Serial femtosecond crystallography on in vivo-grown crystals drives elucidation of mosquitocidal Cyt1Aa bioactivation cascade

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Cyt1Aa is the one of four crystalline protoxins produced by mosquitocidal bacterium Bacillus thuringiensis israelensis (Bti) that has been shown to delay the evolution of insect resistance in the field. Limiting our understanding of Bti efficacy and the path to improved toxicity and spectrum has been ignorance of how Cyt1Aa crystallizes in vivo and of its mechanism of toxicity. Here, we use serial femtosecond crystallography to determine the Cyt1Aa protoxin structure from sub-micron-sized crystals produced in Bti. Structures determined under various pH/redox conditions illuminate the role played by previously uncharacterized disulfide-bridge and domain-swapped interfaces from crystal formation in Bti to dissolution in the larval mosquito midgut. Biochemical, toxicological and biophysical methods enable the deconvolution of key steps in the Cyt1Aa bioactivation cascade. We additionally show that the size, shape, production yield, pH sensitivity and toxicity of Cyt1Aa crystals grown in Bti can be controlled by single atom substitution.
Mosquitoes remain the organisms most harmful to human health, transmitting diseases such as malaria, dengue fever, and filariasis. Disease prevention relies mostly on the control of mosquito vector populations by use of chemical insecticides but these elicit resistance whilst also harming crustaceans, bees, and fish. A safer alternative to chemical is the dissemination of sporulated formulations of the mosquitoicidal bacterium, *Bacillus thuringiensis* subspecies *israelensis* (*Bti*), which upon ingestion by mosquito larvae destroys their midgut, killing them. The active ingredient of *Bti* is a parasporal body that contains three natural sub-micron-sized crystals of four highly efficient mosquito-specific toxins, namely Cyt1Aa, Cry11Aa and co-crystallizing Cry4Aa and Cry4Ba. Following ingestion by mosquito larvae, the crystals promptly dissolve in the alkaline environment of the gut (pH ~11), releasing protoxins that are activated into toxins through proteolytic cleavage of propeptides by gut enzymes. Whereas Cyt1Aa directly interacts with lipids from gut cell membranes, Cry toxins require interaction with membrane-bound receptors. Each activated protein eventually self-assembles into cytolytic oligomers that perforate gut cells, leading to mosquito larval death. Cyt1Aa oligomers can additionally serve as substitution receptors for Cry toxins, enabling them to kill cells even in the absence of mosquito receptors. This synergy explains how *Bti* is able to evade resistance and makes Cyt1Aa the key element of its mosquitoicidal arsenal. However, the molecular determinants of Cyt1Aa crystallization in *Bti* cells and of crystal dissolution in the mosquito midgut remain unclear, and the mechanism by which oligomers form and exert direct toxicity to mosquito gut cells is actively being researched (Fig. 1a). The Cyt1Aa terminus were missing from the initial model, respectively, corresponding to the digested propeptides. Clear residual density allowed us to build most of these residues (H6-L249) (Fig. 1d). At the monomeric level, the Cyt1Aa protoxin displays a conformation overall similar to that of the activated toxin, with two outer layers of α-helix hairpins, αA/αB and αC/αD, respectively covering the hydrophilic and hydrophobic sides of a central five-stranded mixed β-sheet (namely β2-β5-β6-β7-β8; Fig. 1e). Besides the presence of propeptides, the largest structural differences between the protoxin and the toxin are observed at the C-terminus where αE residues display distinct conformations (Fig. 1e). Additionally, the αC/αD hairpin draws away from the β-sheet and from helix αE upon activation (Fig. 1e and Supplementary Fig. 2a, b). While these conformational changes are on the overall subtle, they appear to be concerted at the main-chain level (Supplementary Fig. 2b). At the side chain level, all residues assuming different conformations are found on the αC/αD side of the β-sheet, except D72 (Fig. 1e). In some cases, we observe conformational changes that maintain an H-bond in place, e.g. that which tethers the αC/αD hairpin (Q138) to the tip of β2 (E45) (Supplementary Fig. 2c).

In vivo-grown Cyt1Aa protoxin crystals are remarkably packed, burying 40.1% of surface area at crystal contacts (5484.2 Å² out of 13661.2 Å² of total monomeric areas), compared with only 13.3% (1252.7 Å² out of 9405.5 Å²) in crystals of the activated toxin grown in vitro. Examination at the unit-cell level reveals that the Cyt1Aa protoxin crystallizes as a domain-swapped (DS) dimer with strands β1 (contributed by the N-terminal propeptide) and β2 entwined at the DS interface (interface #1), aligned with a crystallographic two-fold axis (Fig. 2a, Supplementary Fig. 3 and Supplementary Table 1). The DS interface of Cyt1Aa, which is mainly stabilized by H-bonds between carboxylic (E32, E45, D137, E156), amide (Q138, N181) and amine (R30) groups (Fig. 2b), also includes the αC/αD hairpin and the C-terminal propeptide helix αF (Fig. 2a), burying a cumulated surface area of 3077 Å² in each monomer—i.e. 22.5 % of the protoxin accessible area, which is more than all other crystal packing interfaces combined (2414 Å² over nine interfaces; Supplementary Fig. 3 and Supplementary Table 1) or all interfaces in the in vitro crystals of the activated toxin combined (1710 ± 120 Å²) (Fig. 1f). Formation of the DS dimer thus likely precedes crystallization in *Bti* cells. A similar DS interface was previously observed in the in vitro structure of the homologous non-cytolytic Cyt2Ba protoxin (33% identity with Cyt1Aa), but its biological significance could not be ascertained.

Strikingly, residues absent from the activated toxin structure are the main contributors to the packing of the natural crystals. Most notably, the N-terminal propeptide buries 73% of its surface area (2704 over 3691 Å²) across nine interaction zones, contributing 34 H-bonds, 10 salt-bridges and a disulfide bridge at position C7 (Fig. 1g). This disulfide bridge is aligned with the other crystallographic two-fold axis (Fig. 2). Collection of a dataset upon soaking of crystals with 1 mM DTT (“DTT” dataset) indicates that at pH 7, rupture of the C7(Sy)-C7(Sy) disulfide is evident, by a strong negative peak in the Fo-Fc map, is not

**Results**

**The N-terminal propeptide governs in vivo crystallization.** We produced sub-micron-sized crystals of Cyt1Aa in vivo by recombinant expression in an acrystalliferous strain of *Bti* (4Q7). Crystal size, shape, integrity, and diffraction power were assessed by scanning electron microscopy (SEM) (Fig. 1a), atomic force microscopy (AFM) (Fig. 1b), transmission electron microscopy (TEM) (Fig. 1c), and serial synchrotron crystallography, respectively, revealing highly ordered bipyramidal crystals (0.6 × 0.6 × 0.9 μm², corresponding to ~180,000 unit cells) which diffract to ~4.0 Å resolution when exposed to a sub-microfocus synchrotron X-ray beam (0.7 μm FWHM; ESRF-ID13) at 100 K (Supplementary Fig. 1). Using SFX at the CXI-SC3 micro-focused beamline of the Stanford Linear Accelerator Center (SLAC) Linac Coherent Light Source (LCLS), diffraction data extending to 1.86 Å could be collected at room temperature (RT) for the wild-type (WT) Cyt1Aa protoxin at pH 7 (“pH7” dataset, Table 1), from sub-micron-sized crystals injected by a MESH device. Data were phased by molecular replacement using as a starting model the in vitro structure of proteolytically activated Cyt1Aa (PDB entry: “3m50” determined by synchrotron crystallography at 100 K. Therefore, 37 residues from the N-terminus and 7 residues from the C-terminus were missing from the initial model, respectively, corresponding to the digested propeptides. Clear residual density allowed us to build most of these residues (H6-L249) (Fig. 1d). At the monomeric level, the Cyt1Aa protoxin displays a conformation overall similar to that of the activated toxin, with two outer layers of α-helix hairpins, αA/αB and αC/αD, respectively covering the hydrophilic and hydrophobic sides of a central five-stranded mixed β-sheet (namely β2-β5-β6-β7-β8; Fig. 1e). Besides the presence of propeptides, the largest structural differences between the protoxin and the toxin are observed at the C-terminus where αE residues display distinct conformations (Fig. 1e). Additionally, the αC/αD hairpin draws away from the β-sheet and from helix αE upon activation (Fig. 1e and Supplementary Fig. 2a, b). While these conformational changes are on the overall subtle, they appear to be concerted at the main-chain level (Supplementary Fig. 2b). At the side chain level, all residues assuming different conformations are found on the αC/αD side of the β-sheet, except D72 (Fig. 1e). In some cases, we observe conformational changes that maintain an H-bond in place, e.g. that which tethers the αC/αD hairpin (Q138) to the tip of β2 (E45) (Supplementary Fig. 2c).

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Fig. 1 SFX on in vivo-grown crystals enables determination of the Cyt1Aa protoxin structure. a–c Cyt1Aa sub-micron-sized crystals were grown in vivo by recombinant expression in Bti-4Q7, and their quality assessed by scanning electron microscopy (SEM; a scale bar = 200 nm), atomic force microscopy (AFM; b scale bar = 500 nm) and transmission electron microscopy (TEM; c scale bar = 200 nm). d Electron density was visible for the propeptides in the initial 2Fo-Fc electron density map displayed at 1σ. The N-terminal propeptide establishes contact with four symmetry-related molecules. e Tertiary structure of Cyt1Aa. In the right panel, the two chains constitutive of the asymmetric unit of the activated toxin ("3ron [10.2210/pdb3RON/pdb]") are overlaid on the protoxin structure, with residues displaying different side chain conformations shown as sticks. Secondary structure information is overlaid on the models. f Packing in the natural protoxin crystals grown in vivo (left) and in the crystals of the activated toxin grown in vitro (right). g The N-terminal and C-terminal propeptides scaffold the natural crystals.
We engineered a C7S mutation to probe the role of the disulfide bridge of DS dimers. This step therefore likely occurs after the crystal is fully formed. We also mutated the other residues pinpointed by strong peaks in the Fo–Fp maps, D11, E32, E45, Q168, and Y171, hypothesizing that they would be central to crystal formation and dissolution, and possibly function as a control for C7, we mutated the other cysteine of C7S, pH 7 to pH 10.3 (Fig. 2a and Supplementary Fig. 2a). The αA/αB face of the β-sheet is not significantly different from the WT, confirming that this mutation does not affect the pH sensitivity of crystals and that the effect of DTT on crystal solubilization is unrelated to the disulfide bridge chaining of DS dimers. This step therefore likely occurs after the crystal is fully formed. We also mutated the other residues pinpointed by strong peaks in the Fo–Fp maps, D11, E32, E45, Q168, and Y171, hypothesizing that they would be central to crystal formation and dissolution, and possibly function as a control for C7, we mutated the other cysteine of C7S, pH 7 to pH 10.3 (Fig. 2a and Supplementary Fig. 2a). The αA/αB face of the β-sheet is not significantly different from the WT, confirming that this mutation does not affect the pH sensitivity of crystals and that the effect of DTT on crystal solubilization is unrelated to the disulfide bridge. The C7S mutation also revealed a structure nearly indiscernible from the “DTT” structure (Fig. 3 and Supplementary Fig. 2a–e). Thus, neither crystal formation nor crystalline order depends on the disulfide bridge. It has been observed that the presence of cysteine residues in the DS interface as the most sensitive to pH elevation, with the strongest positive and negative peaks in the Fo–Fp maps, D11, E32, E45, Q168, and Y171, hypothesizing that they would be central to crystal formation and dissolution, and possibly function as a control for C7, we mutated the other cysteine of C7S, pH 7 to pH 10.3 (Fig. 2a and Supplementary Fig. 2a). The αA/αB face of the β-sheet is not significantly different from the WT, confirming that this mutation does not affect the pH sensitivity of crystals and that the effect of DTT on crystal solubilization is unrelated to the disulfide bridge. The C7S mutation also revealed a structure nearly indiscernible from the “DTT” structure (Fig. 3 and Supplementary Fig. 2a–e). Thus, neither crystal formation nor crystalline order depends on the disulfide bridge. It has been observed that the presence of cysteine residues in the DS interface as the most sensitive to pH elevation, with the strongest positive and negative peaks in the Fo–Fp maps, D11, E32, E45, Q168, and Y171, hypothesizing that they would be central to crystal formation and dissolution, and possibly function as a control for C7, we mutated the other cysteine of C7S, pH 7 to pH 10.3 (Fig. 2a and Supplementary Fig. 2a). The αA/αB face of the β-sheet is not significantly different from the WT, confirming that this mutation does not affect the pH sensitivity of crystals and that the effect of DTT on crystal solubilization is unrelated to the disulfide bridge.

### Table 1 Data collection and refinement statistics (molecular replacement).

| Wild type, pH 7 | Wild type, DTT | Wild type, pH 10 | C7S, pH 7 |
|----------------|---------------|-----------------|-----------|
| PDB accession code | 6T14 | 6T19 | 6T1A | 6T1C |
| Space group | P6_22 | P6_22 | P6_22 | P6_22 |
| Cell dimensions | 64.8, 64.8, 164.5 | 65.6, 65.6, 164.3 | 65.5, 65.5, 165.5 | 65.6, 65.6, 164.1 |
| a, b, c (Å) | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 | 90, 90, 120 |
| α, β, γ (°) | 1.3 | 1.3 | 1.3 | 1.3 |
| X-ray beam focus (µm) | 1.3 | 1.3 | 1.3 | 1.3 |
| Number of collected frames | 65473 | 39547 | 45117 | 18218 |
| Number of indexed patterns | 8584 | 18882 | 20052 | 7754 |
| Number of merged images | 8462 | 18766 | 19924 | 7683 |
| Resolution (Å) | 43.5–1.86 (1.89–1.86) | 43.5–1.83 (1.86–1.83) | 43.5–1.85 (1.88–1.85) | 43.5–1.97 (2.01–1.97) |
| Number of observations | 2003832 (16132) | 3285436 (30913) | 3123781 (30982) | 1203324 (23089) |
| Multiplicity | 107.8 (18.0) | 160.5 (30.8) | 165.4 (33.6) | 65.8 (26.33) |
| Completeness (%) | 98.7 (100.0) | 99.9 (100.0) | 99.9 (100.0) | 99.9 (100.0) |
| Resolution (Å) | 1.86 (1.91–1.86) | 1.85 (1.90–1.85) | 1.85 (1.90–1.85) | 2.00 (2.05–2.00) |
| Number of reflections | 16099 (1126) | 17339 (1231) | 17513 (1262) | 13698 (722) |
| Rwork/Rfree (%) | 0.217 (0.503)/0.259 (0.451) | 0.231 (0.459)/0.295 (0.547) | 0.237 (0.497)/0.287 (0.494) | 0.220 (0.427)/0.267 (0.473) |
| Number of protein | 1923 | 1941 | 1987 | 1941 |
| Number of water | 251 | 293 | 281 | 276 |
| Number of B-factors (Å²) | 0.007 | 0.007 | 0.007 | 0.007 |
| Number of bond lengths (Å) | 1.146 | 1.083 | 1.103 | 1.120 |
| Number of bond angles (°) | 1.114 | 1.057 | 1.103 | 1.120 |

*Values in parentheses are for highest-resolution shell.

*The number of merged images corresponds to number of crystals used to solve the structure.

**Mapping the role of the disulfide bridge and DS interface.** The Cyt1Aa bioactivation cascade involves dissolution of crystals in the alkaline environment of the mosquito gut. Accordingly, a pH of 11.2 ± 1.0 is required to solubilize 50% of Cyt1Aa WT crystals after 1h incubation (SP₅₀) (Fig. 3 and 4a and Supplementary Table 2). This incubation time was chosen on the basis that the transit time along the mosquito larvae gut is 30–60 min, depending on species. When DTT is added, crystals solubilize at a significantly lower pH, with a SP₅₀ of 9.8 ± 1.0 (Fig. 4a and Supplementary Table 2). The solubilization pattern of C190V crystals does not significantly differ from the WT, confirming that this mutation does not affect the pH sensitivity of crystals and that the effect of DTT on crystal solubilization is unrelated to the disulfide bridge. The C7S mutation also revealed a structure nearly indiscernible from the “DTT” structure (Fig. 3 and Supplementary Fig. 2a–e). Thus, neither crystal formation nor crystalline order depends on the disulfide bridge.
**Fig. 2 Disulfide-bridge chaining of domain-swapped (DS) dimers in the natural Cyt1Aa crystals.**

a) Cyt1Aa dimers associated through a DS interface constitute the building block of natural crystals. These DS dimers are chained one to another by a disulfide bridge at position C7.

b) Fourier difference maps computed between datasets, and phased by the pH7 structure, highlight the most striking conformational changes upon pH elevation (upper panels; \(F_0^{pH10} - F_0^{pH7}\) map) and DTT soak (lower panels; \(F_0^{DDT} - F_0^{pH7}\) map). The difference maps are overlaid on the pH7 protoxin structure, shown as a slate-colored ribbon. Symmetry related molecules are all colored differently, with each molecule having the same color coding in all panels. From left to right, the figure shows the maps contoured at ±3 \(\sigma\) around the disulfide bridge, at crystal packing interface #3 and at the DS interface, respectively. Positive and negative peaks are shown in green and red. Secondary structure information is overlaid on the models.
mutation does not significantly affect pH sensitivity, eliminating the possibility of this residue partaking in the crystal dissolution mechanism (Fig. 3, Supplementary Table 2 and Supplementary Fig. 5). In contrast, the C7S crystals dissolve in the absence of DTT at the same pH as WT crystals soaked with DTT and remain unperturbed by addition of DTT (Figs. 3 and 4). Hence, the disulfide bridge contributed by C7 increases the resilience of WT crystals by diminishing their pH sensitivity. Likewise, the Q168E mutation strongly increases pH sensitivity to lower pHs, with SP50 of 10.0 ± 1.0 and 8.5 ± 1.0 in the absence and presence of DTT, respectively, confirming the suspected involvement of interface #3 (Fig. 2b, Supplementary Fig. 4 and Supplementary Table 1) in pH sensing and in the subsequent dissolution cascade (Fig. 3 and Supplementary Table 2). Irrespective of the presence of DTT, the D11N, E32Q and E45Q mutations do not significantly affect pH sensitivity (Fig. 3, Supplementary Table 2 and Supplementary Fig. 5), indicating that none of the pH-insensitive H-bonds introduced by mutation at the DS interface is on its own sufficient to significantly increase the SP50.

We characterized the first active protoxin unit—i.e., that released upon crystal dissolution—by combined use of electrophoresis and mass spectrometry. By these two methods, we found that a dimer is predominantly released upon dissolution of crystals in the absence of DTT, accompanied by a monomer and by decreasing amounts of 3-mers, 4-mers and 5-mers (Fig. 4b and Supplementary Fig. 6). The dimer is disulfide-bridged at position C7, as demonstrated by the fact that it dissociates into monomers in presence of DTT and β-mercaptoethanol, but not upon heating to 95 °C, and by the observation of a single monomeric species upon dissolution of crystals of C7S, but not C190V (Fig. 4b and Supplementary Figs. 4–7). The observation of WT Cyt1Aa monomers upon dissolution of crystals in the absence of DTT nonetheless establishes that a fraction of the disulfide bridges break upon elevation of pH to 11 (Fig. 4b and Supplementary Fig. 6), consistent with the two alternate conformations observed in the “pH10” structure (Fig. 2b and Supplementary Fig. 4) and with the known pH sensitivity of disulfide bonds which can rupture upon pH elevation following a Cys-S-S-Cys + OH− → Cys-S− + Cys-S-OH oxidation reaction.2,3,16,17 The pH-dependent heat-stability profile of the Cyt1Aa protoxin dimer is also consistent with the presence of a disulfide bridge associating two protoxin monomers in the dimer (Supplementary Fig. 7).18,20,21,22

We therefore investigated the propensity of these three Cyt1Aa species—the disulfide-bridged dimer, the protoxin monomer and the activated toxin monomer—and of their mutated variants to assemble into membrane-bound oligomers (MBO). When monomers of either the 27 kDa protoxin or the 23 kDa proteolytically activated toxin21,22,24 are mixed with POPC liposomes (~100 nm radius) and electrophoresed on 6% SDS-PAGE gels, a ladder pattern characteristic of Cyt1Aa MBO is observed (Fig. 4c). The disulfide-bridged dimer is unable to form MBO upon contact with liposomes (~100 nm radius; LUVs) whereas the C7S mutant, able only to solubilize as protoxin monomers, forms MBO regardless of proteolytic activation (Fig. 3

| Cyt1Aa mutant | Wild-type | C7S | C190V | Y171F | D11N | Q168E | E32Q | E45Q |
|---------------|-----------|-----|-------|-------|------|-------|------|------|
| Crystal size (nm) (mean ± SE) | L1 | W1 | L/W | 925.0 ± 28.3 | 591.2 ± 17.3 | 1.57 ± 0.10 | 746.6 ± 16.8 | 666.8 ± 17.5 | 751.6 ± 14.6 | 1217.2 ± 36.4 | 1086.7 ± 26.6 | 849.4 ± 20.7 | 1153.4 ± 20.8 |
| Productivity | ++ | ++ | ++ | + | ++ | ++ | +++ | ++ | +++ | ++ | +++ | ++ |
| Solubility at 1 h (SP50 ± SE) | 11.18 ± 1.01 | 9.58 ± 1.02 | 11.07 ± 1.01 | 10.90 ± 1.02 | 9.50 ± 1.02 | 9.22 ± 1.02 | 9.95 ± 1.01 | 9.80 ± 1.02 | 11.31 ± 1.02 | 9.94 ± 1.02 |
| Oligomer formation | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Toxicity (in nM) (LC50 ± SE) | 366.9 ± 1.9 | 293.4 ± 1.8 | 460.7 ± 1.6 | 301.9 ± 1.4 | 610.7 ± 1.6 | >30,000 | >30,000 | 3676.0 ± 1.5 | 204.7 ± 1.3 | 1156.9 ± 1.1 |

Fig. 3 Point mutations affect different steps along the Cyt1Aa bioactivation cascade. For each row, different letters indicate significant differences between mutants. Length and width of crystals. Productivity was visually estimated based on the quantity and aspect of the crystal-spore suspension collected. 4SP50 corresponds to the concentration at which 50% of crystals solubilize after 1 h incubation at RT. See Supplementary Table 2 for corresponding statistics. 5Toxin solubilized without DTT, releasing a disulfide-bridged dimer (except for C7S), and with DTT, releasing a protoxin monomer. 6Toxin monomer activated by protease K. 7Capacity of each toxin species to generate a ladder pattern characteristic of membrane-bound oligomers (MBO) on 6% SDS-PAGE. See corresponding gels in Supplementary Fig. 9. 9LC50 corresponds to the concentration lethal for 50% of cell population. See Supplementary Table 2 for corresponding statistics. Scale bar = 0.2 μm. Source data are provided as a Source Data file.
and Supplementary Fig. 9). Thus the disulfide bridge, but not the N-terminal propeptide, can abrogate MBO formation. Thin layer chromatography (TLC) nonetheless indicates that the N-terminal propeptide masks half of the lipid binding interface in the oligomers, with protoxin and toxin MBO respectively featuring ~11.1 ± 2.6 (N = 4) and ~22.1 ± 6.9 (N = 4) phospholipids per Cyt1Aa monomer (Fig. 4d), consistent with previous estimations based on liposome disruption assays. MBOs do not form upon contact of toxins with lipids solubilized in detergent micelles or bilayered in two-times smaller liposomes (~50 nm radius; SUVs) (Fig. 4c), demonstrating a requirement for a fully formed membrane with a minimal radius of curvature. MBO persist after solubilization of their supporting liposomes and, despite an apparent spacing of bands by 11 kDa in SDS-PAGE gels (Supplementary Fig. 10), dissociate into full-size monomers upon heating to 95 °C (Fig. 4d and Supplementary Fig. 6). This observation shows not only that MBO formation entails drastic conformational changes after which Cyt1Aa may not solubilize as a monomer unless heated, but also that there is no further proteolytic cleavage occurring post-insertion into membrane nor...
covalent link between monomers within MBO. MBO formation is unaffected by the C190V and Y171F mutations but is reduced in the E32Q protoxin and abrogated in the E45Q and Q168E protoxins, with full, partial and no restoration upon cleavage of the propeptides, respectively (Fig. 3 and Supplementary Fig. 9). This result indicates that stabilization of the N-terminal propeptide, and by extension of the DS dimer, inhibits MBO formation. That propeptide removal does not rescue MBO formation in E45Q and Q168E indicates that these mutations further impact processes downstream dissociation of dimers into monomers.

Treatment of Cyt1Aa MBO with detergents other than SDS (sodium dodecyl sulfate) prior to electrophoresis on native gels reveals the unique ability of SDS to produce a ladder pattern, effectively breaking down higher order MBO as they migrate through the gel (Supplementary Fig. 11). The smallest and largest MBO fragments migrate at the expected sizes for a Cyt1Aa trimer and 26-mer (~0.6 MDa; Figs. 4c, 1e), respectively, but cross-linking (using either glutaraldehyde or DTSSP (3,3′-dithiobis(sulfosuccinimidyl propionate)) thence after sizing 3 and 12 Å spacing between amine, respectively) prior to SDS-PAGE migration prevents MBO from entering gels, suggesting that they are monodisperse (Supplementary Fig. 12). We note that similar ladder-like profiles, proposed to reflect formation of oligomers of non-fixed stoichiometry by stepwise addition of monomeric units have been reported for the structurally homologous Cyt2Aa24,25 and VVA26 toxins, as well as for pneumolysin27, whose pore-forming domain resembles Cyt1Aa28.

**Cytotoxicity originates from MBOs.** Cyt1Aa WT toxicity was assayed on insect Sf21 cells (0.04–0.4 μM) (Supplementary Fig. 13, Supplementary Movies 1 and 2) and on two mammalian cell lines, namely NIH fibroblast cells (0.04–4 μM) (Supplementary Fig. 13, Supplementary Movies 3 and 4) and HEK293 cells (0.001–30 μM) (Figs. 3 and 4e and Supplementary Table 2), supporting a generalist mode of action whose efficiency likely depends on the cell membrane phospholipid composition. HEK293 cells were chosen to pursue investigations, because their monodisperse nature enables use of a fluorescence-activated cell sorting (FACS) flow cytometer system, combined with propidium iodide (PI) staining as a reporter for cell death, to quantify the cytotoxicity of WT Cyt1Aa and mutants thereof. For each, LC50 iodide (PI) staining as a reporter for cell death, to quantify the monodisperse nature enables use of a dependency on the cell membrane phospholipid composition.

Porous oligomers fully perforate cell membranes. How Cyt1Aa exerts its cytolytic activity remains unclear. It has been proposed, based on electrophysiology data, that Cyt1Aa oligimerizes into cation-selective channels of 6–20 Å diameter5, but we failed to observe the formation of such pores despite conducting similar black lipid membrane (BLM) experiments at Cyt1Aa concentrations ranging from 1.7 to 60 μg/mL−1 (Fig. 5a). Rather, our observations are suggestive of a cooperative membrane-binding process, whereby multiple toxins successively insert and coaggregate in the bilayer (inducing flickering in the measured electrical current) before the latter is ripped apart. We therefore further challenged the porous nature of MBO by exposing Sf21 (insect) (Fig. 5b) and NIH 3T3 (mammal) cells (Fig. 5c) simultaneously to Cyt1Aa toxin at sub-lethal concentration and to fluorescent dextran beads of 1.4–8.5 nm. We found that beads up to 8.5 nm can penetrate cells through Cyt1Aa-induced lesions, excluding the possibility that Cyt1Aa forms a selective pore.

We used AFM on supported lipid bilayers (SLB) to obtain further insights into the large-scale structural dynamics of Cyt1Aa MBO (Fig. 5d and Supplementary Fig. 15). Minutes after the addition of Cyt1Aa WT activated toxin, a first type of MBO—hereafter referred to as membrane-bound aggregates (MBA)—forms from the continuous encounter of monomers freely diffusing at the membrane surface (Fig. 5d–f), with a strong significant positive correlation between the time elapsed since toxin addition and the surface area occupied by the MBA (Fig. 5g). Toxin aggregation eventually leads to the formation of holes at the periphery of MBA (Fig. 5h, i), consistent with cell microscopy assays (Fig. 5b, c).

We monitored the area occupied by MBA and holes as a function of hole depth. The significant negative correlation observed between the hole depth and MBA surface (Fig. 5i), but not between the hole depth and the total “MBA+hole” surface (Fig. 5k), suggests that two types of MBO coexist: porous oligomers and MBA. Full membrane perforation is visible only for holes of ~54 nm diameter, with a ~2300 nm2 surface and ~169 nm circumference, corresponding to a porous oligomer formed by the assembly of ~56 monomers (Fig. 5i). That only a fraction of the porous oligomers displays sufficient depth to fully perforate the membrane (~4.5 nm) suggests that the structural transition between MBA and porous oligomers is independent of
MBA size and could involve formation of a pre-pore (Fig. 5j). Experiments on the Q168E mutant reveal that this mutation affects the first step of toxin insertion into the membrane. Indeed, the Q168E mutant is unable to penetrate the membrane and aggregate to form pores (Fig. 5l), behaving like the BSA control (Fig. 5m).

We attempted to characterize the porous oligomers by TEM, either after negative staining or under cryogenic conditions (cryo-EM). Addition of Cyt1Aa to 100 nm liposomes led to their almost total disruption, with the few remaining LUVs exhibiting membrane leakage (Fig. 5n), and to the release of a homogenous population of 3.50 ± 0.42 nm thick and 30.3 ± 2.3 nm long.
Fig. 5 Cyt1Aa forms oligomers that fully perforate and eventually disrupt lipid bilayers. a Single cation-channel formation was not observed in black lipid membrane (BLM) experiments. b–e Cyt1Aa allows entry in both SF21 (b) and NIH 3T3 cells (c) of co-incubated FITC-labelled (fluorescein isothiocyanate) dextran beads up to 8.5 nm in size. Scale bars = 10 μm. d–f Membrane-bound Cyt1Aa monomers exhibit mobility (d, e) and display the capacity to merge into larger membrane-bound aggregates (MBA). Scale bars = 3 μm (d) and 300 nm (e, f). g A significant positive correlation was observed between the surface of MBA and the time elapsed since toxin addition. h 35 min after toxin addition, membrane perforation is observed at the periphery of MBA. Scale bar = 500 nm. i The depth of holes can reach 4.5 nm, consistent with a full spanning of the lipid bilayer. j, k A significant negative correlation is observed between the surface of holes and their depth (j), but not between the latter and the combined area of the hole and the parent MBA (k). l, m Formation of MBA and holes was neither observed for the activated form of the non-toxic Q168E mutant (l) nor for the BSA control (m). Scale bars = 500 nm. n–q Transmission electron microscopy captures liposome lysis by the toxin (n), and the resulting release of arciform oligomers (o–q). Scale bars = 50 nm (n, q), 20 nm (p) and 100 nm (o). Source data are provided as a Source Data file.

Fig. 6 Proposed model for Cyt1Aa bioactivation cascade. The bioactivation cascade of Cyt1Aa starts with dimerization through a domain-swapped interface, which allows both self-inhibition and in vivo crystallization, and ends with oligomer formation in the membrane of mosquito gut cells. The Cyt1Aa structure is abstracted and colored sequence-wise, from cold (N-terminus) to hot (C-terminus) colors. The magenta square highlights the disulfide bridge between domain-swapped (DS) dimers. Red starbursts indicate electrostatic repulsion, whereas the yellow-blue starburst indicates disulfide bridge disruption. Conformational changes occur in the toxin upon pH elevation, resulting in an untethering of the αC/αD hairpin from the β-sheet. We propose that upon contact with a cell membrane, the protein fully opens at the αC/αD hydrophobic interface and that the two thereafter exposed hydrophobic surfaces appose onto the membrane bilayer, yielding the membrane-bound aggregate (MBA) conformer. Aggregation of MBA conformers eventually results in the formation of holes, at the periphery of MBA, resulting in death of midgut cells.
arciform oligomers, characterized by a mean curvature of 129.3 ± 7.4° (mean ± SD; N = 27) (Fig. 5n–q). These could represent either the open form of a porous oligomer or pieces thereof. In the first case scenario, i.e. assuming conservation of the length of arciform oligomers, porous oligomers would be ~12 mers of ~276 kDa characterized by a 2–5 nm pore, which is inconsistent with our native and SDS-PAGE, AFM and BLM conclusions. Thus, we favor the hypothesis that arciform oligomers represent pieces from the breakdown of larger cytolytic oligomers, such as those observed by AFM.

Discussion

Structural investigations of in vivo-grown sub-micron-sized crystals using SFX, AFM, and complementary methods have shed light on key steps in the Cyt1Aa bioactivation cascade, from in vivo crystallization in Bti cells, to crystal dissolution, proteolytic activation, and membrane insertion and perforation through oligomerization (Fig. 6). Our SFX structures point to the N-terminal propeptide of Cyt1Aa being a key structural element which, by dimerization through a DS interface, intervenes in Cyt1Aa folding, self-inhibition and in vivo crystallization—but not in crystal dissolution. Rather, we unveil the respective roles played by the disulphide-bridge and interface #3, showing how these act together to ensure crystal dissolution occurs only under highly alkaline (and possibly reducing) conditions, such as those found in the larval mosquito midgut. This mechanism differs slightly from that highlighted in naturally occurring nanocrystals of BinAB, wherein no cysteine interface is in play and it is rather pH-induced electrostatic repulsion at crystal interfaces between tyrosine residues and their obligate H-bond acceptors that complement the analogous mechanism between acidic side chains.

Further structural characterization of naturally crystalline prototoxin structures may allow the discovery—and eventually the utilization—of additional mechanisms of in vivo crystallization and controlled pH-dependent solubilization.

It remains unclear whether the mosquito gut is a reducing environment. If so, crystals could dissolve and begin to initiate cytopathy at a pH as low as 9.5, in line with the observation of Cyt1Aa lesions to anterior midgut cells; if not, a disulfide-bridged dimer would be released upon crystal dissolution, which would need to be proteolytically activated. We showed that both this dimer and the protoxin monomer can be processed by proteases, yielding the 23 kDa activated toxin. We also show that upon contact with a lipid membrane, drastic conformational changes take place, most likely due to an opening of the structure at the αc/αD hydrophobic interface with the β-sheet. This interface indeed demonstrates the greatest structural differences observed between the protoxin at pH 7, the protoxin at pH 10 and the activated toxin at pH 8 (Figs. 1 and 2, and Supplementary Figs. 2–4). The hypothesis that an opening of the protein at the αc/αD hydrophobic interface is required for MBO formation was examined by the introduction of a E45Q mutation, intended to render pH-insensitive the tether between the β-sheet and αD (Q138(OE1)), besides stabilizing the DS dimer. The mutation favoured crystal growth but resulted in a 14-fold reduced toxicity for the activated toxin, while eliminating toxicity of the protoxin (Fig. 3). It thus seems plausible that opening of the WT structure at the αc/αD hydrophobic interface with the β-sheet is an important step for membrane insertion and subsequent MBO formation. That the αc/αD hairpin would be involved in MBO formation is in line with its burial at the DS interface in the protoxin structure.

Evidence was also provided that Q168E, buried at crystal packing interface #3, is a key player in crystal formation (markedly different crystal dimensions and production yield upon Q168E mutation), pH-induced dissolution (~1 pH unit difference upon Q168E mutation) and toxic activity (fully abrogated in the Q168E mutant) (Fig. 3). AFM imaging and SDS-PAGE analyses indicate that the mutation produces effects at the membrane insertion level, which we interpret as an indication that this residue, at the tip of β5, is part of the β-sheet structure that plunges into the membrane to form the porous oligomer. This proposition is in agreement with earlier mass spectrometric determination of the extent of membrane-inserted domains in the Cyt1Aa porous oligomers. These studies indeed pointed to residues 42 to 132 forming the hydrophilic part of the pore (β2-αA-αB-β3), and to residues 154 to 234 as being membrane-associated and thus forming the hydrophobic part of the pore. These are all residues located between the C-terminus of αD and the N-terminus of αF (Fig. 1), namely the αD-β4 loop and the β4-β5-β6-β7 β-sheet featuring the short αE helix between β6 and β7. It was also shown that the N- and C-termini of the protein are both exposed on the extracellular side of the pore.

The simplest way to reconcile these two results is to envision a pore wherein β4 (and possibly the αD-β4 loop) contributes one of the strands of the oligomerizing β-sheet structure, with the β4-β5 loop pointing on the intracellular side—in line with our proposal that Q168E is part of the β-sheet structure that plunges into the membrane. Regardless, the absence of β2 and β3 from the membrane-inserted segment of the pores indicates that these must respectively dissociate from β4 and β7 on each edge of the β-sheet, to enable transition to the porous oligomer. It remains to be determined if this step is completed at the monomer to MBA-conformer (i.e. upon interaction with the membrane) or at the MBA-conformer to porous oligomer transition (i.e. upon side-by-side contact between MBA conformers).

The exact structures of the Cyt1Aa MBA conformers and porous oligomers remain to be determined. Our CryoEM results revealed arciform oligomers from which a high resolution structure could not be derived. CryoEM investigations on pneumoly sin, whose pore-forming domain resembles Cyt1Aa and which alike Cyt1Aa yields ladder-like profile on SDS-PAGE gels and arciform oligomers upon insertion into liposomes, suggest that a prerequisite to obtaining a high resolution cryoEM structure of Cyt1Aa porous oligomers will be to carefully devise a lipid/detergent/additive formulation capable of stabilizing their annular architecture, i.e. preventing their fall-off into arciform oligomers. At present, we can nonetheless propose a model that fits all the information developed by us and others on Cyt1Aa pore formation and which alike Cyt1Aa yields ladder-like profile on SDS-PAGE gels, and arciform oligomers upon insertion into liposomes. Our CryoEM results and in vivo cell assay data exclude the possibility that Cyt1Aa exerts toxicity by forming a cation-channel of 6–20 Å diameter (the “pore-forming” model), or by acting as a detergent with molecules bound to—but not inserted into—the membrane, (the “detergent-like” model). Rather, the data taken together suggest that following the untethering of the αc/αD hairpin from the β-sheet due to pH elevation and proteolytic activation, the Cyt1Aa structure opens at this locus upon membrane contact, yielding a new membrane-bound Cyt1Aa conformer—the MBA conformer. Side-by-side contact between the β-sheets of adjacent MBA protomers would enable the observed transition to the porous conformation. Our data show that this cooperative MBA-to-porous conformer transition occurs only after a critical number of molecules is recruited, yielding the observed >54 nm diameter membrane perforation upon concomitant plunging across the bilayer of the β-sheets of ~56 or more associated porous conformers (Figs. 5 and 6)—possibly with their β4-β5 loops and αE helices pointing towards the intracellular side. Importantly, this proposed model reconciles the data hitherto presented to oppose the “pore-forming” and “detergent-like” models, e.g. explaining how the N-terminal...
and C-terminal parts of the toxin can both be exposed on the outer face of the membrane31,32, how can the membrane-inserted segment of porous oligomers feature Cyt1Aa residues spanning Δd to Δp31,36, how the large pores formed by Cyt1Aa can enable transit into midgut cells of large molecules or proteins, such as the 42 kDa BinA toxin30, how the non-porous Cyt1Aa oligomers, here identified as MBA, may serve as substitutes for mosquito receptors of Cry toxins, enabling them to dock on mosquito midgut cell membranes and subsequently assemble into toxic pores even in absence of their specific membrane-bound receptors. Lack of expression of toxin receptors, or expression of defective or soluble variants thereof, is indeed one of the most efficient mechanisms developed by insects to resist Bt toxins32,33. We admittedly leave open the question of the exact structure of Cyt1Aa porous oligomers, with hope that high resolution cryoEM structures of the Cyt1Aa porous oligomer and/or MBA con-

Methods

Plasmid construction, crystal production, and purification. The shuttle vector pWF45 was used to produce wild crystals of wild-type Cyt1Aa toxin34,35. It was also used as a backbone to construct plasmids containing single-point mutated cyt1aa genes. A total of 7 point-mutants of Cyt1Aa were constructed based on the difference-density maps generated from crystallographic data (Fig. 2 and Supplementary Table 4). A detailed description of the mutation strategy is available in Supplementary Note 1. Our extensive analysis of the bibliography indicates that none of these mutants has been constructed in previous studies (Supplementary Table 3 and Supplementary Fig. 16).

Point mutations were inserted into cyt1aa gene sequence by Gibson assembly (the list of primers used for plasmid construction is available in Supplementary Table 4). For each mutant, two fragments were amplified from pWF45 using two different primer couples. The fragments were complementary by their 15′ overhangs with a target Tm of 50 °C. The point mutation was directly inserted into the overhang of the two fragments spanning the cyt1aa gene. For each mutant, two fragments were assembled using the NEBuilder HiFi DNA Assembly (New England BioLabs) by following manufacturer’s instructions. After 90 min of incubation at 50 °C, the constructed plasmids were transformed by the heat shock procedure into chimiocompetent Top10 Escherichia coli strain (New England BioLabs). Colonies were selected on LB agar medium supplemented with ampicillin (100 μg mL−1) and plasmids were extracted by using the NucleoSpin Plasmid extraction kit (Macherey-Nagel). Successful plasmid constructions were validated by double digestion (EcoRI and BamHI) followed by migration on 1% agarose gel stained with SYBR Safe (Invitrogen) and presence of mutations was assessed by Sanger sequencing at the Eurofins Genomics sequencing platform.

Validated plasmids were transformed into the acrystalliferous strain 4Q7 of Bacillus thuringiensis subs. israelensis (Bt; The Bacillus Genetic Stock Center (BGSC), Columbus OH, USA) with an improved electroporation procedure46 using a MicroPuls Electroporator (BioRad). Colonies were selected on LB agar medium supplemented with erythromycin (25 μg mL−1) and used to inoculate an overnight 5 mL LB liquid preculture. Precultures were spread on T3 sporulation medium and incubated at 30 °C for 4 days to promote Bt sporulation and toxin crystal production. Spheres and crystals were collected using a cell scraper, resuspended in water and centrifuged once at 10,000 g for 45 min. The pellet was resuspended in water and purified using a discontinuous sucrose gradient (20%–40%). After 16 h of ultracentrifugation at 23,000 g and the crystals were recovered from the 67–72% interface. Several rounds of centrifugation and resuspension in water were performed to discard as much sucrose as possible. Crystal purity was verified by SDS-PAGE on 12% gels. Purified crystals were conserved in ultrapure water at 4 °C until use.

Crystal visualization by SEM. For crystal visualization by AFM, 5 μL of purified Cyt1Aa crystals conserved in ultrapure water were deposited on freshly cleaved mica or carbon coated nickel grids and then imaged using a NanoScope V (Bruker) controller. Imaging was done in the tapping mode (TAP) with a target amplitude of 500 mV (about 12 nm oscillation) and a variable setpoint of 1%–79% with an ultracentrifugation. TESP-A2 cantilevers (k = 42–41 N/m, Fq = 320 kHz, nominal tip radius = 7 nm, Bruker probes, Camarillo, CA, USA) were used and images were collected at −1 Hz rate, with 512 or 1024 pixel sampling. Images were processed with Gwydion47, and if needed stripe noise was removed using DeStripes48.

Crystal preparation and injection via MESH-on-a-stick. SX experiments were performed at the Coherent X-ray Imaging (CDI) instrument of the Linac Coherent Light Source (LCLS) at the SLAC National Accelerator Laboratory (Stanford University, California, USA). Crystals of Wild-Type (WT) Cyt1Aa ("pH7" dataset) and of the C7S mutant ("C7S" dataset) were suspended into spion buffer (MES 0.1M, NaCl 0.1M, Glycine 50%, pH 6.5) and delivered across the X-ray beam at a concentration of 1.6 % (grams of crystals in 100 mL of buffer solution) in the SCC flow chamber at room temperature and under vacuum using the real-time space electronic sample holder (MESH) method, described more fully in Sierra et al.11. Cyt1Aa WT crystals were also injected in (i) spin buffer supplemented with 1 mM of freshly prepared DTT, to investigate the structural effects of disruption of disulide bonds between N-terminal propieceptides—"DTT" dataset; and in (ii) an alkaline buffer (CAPS 0.1M, NaCl 0.1M, Glycine 50%, pH 10.3) 30 min prior to injection to characterize the structural changes that drive crystal solubilization at high pH—"pH10" dataset. These redox and pH conditions were chosen after verification that crystals do not dissolve on the timescale of hours.

Specific to MESH injection, a continuous 1.5 m long polyamide-coated fused-silica capillary of 100 μm inner diameter and 300 μm outer diameter was used to deliver the sample into the SCC vacuum chamber. Approximately 800 μL of sample slurry with glyceral additive was pipetted in a microcentrifuge tube, which was then placed in a small pressurized sample holder. The capillary and the platinum wire used as an electrode were fed through the pressure cell and immersed in the slurry. A low backing pressure of 5 psi nitrogen gas was applied in the sample holder to aid the injection. The voltage was applied by a Stanford Research Systems PS305 (Sunnyvale, CA) high voltage source and was held between 4300 and 4500 V (currents < 1 μA) while the counter electrode was grounded. The flow rate was not directly measured, but we estimate that the sample consumption was approximately 2 μL min−1, as indicated by the decrease of leftover sample volume. The four structures presented herein were collected in less than 12 h of continuous beamtime and consumed less than a millilitre of sedimented crystals.

Data collection, processing, and structure refinement. Datasets were collected with a XFEL beam focussed to a 1.3 μm FWHM spot and characterized by a wavelength of 1.28 Å (proposals P125 and P141). The sample chamber was at room temperature, under vacuum. We attempted to index all collected images with DIALS49, using the cctbx.xfel graphical user interface46. The final "pH7"-"pH10", "pH10" and "C7S" datasets, respectively, consisted of 8462, 18766, 19924, and 7683 indexed patterns. Data were merged using cxi.merge48, with negative intensities included, and resolution cut-offs were determined based on completeness (>99%), redundancy (>60) and CC1/2 (>0.5); in all datasets, <1/sigl> in the highest resolution shells are greater than p<0.001. The ph10 datasets were phased the p<0.001 shell and the pH7 shell using Phaser, using as a starting model the activated structure of Cyt1Aa ("3Ron [10.2210/pdb3RON/pdb]"); the C7S mutant ("6T19; [10.2210/pdb6T19/pdb]"); 98.75, 1.65, 1.00 and 1.67 for the "C7S" dataset (6T1A; [10.2210/pdb6T1A/pdb]); 98.35, 1.65, 1.00, 4.51, and 1.75 for the "pH10" structure (6T1A; [10.2210/pdb6T1A/pdb]); and 99.17, 0.83, 0.0, 2.05, and 1.36 for the "C7S" structure (6T1A; [10.2210/pdb6T1C/pdb]). Data collection, processing, and refinement statistics are shown in Table 1. We obtained experimental insights into pH and redox induced conformational changes by calculating structure factor amplitude maps (Fo–Fo) between the "pH7", "pH10", "C7S" and "C7S" datasets. To improve the estimate of structure factor amplitude differences, Fo–Fo maps were g-weighted.
as described[15] and produced using a CNS[16] custom-written script[15]. Application of the Q-weighting scheme to the diffraction datasets was essential to eliminate noise and amplify the difference signal. Fourier difference maps and distance difference (DDM) calculations were performed using custom-written scripts.

**Crystal solubilization assays.** To assess the stability of the crystals formed by Cyt1Aa WT and its different mutants, we determined at which pH the crystals solubilize (Figs. 3 and 4a and Supplementary Fig. 5). The crystal suspensions were centrifuged at 11,000 g for 2 min and the pellets were resuspended in 50 μL of 0.1 M Na2CO3–NaHCO3 solutions buffering at pH ranging from 9.0 to 11.8, or of 0.1 M NaHCO3–NaH2PO4 buffering from 8.5 to 10.5 μL, and acti-
vation by proteolysis (namely the activated monomer) (Fig. 4b, Supplementary Fig. 6). Crystals of WT Cyt1Aa and of all mutants were solubilized for 1h at RT in 0.1 M Na2CO3 buffer pH 11.8 and then were centrifuged at 11,000 g for 1 min. The supernatant contained the solubilized proteins (condition 1). Addition of DTT allowed the breakage of disulfide bonds between protein subunits, if any (condition 2). Soluble proteins were activated into toxins by the addition of proteinase K or trypsin (condition 3). For further experiments, toxin was activated by proteinase K or trypsin (condition 3).

**Determination of the different forms of Cyt1Aa.** We investigated the three Cyt1Aa species possibly formed, in the mosquito larvae gut, upon dissolution of crystals and dissociation at the DS interface (namely, the disulfide bridged-dimer), possible breakdown of disulfide bridges (namely, the protoxin monomer), and activation by proteolysis (namely the activated monomer) (Fig. 4b, Supplementary Fig. 6). Crystals of WT Cyt1Aa and of all mutants were solubilized for 1h at RT in 0.1 M Na2CO3 buffer pH 11.8 and then were centrifuged at 11,000 g for 1 min. The supernatant contained the solubilized proteins (condition 1). Addition of DTT allowed the breakage of disulfide bonds between protein subunits, if any (condition 2). Soluble proteins were activated into toxins by the addition of proteinase K or trypsin (condition 3).

**Heat stability of protoxin.** The stability of WT Cyt1Aa protoxin dimers was assayed at different pH and increasing temperatures (Supplementary Fig. 7). Crystals of Cyt1Aa WT were solubilized in 0.1 M Na2CO3 buffer pH 11.8, cen-
trifuged at 11,000 g for 1 min containing solubilizable protoxin dimers was collected. Suspensions were diluted 50 times in buffers at different pHs. Na2CO3–NaHCO3 buffer solutions (0.1 M) were used for pH 9, 10, and 11, and NaHCO3–NaH2PO4 buffer solutions, for pH 7, 8, and 9, following guidelines from the Sigma Aldrich Buffer Reference Center. Complete buffer exchange was afforded by two steps of concentration using a AMICON ultra-filtration unit. Briefly, the protoxin sus-
pension at pH 11 was concentrated 50 times using a 10 kDa cutoff (Sigma Aldrich, France), and then diluted 50 times in the buffer of interest (pH 7, 8, 9, or 10); this two-step procedure was repeated twice to achieve a complete buffer exchange. Suspensions were then added to Laemmli buffer devoid of DTT. To test the sta-
Biology of the disulfide-linked dimer, each sample at each pH was heated for 5 min at 95, 100, 105, 110, 115, 120, or 125 °C prior to loading on a SDS-PAGE 12% gel. Each temperature was tested in triplicate. Gels were stained by overnight incubation in InstantBlue (Sigma Aldrich, France), washed twice in ultrapure water and digitalized using a ChemiDoc XR+ imaging system controlled by the Image Lab software version 6.0.0 (BioRad, France).

**MALDI-ToF mass spectra.** For SDS-PAGE experiments, unheated samples were electrophoresed on 12% SDS-PAGE gels after addition of Laemmli buffer devoid of DTT. After staining by overnight incubation in InstantBlue (Sigma Aldrich, France), gels were washed twice in ultrapure water and migration results were digitalized using a ChemiDoc XR+ imaging system controlled by Image Lab software version 6.0.0 (BioRad, France).

**FITC-dextran cells exposure.** The adherent insect S21 and NIH 3T3 cell lines were used to visualize the Cyt1Aa-induced morphological changes, determine the dimension of membrane lesions and assess the specificity of Cyt1Aa mode of action (Fig. 5b, c and Supplementary Fig. 13). S21 and NIH cells were exposed to increasing concentrations of activated Cyt1Aa toxin, during 30 min at 27 °C and 37 °C respectively. Cells were then incubated with a solution of fluorescein isothiocyanate-dextran (FITC-dextran, or FD) from Sigma at 4.5 mg mL −1. We selected two different dextran particles radius (FD-4, 1.4 nm and FD-150, 8.5 nm). Cells were imaged using a laser scanning confocal microscope (LSM), Zeiss LSM 510, using an argon laser at 488 nm for the FITC-dextran solution (Laboratory for Fluorescence Dynamics, University of California Irvine, Irvine, CA). The emission was measured using a 590–550 bandpass filter (BLM experiments). The porous nature and permeability characteristics of Cyt1Aa MBO were tested by the BLM electrophysiological approach (Fig. 5a). Potassium Chloride (KCI) and N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) were purchased from Sigma Aldrich Chemical Co. Inc. The multi-channel recording apparatus consisted of a two compartment Teflon chamber (~5 mL each) separated by a Teflon compartment with 300 μm diameter aperture for membrane formation. The inner chamber was washed with 1% Asolectin in PBS and 1% BSA in pH 7.4

**Insect and mammalian cells.** The effect of the Cyt1Aa toxin was tested on three different cell lines, namely an insect cell line (S21 cell line originating from Spodoptera frugiperda) and two mammalian cell lines (NIH 3T3 mouse fibroblasts and HEK293 human kidney embryonic cells). S21 cells were purchased from The American Type Culture Collection (Rockville, MD) and cultured in SF-900 III (Serum-Free Medium) (GIBCO, insect culture media from Invitrogen), supplemented with Antibiotic Antimycotic Solution (100 U mL −1 penicillin, 100 μg mL −1 streptomycin, 0.25 μg mL −1 amphotericin B, Sigma) at 27 °C. NIH 3T3 cells (brand name "CRL-1668") were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in high glucose DMEM/MACS Quant VYB FACS (Miltenyi Biotec) at a flow rate of 1000 cells s −1. They were sorted according to their fluorescence at 617 nm (channel B2—built-in filter 589–639 nm range) upon laser excitation at 488 nm. Raw data were treated and extracted using the MACSQuantify software v2.11. Cell mortality was calculated by dividing the number of cells counted positive for PI staining by the total number of sorted cells (Supplementary Fig. 14). Mortality data were corrected using Abbott’s formula to account for natural mortality in the control[56]. The best fitted model was selected among four logistic regression models for binomial distribution (logit, probit, complementary log-log transformation (cloglog) and Cauchy distribution) by comparing their deviance using a script modified from[54]. Differences in SP 50 between mutants were considered significant when 95% CI did not overlap[55].
of the chambers were filled with buffer solution, 300 mM KCl, 10 mM CAPS at pH 9. Half a microliter of oligomers (1% Asosclon in 4% Butanol in n-Decane) was added to the loading buffer and incubated for 1 h. The resulting mixture was loaded into the chamber. The relative intensities of bands corresponding to toxin monomer (lane 3) and the activated toxin monomer (lanes 4, 10). These were subjected to different treatments including addition of DTT at 10 mM (lanes 3 and 7), treatment with LDAO detergent at 2 times its CMC (Fig. 4c, lanes 1–7) or heating to 95 °C (lane 9) for 1 h. Samples were thereafter loaded on native PAGE (6% acrylamide), SDS-PAGE (6%) (Supplementary Fig. 10). Gels were stained as described above and digitalized using the Image Lab software, version 6.0 (BioRad, France). The distance between each band in the oligomer population was determined using the software ImageJ v1.51k. The linear correlation between (i) the size of the oligomer and the elapsed time since toxin addition (Fig. 5g); (ii) the hole depth and the hole surface (Fig. 5f); and (iii) the hole depth and the hole size (Fig. 5c) was tested using non-parametric Spearman’s Rho rank correlation coefficient, as implemented in the software R 3.5.2.2. Plot correglograms and 95% confidence regions were generated using the "ggpubr", "corplot" and "Hmisc" libraries.

**Oligomer formation and stoichiometry characterization.** To test the different conditions necessary for obtaining oligomers of Cyt1Aa and its toxin liposomes, were prepared by the standard film-hydration method as previously described. Briefly, liposomes were produced by drying L-a-phosphatidylcholine (POPC; Avanti Polar Lipids, France) under nitrogen flow to obtain a thin lipid film. Residual chloroform was eliminated by overnight vacuum. The lipid film was resuspended in phosphate-NaCl buffer (0.1 M K2HPO4/KH2PO4, 0.15 M NaCl, pH 7.4) by vortexing for 5 min. The multilamellar vesicles obtained were freeze-thawed (100–310 K) 20 times to obtain LUVs. Size calibration was performed either by sonication in an ice-cold water bath for 5 min (yielding ~50 nm radius small unilamellar vesicles or SUVs) or by extrusion using a mini-extruder (Avanti Polar Lipids, USA) with 200 nm polycarbonate filters yielding ~100 nm radius large unilamellar vesicles or LUVs). Homogenous size distribution was verified by dynamic light scattering (DLS) on a Wyatt DynaPro NanoStar. Prepared liposomes were directly used or stored for a maximum of 4 days at 4 °C until use.

The capacity of the three different forms (disulfide-bridged dimer, protoxin monomer and toxin monomer) of WT and mutants Cyt1Aa to generate oligomers upon contact with membranes was determined using SDS-PAGE 6% (Fig. 4c). Samples were mixed with Laemmli buffer devoid of DTT, electrophoresed for 90 min at 140 V, and revealed using InstantBlue (Sigma Aldrich, France). The three WT Cyt1Aa species that possibly co-exist in the mosquito larvae gut were tested, namely the disulfide-bridged protoxin dimer (Fig. 4c, lanes 1–2), the protoxin monomer (lane 3) and the activated toxin monomer (lanes 4–10). These were subjected to different treatments including addition of DTT at 10 mM (lanes 3 and 6); exposure to LUV (lanes 2, 3, 5–7, 9 and 10) or SUV (lane 8); heating to 95 °C (lane 7); treatment with LDAO detergent at 2 times its CMC (Critical Micelle Concentration) (lane 9); or heating to 95 °C for 1 h (lane 10). Kinetics of oligomerization were assayed by exposing the protoxin and toxin monomers to LUVs for 15 different durations (2, 5, 7, 10, 12, 15, 30, 45 min and 1, 2, 3, 4, 5, 7 and 10 h), each rinse, 40 μL is removed and subsequently 40 μL of fresh buffer is added). Before adding the protein, a drop of 40 μL of fresh buffer was deposited on the supported bilayers on the mica. Native membranes were imaged first and a proper fusion was observed in the areas of uninterrupted lipids, the drop was replaced by 40 μL of fresh buffer and 4 μL of the toxin at a concentration of 4 mg mL−1 was injected in the drop. ScanAssay fluid cantilevers (k = 0.7 N m−1, Fq = 150 kHz, nominal radius tip = 20 nm, Bruker probes, Camarillo, CA, USA) were used. 512 × 512 pixel images were collected at ~1 Hz rate, with scan sizes varying from 7 to 1.7 μm in length, using the ScanAssay mode in a semi-automatic condition where both the gain and the set-point (from 40 to 120 mV) were manually adjusted. The default ramp size for the peak-force mode was kept at 150 nm.

TIFF images were imported into the software ImageJ v1.51k to calculate the surface of each oligomer as a function of elapsed time since toxin addition. Considering that the data did not follow a normal distribution (Shapiro–Wilk test, W = 0.92, p < 2.2 × 10−16, W = 0.96, p = 0.12; W = 0.91, p < 2.2 × 10−16), the linear correlation between (i) the size of the oligomer and the elapsed time since toxin addition (Fig. 5g); (ii) the hole depth and the hole surface (Fig. 5f); and (iii) the hole depth and the hole + MBA surface (Fig. 5k) was tested using non-parametric Spearman’s Rho rank correlation coefficient, as implemented in the software R 3.5.2.2. Plot correglograms and 95% confidence regions were generated using the “ggpubr,” “corplot” and “Hmisc” libraries.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Structures and structure factor amplitudes have been deposited in the PDB databank under accession codes 6T1A (“pIIF,” [10.2210/pdbT11i/pdb]), 6T1B (“DTT,” [10.2210/pdbT10l/pdb]), STA (P3H0, [10.2210/pdbTIA/pdb]) and 6TIC (“CSS mutant,” [10.2210/pdbTIC/pdb]). The source data for Figs. 3, 4a–g, 5d–h and 5j–q and for Supplementary Figs. 5, 7–12 and 14b are provided as a Source Data file. Other data are available from the corresponding author upon reasonable request.

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**References.**

1. Vachon, V., Laprade, R. & Schwartz, J. L. Current models of the mode of action of Bacillus thuringiensis insecticidal crystal proteins: a critical review. J. Invertebr. Pathol. 111, 1–12 (2012).

2. Sohneer, M., Lopez-Diaz, V. A. & Bravo, A. Cyt toxins produced by Bacillus thuringiensis: a protein fold conserved in several pathogenic microorganisms. Peptides 41, 87–93 (2013).

3. Butko, P. Cytoytic toxin Cry1A and its mechanism of membrane damage: data and hypotheses. Appl. Environ. Microbiol. 69, 2415–2422 (2003).

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4. Knowles, B. H. et al. A cytolytic δ-endotoxin from Bacillus thuringiensis var. israelensis forms cation-selective channels in planar lipid bilayers. FEBS Lett. 244, 259–262 (1989).
5. Drobniewski, F. A. & Ellar, D. J. Investigation of the membrane lesion induced in vitro by two mosquitoicidal δ-endotoxins of Bacillus thuringiensis. Curr. Microbiol. 16, 195–199 (1988).
6. Knowles, B. H. & Ellar, D. J. Colloid-osmotic lysis is a general feature of the membrane lesion of Bacillus thuringiensis δ-endotoxins with different insect specificity. Biochim. Biophys. Acta 924, 509–518 (1987).
7. Manceva, S. D., Pusztai-Carey, M., Russo, P. S. & Butko, P. A. A detergent-like mechanism of action of the cytolytic toxin CryIA from Bacillus thuringiensis var. israelensis. Biochemistry 44, 589–597 (2005).
8. Wu, D. & Federici, B. A. A 20-kilodalton protein preserves cell viability and promotes CryIA cytotoxicity during sporulation in Bacillus thuringiensis. J. Bacteriol. 175, 5276–5280 (1993).
9. Coquelle, N. et al. Raster-scanning serial protein crystallography using micro- and nano-focused synchrotron beams. Acta Crystallogr. D. 71, 1184–1196 (2015).
10. Liang, M. et al. The coherent X-ray imaging instrument at the Linac coherent light source. J. Synchrotron Radiat. 22, 514–519 (2015).
11. Serra, R. G. et al. Concentric-flow electromagnetic injector enables serial crystallization of ribosome and photosystem II. Nat. Methods 13, 59–62 (2016).
12. Cohen, S. et al. Cry1Aa toxin: crystal structure reveals implications for its membrane-perforating function. J. Mol. Biol. 413, 804–811 (2011).
13. Bennett, M. J., Choe, S. & Eisenberg, D. Domain swapping: entangling complexes of Cry11Aa during spore formation from Bacillus thuringiensis var. israelensis. Biochemistry 44, 589–597 (2005).
14. Li, J., Koni, P. A. & Ellar, D. J. Structure of the mosquitoicidal δ-endotoxin CryB from Bacillus thuringiensis sp. kyushuensis and implications for membrane pore formation. J. Mol. Biol. 257, 129–152 (1996).
15. Walker, E. D. Effect of low temperature on feeding rate of Ae. aegypti larvae and efficacy of Cry11Aa toxin from Bacillus thuringiensis var. israelensis (H-14). J. Am. Mosq. Control Assoc. 11, 107–110 (1995).
16. Florence, T. M. Degradation of protein disulfide bonds in dilute alkali. Biochem. J. 189, 507–520 (1980).
17. Settadhi, A. T. et al. Effect of pH on the oxidation-reduction properties of thioredoxins. Biochemistry 42, 14877–14884 (2003).
18. Monahan, F. J., German, J. B. & Kinsella, J. E. Effect of pH and temperature on processing from both the N- and C-termini. J. Biol. Chem. 267, 11742–11748 (1992).
19. 44. Siddiqui, S. A. S. & Ellar, D. J. Maximal toxicity of cloned Cry1A δ-endotoxin from Bacillus thuringiensis subsp. israelensis requires proteolytic processing from both the N- and C-termini. Microbiology 141, 3141–3148 (1995).
20. Canton, P. E., Lopez-Diaz, J. A., Gill, S. S., Bravo, A. & Soberon, M. Membrane binding and oligomer membrane insertion are necessary but insufficient for Bacillus thuringiensis Cry1Aa toxicity. Peptides 35, 286–291 (2014).
21. Lopez-Diaz, J. A., Emilianio Canton, P., Gill, S. S., Soberon, M. & Bravo, A. Oligomerization is a key step in Cry1Aa membrane insertion and toxicity but not necessary to synergize Cry11Aa toxicity in Ae. aegypti larvae environment. Environ. Microbiol. 15, 3030–3039 (2013).
22. Butko, P., Huang, F., Pusztai-Carey, M. & Surewicz, W. K. Membrane permeabilization induced by cytolytic δ-endotoxin CryA from Bacillus thuringiensis var. israelensis. Biochemistry 35, 11335–11346 (1996).
23. Promdonkoy, B. et al. Amino acid substitutions in αA and αC of Cry2Aa2 alter hemolytic activity and mosquito-larvicidal specificity. J. Bacteriol. 133, 287–293 (2008).
24. Promdonkoy, B. & Ellar, D. J. Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from Bacillus thuringiensis. Biochem. J. 374, 255–259 (2003).
25. Wen, Y.-P., Lin, Y.-P., Hsu, C.-I. & Lin, J.-Y. Functional domains of a pore-forming cardiototoxic protein, volvatoxin A2. J. Biol. Chem. 279, 6805–6814 (2004).
26. Lawrence, S. L. et al. Crystal structure of Streptococcus pneumoniae pneumolysin provides key insights into early steps of pore formation. Sci. Rep. 5, 14352 (2015).
27. Van Pee, K. et al. CryoEM structures of membrane pore and pre pore complex reveal cytolytic mechanism of Pneumolysin. eLife 6, 23644 (2017).
28. Colletier, J. P. et al. De novo phasing with X-ray laser reveals mosquito lysis in the BInAB structure. Nature 539, 43–47 (2016).
29. Federici, B. A., Park, H. W., Bideshi, D. K., Wirth, M. C. & Johnson, J. J. Recombinant bacteria for mosquito control. J. Exp. Biol. 206, 3877–3885 (2003).
30. Du, J. P., Knowles, B. H., Li, J. & Ellar, D. J. Biochemical characterization of Bacillus thuringiensis cytolytic toxins in association with a phospholipid bilayer. Biochemistry 28, 198–203 (1989).
31. Rigden, D. J. Does distant homology with Evf reveal a lipid binding site in Bacillus thuringiensis cytolytic toxins? FEBS Lett. 583, 1555–1560 (2009).
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Author contributions
J.-P.C designed and coordinated the project; G.T., A.-S.B., E.A.A., N.B., H.W.P. and B.F. designed and constructed plasmids; G.T., A.-S.B., E.A.A., N.B., F.L. and I.D.Y. produced Cry1Aa crystals in vivo; I.S. performed crystal visualization by SEM; D.F., J.-M.T., and J.-L.P. performed crystal visualization by AFM; A.-S.B. and J.-P.C. secured beamtime at the ESRF; G.T., A.-S.B., E.A.A., T.G., M.R., M.Bu., and J.-P.C. performed serial data collection at the ESRF; G.T., A.-S.B., E.A.A., D.C., M.W., M.S., and J.-P.C. secured beamtime at the LCLS; G.T., A.-S.B., E.A.A., A.S.B., R.G.S., I.D.Y., S.B., D.C., M.R.S., N.K.S., M.S.H., and J.-P.C. performed serial data collection at the LCLS; J.-P.C. performed atomic model building, refinement and structure interpretation; A.S.B., I.D.Y., T.G., N.C., M.Bu., N.K.S. produced new processing tools or devices; A.S.B., I.D.Y., M.K.S., and J.-P.C. performed solubilization assays; G.T. and E.A.A. performed heat stability assays; G.T., J.B., MT.F.L., M.D., E.G., R.S., N.Z., and B.F. performed cytotoxicity assays; A.B., M.Ba., and I.G. conducted transmission electron microscopy imaging; J.-M.T. and J.-L.P. conducted atomic force microscopy imaging on membranes; G.T., J.A.B., and M.W. conducted black lipid membrane experiments; G.T. performed the statistical analyses; G.T. and J.-P.C. prepared the manuscript with input from coauthors.

Competing interests
The authors declare no competing interests.

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