the corresponding positions, did not cause any leakage (Fig. 7A).

In contrast to D1, the D2 and D2' are formed by the two β5 strands. To verify whether D2 (or D2') occurs in the presence of detergents or lipids, we thus performed FTIR experiments on CTX A3 in the presence of PG and PA vesicles. Consistent with previous FTIR studies (15), the β-sheet content of CTX A3 was enhanced upon toxin interaction with negatively charged lipids (Fig. 7B). The increase in the β-sheet content of CTX A3 is because of the alignment of β5 of each interacting monomer forming D2 (Fig. 6B). Previously, this result was interpreted as
after the addition of each toxin. CTX A5.

Alignment of two highly rigid CTX A3; that is, the formation of D2 by the parallel alignment of two membranes may be promoted by the formation of D2 dimer. A ribbon diagram of the four interconnecting CTX A3 molecules, of which blue and green represent D2, green and magenta represent D1, and magenta and light blue represent D2, are also shown. The unit cell and its axes are shown with yellow lines. B, view is parallel to the α-c plane of the crystal, showing the packing of four quasi-micelles of the SDS molecules depicted as ball-and-stick models. Three molecules of CTX A3 forming a D2 (light blue and magenta) and a D1 (magenta and green) are represented as ribbon diagrams. A schematic diagram is shown to suggest that inverted micelles or the presumed fusion intermediates can be formed by the interaction of D2 dimer with neighboring SDS molecules.

Fig. 7. Vesicle leakage and ATR FTIR study of CTX A3 and CTX A5. A, the leakage of PS vesicles is plotted as a function of time after the addition of each toxin. Black line, native CTX A3; blue line, Met-24 modified CTX A3; green line, Met-26 modified CTX A3; cyan line, Met-24 and Met-26 modified CTX A3; red line, CTX A5. Concentrations of CTXs and vesicle were 0.08 and 5 μM, respectively. B, Fourier self-deconvolution FTIR spectra (enhanced factor K = 2.2 and σ = 14 Å cm⁻¹) in the amide I region of CTX A3 (black line) in the absence and presence of PG (red line) and PA (green line).

Fig. 8. Two views of the packing of SDS molecules in the crystal suggest a structural model for the involvement of CTX dimer in the CTX-induced vesicle aggregation and fusion intermediate. A, view along the b axis of the crystal, revealing the arrangements of the SDS molecules in three layers. The schematic diagram is shown to suggest that the aggregation of two membranes may be promoted by the formation of D2' dimer. A ribbon diagram of the four interconnecting CTX A3 molecules, of which blue and green represent D2', green and magenta represent D1', and magenta and light blue represent D2', are also shown. The unit cell and its axes are shown with yellow lines. B, view is parallel to the α-c plane of the crystal, showing the packing of four quasi-micelles of the SDS molecules depicted as ball-and-stick models. Three molecules of CTX A3 forming a D2 (light blue and magenta) and a D1 (magenta and green) are represented as ribbon diagrams. A schematic diagram is shown to suggest that inverted micelles or the presumed fusion intermediates can be formed by the interaction of D2 dimer with neighboring SDS molecules.

a lipid-induced conformational change of the CTX A3 monomer (14, 15). However, there is no detectable change in the β-sheet content of the CTX A3 monomer obtained from the solution NMR and the one crystallized in the presence of SDS (Fig. 4A). Moreover, CTXs are highly stable polypeptides, as they retain most of their biological activities after boiling (37). It is, therefore, implausible that CTX A3 could undergo a conformation change that could result in an enhancement of β-sheet content. It is also unlikely for L2, which is the most flexible CTX loop, to cause an increase in β-sheet content, since with the aid of WAT1 it retains its α-shaped conformation (Fig. 4, A and B), both in the presence of the model membrane in the solution (14) and the SDS molecules in the crystal. The current crystal structure, therefore, provides an alternative explanation for the significant increase in the β-sheet content (Fig. 7B) of the highly rigid CTX A3; that is, the formation of D2 by the parallel alignment of two β5 strands. In contrast to CTX A3, there is no detectable increase in the β-sheet content for CTX A5 (data not shown).

Interestingly, we have recently solved the crystal structure of CTX A5 in complex with SDS.² In the crystal, the CTX A5 molecules form only one type of dimer that resembles the one crystallized in the absence of SDS (10), at the interface of which β5 and L3 of each interacting monomer are involved in hydrophilic and hydrophobic interactions. Because the β5 of each

² J.-H. Liu, K.-Y. Chien, W.-g. Wu, and C.-D. Hsiao, unpublished results.
(Figs. 6C and 8A). This structural evidence strongly suggests that the SDS molecules caused the tight packing of the toxin molecules within the layers, because the packing of solely D2 dimers between the layers is loose and has a significantly higher level of solvent content as compared with the SDS-rich regions (Fig. 8A). More interestingly, as indicated in the schematic diagram of Fig. 8, the structure suggests how the formation of D2 dimer may promote the membrane aggregation.

Oligomer—The significance of D1 can now be understood not only in its L2 coupling of each interacting monomer but also in its mediation between two crystallographically independent D2s (D2 and D2') in the formation of the CTX A3 “tetramer” (Figs. 8A and 9A) in terms of CTX A3 oligomerization. This observation in the crystal is consistent with the biophysical data (Fig. 2A), indicating that CTX A3 forms an oligomer (>dimer) in the presence of negatively charged lipids. Alternatively, the interactions of the four CTX A3 molecules constituting a D1, D2, and D2' within and between SDS layers (Fig. 8A) may represent the building blocks for CTX A3 oligomerization and eventual transient pore formation of CTX A3. Interestingly, the crystal packing also shows the pore-like structure formed by D1, D2, and D2' (Fig. 9B). Due to the demonstrated interaction of anionic lipid with D1 and D2, the putative pore can be seated into lipid bilayer if D2' is further converted into D2 by binding to more SDS molecules.

A Model for the CTX A3-Membrane Interaction—Based on the existence of 3–4 interacting CTX A3 monomers in the crystal, we propose the following chain of events taking place when CTX A3 interacts with the cell membrane with anionic phospholipids. (a) For the CTX-induced aggregation and fusion of anionic lipid vesicles such as cardiolipin, the initial contact of the L1-L3 loops with the negatively charged head group followed by the D2' dimer formation induces aggregation of vesicles. After the formation of D2 dimer through the binding of additional lipids to D2' dimer, D2 dimer may rearrange to allow the formation of inverted micelles as fusion intermediate. (b) For the CTX-induced pore formation of anionic lipid membrane such as phosphatidylserine, the initial contact of the L1-L3 loops with the negatively charged head groups of the cell membrane facilitates the formation of D1 (Fig. 6A), leading to possible destabilization of the membrane. This process is further intensified by interactions of the hydrophobic residues, notably Leu-6 and Pro-8 from L1, Ala-28, Pro-30, and Val-32 from L2, and Leu-47 and Leu-48 from L3, with acyl moieties of the phospholipids (Fig. 6D). The four-methionine cluster (Met-24 and Met-26 from each monomer) in the internal groove of D1 helps to facilitate the toxin-membrane interaction. At this point, D2 together with D1 could lead to CTX A3 oligomerization and a pore formation (Fig. 9B). However, unlike pore structures observed by pore-forming toxins (38), the architecture of CTX A3 does not suggest that the toxin molecules can make up a stable one. Nonetheless, there is evidence of CTX A3-induced pore formation in negatively charged liposomes (this work) and in bullfrog atrial myocytes (39), which is non-specific and reversible, lasting a few seconds.

In summary, our structural and biophysical data provide a mechanism for CTX A3 oligomerization via anionic lipid-induced dimer formation, which likely leads to the formation of a pore or inverted micelles, depending on the type of negatively charged phospholipid membranes used for the study. The approach also underscores the importance of the SDS-protein co-crystallization, from which the present information concerning CTX A3-membrane interaction has been elicited.

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