Potential Prepore Trimer Formation by the *Bacillus thuringiensis* Mosquito-specific Toxin

**MOLECULAR INSIGHTS INTO A CRITICAL PREREQUISITE OF MEMBRANE-BOUND MONOMERS**

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The insecticidal feature of the three-domain Cry δ-endotoxins from *Bacillus thuringiensis* is generally attributed to their capability to form oligomeric pores, causing lysis of target larval midgut cells. However, the molecular description of their oligomerization process has not been clearly defined. Here a stable prepore of the 65-kDa trypsin-activated Cry4Ba mosquito-specific toxin was established through membrane-mimetic environments by forming an ~200-kDa octyl-β-D-glucoside micelle-induced trimer. The SDS-resistant trimer caused cytolsis to S/9 insect cells expressing Aedes-mALP (a Cry4Ba receptor) and was more effective than a toxin monomer in membrane perturbation of calcein-loaded liposomes. A three-dimensional model of toxin trimer obtained by negative-stain EM in combination with single-particle reconstruction at ~5 nm resolution showed a propeller-shaped structure with 3-fold symmetry. Fitting the three-dimensional reconstructed EM map with a 100-ns molecular dynamics-simulated Cry4Ba structure interacting with an octyl-β-D-glucoside micelle showed relative positioning of individual domains in the context of the trimeric complex with a major protrusion from the pore-forming domain. Moreover, high-speed atomic force microscopy imaging at nanometer resolution and a subsecond frame rate demonstrated conformational transitions from a propeller-like to a globularly shaped trimer upon lipid membrane interactions, implying prepore-to-pore conversion. Real-time trimeric arrangement of monomers associated with 1-α-dimyristoylphosphatidylcholine/3-{[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid bicelle membranes was also envisaged by successive high-speed atomic force microscopy imaging, depicting interactions among three individual subunits toward trimer formation. Together, our data provide the first pivotal insights into the structural requirement of membrane-induced conformational changes of Cry4Ba toxin monomers for the molecular assembly of a prepore trimer capable of inserting into target membranes to generate a lytic pore.

**Background:** The molecular description of oligomeric pore formation by *B. thuringiensis* insecticidal toxins remains unclear.

**Results:** Cry4Ba mosquito-active toxins assemble into a stable prepore trimer upon interaction with non-ionic micelles or lipid membranes.

**Conclusion:** A membrane-bound state of monomers is required for facilitating a potential trimer assembly.

**Significance:** This study reveals a requirement of membrane-bound monomers for forming a prepore trimer capable of perturbing target membranes.

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been shown that the activated toxins bind specifically to various types of receptors lining the apical brush-border membrane of target midgut cells (5). For instance, in our previous works, two different glycosylphosphatidylinositol-anchored proteins, i.e. glycosylphosphatidylinositol-anchored alkaline phosphatase (ALP) and glycosylphosphatidylinositol-anchored aminopeptidase N, were identified as potential receptors mediating Cry4Ba toxicity in *Aedes* mosquito larvae (6–8). The toxin-receptor interactions are believed to facilitate toxin insertion into the target cell membrane to form ion leakage pores, resulting in osmotic cell lysis and, eventually, death of the insect larvae (1, 4, 5). However, the exact mechanisms underlying the toxicity exerted by these insecticidal *Bt*-Cry toxins still remain to be explored.

Crystal structures of almost all major specificity classes of the *Bt*-Cry toxins, including the Cry4Ba mosquito-specific toxin, reveal a strong overall structural resemblance suggestive of the same general mechanism of toxicity (2, 9, 10). All individual structures display a wedge-shaped form (estimated dimensions, 55 × 65 × 75 Å) with three structurally distinctive domains (DI–III): from the N to the C terminus, an α-helical bundle (DI), a β sheet prism (DII), and a β sheet sandwich (DIII). Unlike DIII, whose function is still ambiguous, both DI and DII have been clearly defined regarding membrane pore formation and receptor recognition, respectively (2, 4, 9). In our earlier studies, the role of Cry4Ba-DI in membrane-inserted pore formation has been studied intensively. Of particular interest, we have strengthened the proposed “umbrella-like” mechanistic model (2, 11) by providing direct evidence for liposomal membrane-perturbing activity of the purified Cry4Ba pore-forming fragment, i.e. α4-loop–α5 hairpin (12). Additionally, one highly conserved residue, Asn183, located in the middle of the transmembrane α5, has been found to play an important role in Cry4Ba toxicity and is essentially involved in toxin-pore oligomerization (13). Moreover, two highly conserved aromatic residues (Tyr249 and Phe264) of Cry4Ba-α7 have been found to play a vital role in toxin-membrane interactions, conceivably needed for lipid-induced conformational transitions prior to an efficient insertion of the transmembrane α4-loop–α5 hairpin into the lipid bilayers (14, 15). More recently, we have demonstrated that the polarity of the Cry4Ba α4–α5 loop residue Asn166 is an important element for ion permeation through the toxin-induced pore (16). We have also verified the functional changes in cell morphology under a light microscope. Cell via- 

**Experimental Procedures**

**Toxin Preparations**—The 130-kDa Cry4Ba-R203Q mutant protoxin in which one trypsin cleavage site at Arg203 was removed, therefore producing a 65-kDa active fragment upon trypsin digestion and retaining high *Aedes* larval toxicity (25), was overexpressed as cytoplasmic inclusions in *Esherichia coli* JM109 upon isopropyl-β-d-galactopyranoside induction. Toxin activation was done by digesting the protoxin presolubilized in carbonate buffer (50 mM Na2CO3/NaHCO3 (pH 9.0)) with trypsin (1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, Sigma) as described elsewhere (25). The 65-kDa activated toxin was purified via FPLC using Superdex 200 or Suprose 12 HR10/30 and concentrated to 1–5 mg/ml by ultrafiltration. Protein concentrations were determined by Bradford assay.

**Toxin Oligomerization Assays**—Purified toxins (0.5–1.0 mg/ml) in carbonate buffer (pH 9.0) were incubated with and without OG micelles under different conditions (critical micelle concentrations (1×, 5×, and 10× CMC corresponding to 0.6, 3, and 6% w/v, respectively), incubation times (1 and 24 h), and temperatures (4, 25, and 37 °C) and then analyzed on a seminative PAGE system containing SDS only in gel and running buffers. Samples were prepared in loading dye buffer (lacking SDS or containing 0.2–2% SDS in certain experiments) with and without 25 mM DTT under various heat treatment conditions (none, 60, and 95 °C) for 10 min.

**Detergent Concentration Determination**—An OG concentration in elution fractions collected during trimer purification was determined by measuring glucoside content using a sensitive phenol/sulfuric acid assay (26).

**Cytotoxicity Assays**—The FPLC-purified toxin monomer or trimer was tested against S/9 insect cells (8 × 10⁴ cells/ml in a 24-well plate) expressing recombinant *Aedes aegypti* membrane-bound ALP (Aedes-mALP) (6) or chloramphenicol acetyltransferase (CAT). Cell susceptibility was monitored for changes in cell morphology under a light microscope. Cell viability was assessed with the PrestoBlue™ fluorescent-based assay (Life Technologies). Statistical analysis was performed via the t test function of Microsoft Excel to determine the level of significance.

**Membrane Perturbation Assays**—Calcein-entrapped large unilamellar vesicles (LUVs, ~80 nm in mean diameter) were prepared from a lipid mixture (Avanti Polar Lipid) of phos-
Trimerization of Mosquito-active Toxin

It has been documented that Cry-oligomer formation in vitro by incubating toxin monomers, either with receptor-free liposomes (13, 20), with brush-border membrane vesicles containing a specific toxin-receptor (18, 23), or even with a receptor fragment (18, 38), suggesting an involve-

Modeling of the toxin to lipid vesicles was performed in 400 μl of carbonate buffer (pH 9.0) containing LUVs (~1 or ~10 nmol). Amounts of toxin monomer (~0.04, ~0.15, ~0.45, or ~0.75 nmol) or trimer (~0.04 nmol) were incubated in the LUV suspension for 30 min at room temperature and then centrifuged at 18,000 × g for 20 min. The toxins present in the pellet fractions were analyzed by SDS-PAGE. Viscosity aggregation assays were carried out in a quartz microcuvette with a 1-cm light path as described previously (28) with some modifications. Turbidity changes upon toxin-induced aggregation were followed continuously for 30 min at 550 nm using 800 μl of carbonate buffer (pH 9.0) pre-equilibrated with LUVs (2 or 20 nmol) for 1 min prior to toxin addition (0.15- to 1.4-nmol monomers or 0.05- to 0.45-nmol trimers).

Negative-stain Electron Microscopy—An aliquot of the purified toxin oligomer solution (~1 μg/ml) in 20 mM Tris-HCl buffer (pH 8.0) was placed on a carbon film-coated 300-mesh copper grid which had been glow-discharged. The toxin sample on the grid was negatively stained with 2% (w/v) uranyl acetate solution and imaged using a Tecnai G2 transmission electron microscope equipped with a Twin lens operating at 120 kV. Pairs of images of the same specimen area taken at 0° and 45° tilt angles were recorded with a GATAN 794 format (2048 × 2048 pixels) at ×62,000 magnification.

Image Analysis and Three-dimensional Reconstruction—Micrographs were processed with the EMAN2 software package (29). To obtain class averages and initial two-dimensional projections, particles with a three-bladed shape from the negative-stain images were selected, in windows of 128 × 128 pixels (~220 × 220 Å), from the untilted micrographs using the Boxer program in the EMAN2 package. The particles were subsequently subjected to rotational and translational alignments and classified without symmetry enforcement, and then we performed 10 cycles of average iterations. For single-particle three-dimensional reconstruction, pairs of particles were selected simultaneously from the untitled and 45° tilted images, windowed, and used for reconstruction of the initial model with an imposed symmetry of C3. The refined three-dimensional model was obtained after 10 iterations of local refinement until convergence. The resolution of the three-dimensional modeled structure was analyzed using the Fourier shell correlation (0.5 criterion) (30), and the final three-dimensional map was low pass-filtered at 3 nm using Chimera software (31).

Molecular Dynamics (MD) Simulations and Model Fitting—All-atom MD simulations of the Cry4Ba monomeric structure (PDB code 4MOA) interacting with an OG micelle were performed for 100 ns. An OG molecule was modeled via the ParamChem server (32), and a spherical micelle consisting of 80 OG molecules was constructed via the Visual Molecular Dynamics program (33). The final system (95,150 atoms), which included the Cry4Ba monomer interacting with an equilibrated OG micelle in a 150 mM KCl solution box (7.7 × 9.4 × 13.9 nm), was energy-minimized, heated to 300 K, equilibrated at 1 atm, and finally run as unrestrained MD simulations for 100 ns via Nanoscale Molecular Dynamics program (34). Analyses of MD trajectories were done via the Visual Molecular Dynamics program. Fitting of the 100-ns MD-simulated structure into the three-dimensional reconstructed EM trimer model was done using Chimera software (31).

Toxin Adsorption on a Mica Surface—Purified toxin monomers and/or oligomers (100–350 nm) were deposited onto a 3-aminopropyltriethoxysilane-modified mica surface that was prepared by incubation of the freshly cleaved mica surface with 0.01% 3-aminopropyltriethoxysilane, and excess toxins were removed with washing by 50 mM carbonate buffer (pH 9.0) before HS-AFM imaging.

Toxin Reconstitution in Bicelle Vesicles—Bicelles were prepared from the lipid/detergent mixture of DMPC/CHAPSO at a 3:1 molar ratio following the method described previously (35). The mixture of bicelle vesicles (~0.3 mg) and toxin (0.2–1.0 mg) was incubated on ice for various incubation times prior to deposition on a freshly cleaved mica surface to obtain membrane-bound toxins on mica-supported lipid bilayers. For membrane binding studies, toxin monomers or trimers (~1 pmol) were added onto the prepared mica-supported lipid bilayers and incubated for 30 min, and then unbound toxins were washed out before HS-AFM imaging.

High-Speed AFM Imaging—HS-AFM measurements were performed under 50 mM carbonate buffer (pH 9.0) at room temperature using the laboratory-built high-speed AFM apparatus (36). Detailed descriptions on the HS-AFM setup have been given previously (37). Miniature cantilevers (Olympus) used for imaging that were fabricated to contain an electron-beam deposition tip with an apex radius of 4–5 nm (37) have a spring constant of 0.1–0.2 N/m and a resonant frequency of ~1 MHz in water. The setpoint imaging amplitude was ~95% of the free oscillation amplitude. The imaging rate varied from 1–10 frames/s depending on the scan ranges.

Results

Micelle-induced Trimerization of the 65-kDa Cry4Ba Active Toxin—It has been documented that Cry-oligomer formation can be induced in vitro by incubating toxin monomers, either with receptor-free liposomes (13, 20), with brush-border membrane vesicles containing a specific toxin-receptor (18, 23), or even with a receptor fragment (18, 38), suggesting an involve-
Trimerization of Mosquito-active Toxin

A

(i) OG-CMC

(ii) Incu. Temp.

(iii) SDS Conc.

(iv) Heat Treatment

B

(i) AU

(ii) 1 1 1 3

(iii) 0.2

(v) Elution volume (ml)

200

65

FIGURE 1. Micelle-induced Cry4Ba trimerization. A, seminative PAGE (Coomassie Brilliant Blue-stained 10% gel) of the 65-kDa purified Cry4Ba toxin incubated (incu) with OG micelles for 1 h under different conditions (i–iv). B, size-exclusion FPLC chromatograms of the purified Cry4Ba toxin after incubation at 37 °C for 2 h in the absence (i) or presence (ii) of 10× CMC-OG micelles. Inset, seminative PAGE (10% gel) of protein fractions collected from each peak as indicated. M, broad-range protein markers; AU, arbitrary units.

The trimeric complex can function as a prepore, and the resulting parameters obtained from its logarithmic dose-response curve in Fig. 2 corresponding to the number of cell deaths, a cell viability fluorescence assay was performed. The cell mortality of Aedes-mALP-transfected cells when exposed to Cry4Ba monomers or trimers (Fig. 2A, i and ii) or trimers (Fig. 2A, iii and iv). Additionally, to quantify the number of cell deaths, a cell viability fluorescence assay was performed. The cell mortality of Aedes-mALP-transfected cells was found to be considerably higher than that of CAT-transfected cells when exposed to Cry4Ba monomers (~31% versus ~12%) or trimers (~34% versus ~19%) (Fig. 2B).

Further assessment to determine the ability of the FPLC-purified Cry4Ba trimer to perturb receptor-free LUVs in comparison with the toxin monomer was also carried out via encapsulated calcein release assays. The release activity of both trimers and monomers against the calcein-loaded LUVs was initially evaluated at different toxin concentrations, and concentration versus release activity profiles were generated. Each dose-response curve in Fig. 2C was fitted with the Hill equation, and the resulting parameters obtained from its logarithmic expression, log Y = n log [T] − log EC50 (Fig. 2C, insets), were ~0.14 and ~1.36 μM for EC50 with Hill coefficients (n) of ~1 for trimers and monomers, respectively. Accordingly, the purified trimer was much more effective for membrane permeability of the receptor-free LUVs than the toxin monomer (~10 times at the EC50 concentration). In addition, the initial rate of calcein leakage produced during the first 20 s after addition of the toxin trimer was found to be much faster than that of the monomer at the same toxin concentration (Fig. 2D). Interest-

ment of either toxin-membrane or toxin-receptor interactions in the Cry-oligomer assembly. Here the notion of toxin-membrane interactions in promoting toxin oligomerization was tackled by using non-ionic OG micelles as a membrane-mimetic environment. The 65-kDa Cry4Ba purified toxin was incubated with OG micelles under different conditions, i.e., varying temperatures and CMCs, prior to determination of the formation of an oligomeric complex by the use of modified SDS-PAGE (a seminative system containing SDS only in gel and running buffers and no boiling of samples) (Fig. 1A) along with size exclusion chromatography (Fig. 1B).

When the 65-kDa purified toxin was incubated with OG micelles, an oligomer with an apparent relative molecular mass of ~200 kDa was observed in both systems used (Fig. 1A, i and ii). Therefore, the ~200 kDa species could represent a trimeric form of Cry4Ba toxin, with a persisting quaternary structure under the conditions used for the modified SDS-PAGE (exposure to 0.1% SDS in the gel and electrode buffers). It is noteworthy that OG micelles at 10× CMC (6% w/v or 200 mM) gave the highest yield trimer when compared with that at 1× or 5× CMC (Fig. 1A, i). Conversely, incubation of the purified toxin in the absence of OG micelles showed no detectable form of the 200-kDa trimer (Fig. 1A, i, and B, i), an excess toxin

monomer (5 mg/ml) has been incubated for 7 days at either 4, 25, or 37 °C. It should be also noted that this micelle-induced trimerization (at 10× CMC-OG) did not seem to be affected by the three different incubation temperatures used (4, 25, and 37 °C) because a comparable high yield of the trimer was obtained (Fig. 1A, ii). In addition, this 200-kDa OG micelle-induced trimer can be FPLC-purified as a highly stable trimer (Fig. 1B, iii) that was found to be relatively free from OG micelles (data not shown). Together, these results indicate that a lipid membrane-like environment is required for facilitating the trimer assembly of the Cry4Ba toxin.

To further determine what could be a key driving force in facilitating intermolecular interactions for the Cry4Ba-trimer assembly, effects of reducing agent (DTT), strong ionic detergent (SDS), or high temperatures on the trimer dissociation were examined. Under the conditions used for sample loading buffer, the 200-kDa trimer was still observed in the presence of DTT (Fig. 1A, iii and iv) and did not disappear upon treatment with SDS (up to 2%, Fig. 1A, iii). Moreover, the trimeric band remained detectable after exposure to temperatures up to 60 °C for 10 min but disappeared completely when heated at 95 °C (Fig. 1A, iv).

Perturbation Effects of the Trimeric Prepore on Cell and Liposomal Membranes—To determine whether the SDS-resistant trimeric complex can function as a prepore, S9 recombinant cells expressing Aedes-mALP, a potential receptor for Cry4Ba toxin-mediated cytolyis (6), were used for the assessment of cytolytic activity of the FPLC-purified trimer. The results, after 3-h incubation with either the monomers or trimers (100 μg/ml each), showed that the Aedes-mALP-transfected cells underwent morphological changes and subsequent cell lysis, as can be seen by the swelling of the cells (Fig. 2A, i and ii). On the contrary, the morphology appeared normal in mock-transfected control cells (expressing CAT) incubated with either Cry4Ba monomers or trimers (Fig. 2A, iii and iv). Additionally, to quantify the number of cell deaths, a cell viability fluorescence assay was performed. The cell mortality of Aedes-mALP-transfected cells was found to be considerably higher than that of CAT-transfected cells when exposed to Cry4Ba monomers (~31% versus ~12%) or trimers (~34% versus ~19%) (Fig. 2B).
ingly, this potential prepore trimer also exhibited faster kinetics of the dye release activity than the monomer at a 10-fold difference in toxin concentrations (0.1 μM trimer versus 1.0 μM monomer). On the other hand, the control had no effect on dye leakage (Fig. 2D), indicating that the leakage of entrapped calcein did not arise from instability of the LUV membrane. It is worth mentioning that neither the monomeric nor the trimeric form were able to induce LUV aggregation (data not shown), implying that membrane perturbation by both the monomer and trimer is unlikely to be aggregation-dependent.

Attempts were also made to determine the membrane-binding capability of the prepore trimer in comparison with its monomer via toxin-LUV cosedimentation and HS-AFM imaging of membrane-bound toxins. The results from cosedimentation assays demonstrated that toxin trimers (0.1 μM) can be effectively pulled down to comparable levels by LUVs at both final lipid concentrations (2.5 and 25 μM) (Fig. 2E, lanes 5 and 7). Conversely, toxin monomers (0.1 or 0.4 μM) were inefficiently cosedimented with LUVs at lipid concentrations of 2.5 μM (Fig. 2E, lanes 1 and 2). Additionally, much higher monomer concentrations (1.2 or 2.0 μM) were required for cosedimentation with LUVs compared with the preformed trimer (Fig. 2E, lanes 3, 4, and 6). Similar results were observed by means of HS-AFM imaging, which showed that the number of trimers bound on the mica-supported bicelle membrane is greater than that observed for the membrane-bound monomeric molecules (Fig. 2F).

**FIGURE 2.** Effects of the Cry4Ba monomer and its trimer on insect cell and liposomal membranes. A, light microscopic appearance of Sf9 insect cells expressing Aedes-mALP or CAT (a mock-transfected control). i and ii show Aedes-mALP-transfected cells whereas iii and iv show mock-transfected cells after treatment with the toxin monomer and trimer (~100 μg/ml each) for 3 h, respectively. B, cell mortality (percent) determined from A as assessed by PrestoBlue™ viability assay. Error bars denote mean ± S.E. from three independent experiments, each performed in triplicates. The level of statistical significance was assigned at p < 0.01 compared between Aedes-mALP- and CAT-expressed cells. C, dose response curve for membrane permeability of calcein-loaded LUVs induced by toxin monomer (i) or trimer (ii). Insets, the plot of fractional release activity, Y (P(T)/1 − P(R)), versus toxin concentration, [T]. Error bars indicate mean ± S.E. from two independent experiments where each toxin concentration was performed in triplicate. D, traces represent fluorescence intensity as function of time t after 10 min-incubation with individually tested toxins (black arrow), trimers (T) at 0.1 and 0.2 μM, and monomers (M) at 0.2 and 1.0 μM. Carbonate buffer (pH 9.0) was used as a negative control. E, SDS-PAGE (Coomassie Brilliant Blue-stained 10% gel) of toxin monomers (0.1, 0.4, 1.2, or 2.0 μM) or trimers (0.1 μM) cosedimented with LUVs (2.5 or 25 μM). Pellet samples prepared in loading dye buffer containing 0.2% SDS and 25 mM DTT were heated at 95 °C for 10 min prior to analysis. Lanes 1–4 and 6 represent the 65-kDa monomers from starting toxins of 0.1, 0.4, 1.2, and 2.0 μM, respectively. Lanes 5 and 7 represent a 65-kDa dissociated form of trimers upon denaturing conditions. M, broad-range protein markers. F, HS-AFM images of the membrane-bound monomers (i) or trimers (ii) on a mica-supported DMPC/CHAPSO bicelle membrane. Membrane-bound monomeric and trimeric molecules are indicated by red and blue circles, respectively.
Trimerization of Mosquito-active Toxin

Three-dimensional Reconstructed Model of the Cry4Ba Prepore—For structural analysis of the Cry4Ba prepore trimeric complex, negative-stain EM in combination with single-particle analysis was performed. In negative-stain EM images, the FPLC-purified trimer samples were relatively monodispersed in size (15–20 nm), with a clear indication of a three-bladed shape of the particles (Fig. 3A). Preliminary two-dimensional reconstructions of 901100 particles resulted in a convincing trimeric structure that, in the absence of enforced symmetry, displayed a clear three-subunit arrangement with 3-fold rotational symmetry (Fig. 3A, inset).

To calculate three-dimensional maps, we recorded image pairs at the same location of the specimens at 45° tilt and 0° by which 509 pairs of tilted and untilted particles were classified into 30 classes. An initial three-dimensional prepore model was built from selected representative classes with a merged volume of 3.27 × 105 Å3. The resolution of the final three-dimensional reconstructed prepore structure was calculated at ~50 Å (Fig. 3B) using the Fourier shell correlation 0.5 criterion. The trimeric prepore clearly displays a propeller-like shape with three distinctive blades, having a diameter of ~12 nm at its broadest point in the top half and ~3 nm at its narrowest toward the bottom, forming a protruding core (Fig. 3C). The entire prepore complex is ~8 nm in height. The blades as viewed from the top have a diameter of ~4 nm (Fig. 3C). The reconstructed volume is ~275 kDa in mass, as calculated with an average protein density of 0.84 Da/Å3 (39). This corresponds to ~1.4 times the expected molecular mass of the trimeric volume of the Cry4Ba prepore (3 × 67.5 = 202 kDa). The larger size might be due to effects from the negative stain grains, as suggested previously by other EM studies (40).

Attempts were also made to determine the domain organization of individual subunits within the trimeric complex by MD simulations and model fitting. When three modeled monomers of the 65-kDa full-length Cry4Ba atomic structure interacting with an OG micelle from 100-ns MD simulations in OG micelles were docked into the EM density map. The yellow circles denote locations of Cys27 in DI and Cys525 in DIII (left). DII and DIII of individual monomers are surface-represented (right).

FIGURE 3. Single-particle EM of the Cry4Ba prepore trimer. A, typical view from a raw image of negatively stained Cry4Ba trimers. Manually selected subsets of individual trimer particles are highlighted with white circles. Inset, representative class averages and their corresponding two-dimensional (2D) projections without symmetry enforcement, revealing a trimeric organization. B, resolution determination of the final three-dimensional reconstruction of the Cry4Ba trimer. Fourier shell correlation (FSC) drops below 0.5 at ~ 50 Å resolution. C, surface-rendered three-dimensional reconstructions of the trimer complex with the 3-fold rotational symmetry axis oriented along the y direction. D, fitting of the atomic structure into the three-dimensional reconstructed EM map of the Cry4Ba trimer. Three copies (with different shades of colors) of the 65-kDa Cry4Ba crystal structure from 100-ns MD simulations in OG micelles were docked into the EM density map. The yellow circles denote locations of Cys27 in DI and Cys525 in DIII (left). DII and DIII of individual monomers are surface-represented (right).
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Molecular Organization and Conformational Transition of Preparite Trimer—HS-AFM with nanometer and subsecond spatiotemporal resolution (41) was also performed to directly visualize the molecular organization and conformational transition of the Cry4Ba prepre trimer on a 3-aminopropyltriethoxysilane-modified mica surface with cross-section profiles. The highest magnification image in B illustrates a propeller-like trimer. C, HS-AFM images of the membrane-bound trimer on a mica-supported DMPC/CHAPSO bicelle membrane with a cross-section profile. Inset, a high-magnification image showing an apparent globularly shaped trimer. D, time-lapse HS-AFM images of the membrane-bound trimer on a mica-supported bicelle membrane, showing structural dynamics followed at a scan rate of 12.5 frames/s.

Discussion

So far, the step at which the Bt-Cry oligomerization pathway might occur, either in solution (18, 23–24, 42) or in a membrane-bound state (13, 20), remains to be clearly elucidated. In this study, we demonstrated that no defined oligomeric complex was observed for the purified trypsin-activated Cry4Ba toxin in carbonate-based solution (toxin solubilization buffer (pH 9.0)), as analyzed via a seminative PAGE system and size exclusion chromatography. Therefore, these results indicate that the 65-kDa Cry4Ba monomer is unable to self-assemble to form an oligomeric complex in solution, implying a need of a particular driving force in promoting a molecular recognition between toxin monomers required for the oligomeric assembly.
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A

B

C

FIGURE 5. Trimeric arrangement of membrane-bound Cry4Ba toxin monomers. A, HS-AFM images of the membrane-bound monomers on a mica-supported DMPC/CHAPSO bicelle membrane with a cross-section profile. B and C, time-lapse HS-AFM images of two independent experiments captured at a scan rate of 1 frame/s, illustrating three membrane-bound monomers (encircled) in close proximity to one another for forming the trimer complex in a step-like process within ~10 s. Insets in C, enlarged regions with cross-section profiles.

In other words, a proper conformational transition of the toxin monomer could be a critical prerequisite for formation of the oligomeric pore complex.

We clearly demonstrated that the 65-kDa monomers were able to assemble into a ~200-kDa stable trimer upon incubation with non-ionic OG micelles. In addition, the amount of toxin trimer obtained appeared to be CMC-dependent, implying that a higher OG-CMC could perhaps provide a better membrane-like environment. It has been shown recently that the chymotrypsin-treated Cry4Ba toxin, upon interacting with lipid vesicles, was able to assemble into a ~250-kDa oligomer without the need of the Aedes-cadherin receptor fragment, rather dissimilar to Cry11Aa (another mosquito-active toxin from Bti) (38) and other lepidopteran active Cry1A toxins that require the cadherin-binding for oligomerization (18). Therefore, this could corroborate our findings that toxin-membrane interactions are most likely to be involved in the Cry4Ba-trimer assembly.

We also revealed that the Cry4Ba trimer assembly is not covalently mediated by intermolecular disulfide bonds and that the only two Cys residues (Cys27 in DI and Cys325 in DIII, Fig. 3D) are unlikely to be involved in Cry4Ba trimer formation. Moreover, this trimeric complex seems to be relatively stable at a moderately high temperature as well as resistant to SDS-induced dissociation. Although, at this stage, a detailed description of an SDS-resistant intermonomeric interface is still unclear, 200-kDa OG-induced trimer formation could likely be mediated by intermolecular non-covalent interactions. It has been suggested that multiple polar interactions, especially a network of hydrogen bonds and ionic interactions, appeared to display a key role in the stability of oligomer assemblies to the dissociation by SDS (43). We have demonstrated previously, via combined MD and continuum solvent studies, that the Cry4Aa toxin, which is very much related to Cry4Ba, could form a stable trimer in aqueous solution, as primarily attributed to the inter-subunit interactions through certain polar uncharged and charged residues in the pore-forming domain DI (44). We have also shown, via mutagenesis studies, that one highly conserved polar residue, Asn183, situated in DI-a5, exerts a vital role in Cry4Ba trimer prepore formation and, therefore, larvicidal activity (13). Therefore, a major driving force in facilitating the molecular recognitions between micelle-associated monomers required for trimer assembly could possibly be a network of H-bonds and ionic interactions.

Results from cytotoxicity assays indicated that the SDS-resistant Cry4Ba trimer obtained via OG micelle induction appeared to be a potential preformed trimer because it was able to interact with Aedes-mALP (a potential Cry4Ba receptor expressed on S9 cells) to exert cytolytic activity to an extent similar to that seen with the monomer. Although it is still unclear whether the similar cytotoxicity observed between trimers and monomers is due to the role of such a Cry4Ba receptor expressed on the target cells, the preformed trimer clearly induced membrane permeability of receptor-free LUVs and much more efficiently than the toxin monomer. Moreover, hyperbolic kinetics of calcein release can be observed with this potential prepore trimer even at a 10-fold lower concentration than the monomer, which, instead, displayed an apparent sigmoidal pattern containing an initial lag phase. Therefore, these findings corroborate that the OG micelle-induced trimer is indeed a potential prepore state that can readily incorporate into the lipid membrane, whereas the toxin monomers have to insert into the lipid membrane prior to assembly into a prepore/active pore trimer for inducing membrane permeability. Another possibility is that the membrane-interacting capability of the prepore trimer with the receptor-free LUVs is greater than that of the toxin monomer, which may, perhaps, require a specific docking molecule for promoting interactions with the membrane surface. In this context, the results from toxin-lipid interaction assays via toxin-LUV cosedimentation as well as HS-AFM imaging substantiated that the Cry4Ba preformed trimer indeed exhibits a much higher binding capability than the monomer toward artificial membranes, thereby acting faster to induce LUV permeability.
Previously, although two symmetrical trimeric conformations of the membrane-associated Cry4Ba complex, i.e. propeller-like and pinwheel-like, could be revealed by electron cryotomography, these two-dimensional data were inadequate to provide more insights into the molecular organization of the pore architecture (21). In this study, although the three-dimensional reconstructed model revealed only the propeller-like shape, its reconstructed EM map fitted with a 100-ns MD simulated Cry4Ba structure interacting with an OG micelle could give an idea about the relative positioning of individual domains in the context of the trimeric prepore complex. In line with several previous studies as well as the established mechanistic models for the membrane-bound state of Bt-Cry toxins, a defined hairpin structure of α4-loop-α5 within DI is conceivably required for membrane insertion and pore formation (3, 4, 9, 12). However, the fact that the hydrophobic faces of the outer amphipathic helices of DI face inwards, these three-domain Cry toxins must go through conformational changes, particularly within DI, to convert this pore-forming domain into a transmembrane pore in which the hydrophobic surfaces would be in close contact with the membrane lipids (3, 9). Accordingly, the individual protomeric subunits within the propeller-shaped trimer that shows an apparent protrusion from the pore-forming domain could be expected to have an overall conformation different from the water-soluble monomer.

One caveat in this study with the use of HS-AFM is that its images, particularly measured in the x-y plane, might contain some structural features that are not present on the sample in actuality because their measurements are often influenced by tip sample convolution effects. Even with such common artifacts arising in the particle image diameter, a significant increase observed in height (z axis) of the Cry4Ba trimer could still possibly be a sign of conformational differences between the monomer and each subunit in the trimeric propeller-shaped complex. Therefore, this suggests a change in toxin conformation upon trimeric organization. Regardless of the molecular details of this complex that could not be revealed at this stage, such a conformational change may perhaps occur through a protrusion of a transmembrane part, possibly either the α4-loop-α5 hairpin (11) or lipid-sensing α7 (15), from the pore-forming domain. However, our findings via HS-AFM were rather different from those of the previous AFM studies of Cry1Aa (45) and Cry4Ba (46) via a conventional tapping mode because a tetramer rather than a trimer has been proposed as the preferred oligomerization state of the membrane-associated pore complex, although there is no direct evidence for its functional relevance.

Moreover, our HS-AFM imaging at nanometer resolution and a subsecond frame rate clearly demonstrated conformational transitions from a propeller-like to globularly shaped trimer upon lipid membrane interactions, conceivably implying prepore-to-pore conversion. Albeit probe artifacts, a significant increase in size (diameter) together with a height decrease (~2 nm) observed for the globularly shaped complex may perhaps reflect an opening (increased diameter) and/or membrane-inserting (decreased height) feature of the trimeric pore. Nevertheless, the detailed perception of the two different trimeric conformations (prepore and membrane-associated pore) remains to be further verified by performing a more in-depth structural characterization.

The real-time trimeric arrangement of monomers associated with bicelle membranes can also be visualized by our successive HS-AFM imaging, depicting interactions among three individual subunits toward trimer assembly. This important evidence therefore further strengthens the notion proposed here for the structural requirement of a membrane-associated state of the Cry4Ba active toxin for molecular assembly of a potential prepore trimer that would subsequently transform into a membrane-lytic pore. Likewise, other recent studies also attempted to give details for an oligomerization pathway of the Cry1Aa toxin using single-molecule analysis via photobleaching (20). Despite the fact that the toxin molecule itself could not be visualized directly, the authors suggested that oligomerization of the Cry1Aa toxin is a highly dynamic process occurring after insertion of monomers into the lipid membrane to form an oligomeric complex (20). Taken together, our findings provide pivotal insights, for the first time, into the structural requirement of membrane-induced conformational changes for the formation of a potential prepore trimer of such insecticidal pore-forming proteins from Bt biopesticides.

Author Contributions—W.S., T.U., T.A., and C.A. designed research. W.S. performed most of the experiments for her Ph.D. study and performed research. A.A., S.S., C.K., and T.U. performed research. W.S., C.K., T.U., T.A., and C.A. analyzed data. W.S. and C.A. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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Protein Structure and Folding: Potential Prepore Trimer Formation by the Bacillus thuringiensis Mosquito-specific Toxin: MOLECULAR INSIGHTS INTO A CRITICAL PREREQUISITE OF MEMBRANE-BOUND MONOMERS

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