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Rapid report

A trimeric building block model for Cry toxins in vitro ion channel formation

Jaume Torres [a,*], Xin Lin [a], Panadda Boonserm [b]

[a] School of Biological Sciences, Nanyang Technological University, 60, Nanyang Drive, Singapore 637551, Singapore
[b] Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Phuttamonthol, Nakornpathom 73170, Thailand

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Abstract

The crystal (Cry) insecticidal toxins, or δ-endotoxins, are lethal to a wide variety of insect larvae, and are therefore very important in insect control. Toxicity has been explained by formation of transmembrane oligomeric pores or ion channels and, more recently, by the ability of the monomeric toxin to subvert cellular signaling pathways. The structure, topology, and precise role of the putative pore in toxicity are not known. However, in vitro biophysical studies suggest that helices α4 and α5 in domain I insert into the lipid bilayer as an α-helical hairpin. Mutagenesis studies have assigned an important role to α5 in maintaining oligomerization, and to α4 in channel formation. To detect the possible homo-oligomerizing tendencies of these two helices, we have used the evolutionary conservation data contained in sixteen Cry homologs in order to filter non-native interactions found during a global conformational search. No conserved homo-oligomer was found for α4, but a right handed trimeric α5 model was present in the simulations of all Cry sequences. We propose a model for Cry toxin oligomerization based on sequence analysis and available mutagenesis data.

Keywords: Cry toxin; *Bacillus thuringiensis*; Pore; Biopesticide; Molecular dynamics; Membrane

The crystal (Cry) insecticidal toxins produced by the Gram positive soil bacterium *Bacillus thuringiensis* (Bt) are toxic to a wide variety of insect larvae [1]. Several of these Cry toxins have been crystallized [2–5] and contain three distinct domains: the N-terminal domain I, formed by 7 α-helices, α1 to α7, and domains II and III, important for binding specificity and structural integrity [6].

Toxicity requires solubilization of the pro-toxin crystals in the highly alkaline insect midgut lumen, followed by activation by intestinal proteases. These activated toxins bind to specific receptors on the midgut brush border membrane (BBM) of the larvae.

Two mechanisms have been proposed to explain Cry toxin larvicidal activity. One is based on data from non-susceptible insect cells that express the *Manduca sexta* Cry1Ab toxin receptor, Bt-R1. In this system, toxicity was associated to the sequential activation, by the toxin monomer, of a cell signaling pathway which ultimately leads to cell cytoskeleton destabilization [7].

The above mechanism is in contrast with what has been the prevailing paradigm to explain toxicity for the last 20 years, which basically invokes the formation of a lytic transmembrane pore [8] mediated by domain I [9]. In this pore model, oligomerization is necessary [10] for toxicity, although not sufficient [11,12].

Indeed, SDS-resistant oligomers, mainly trimers, have been observed after the toxin binds BBM vesicles [12–14] or synthetic-lipid liposomes [11]. Oligomers of ambiguous oligomeric size, trimers or perhaps tetramers, have been observed using Atomic Force Microscopy (AFM) in model lipid bilayers [15,16]. More recently, a clear trimeric form has been reported using electron crystallography data obtained from 2D crystals [17].

A "pre-pore" tetrameric oligomer of ~250 kD, that would form after interaction of Cry1 toxins with the cadherin receptor, has also been reported [18–20]; this tetramer showed enhanced membrane insertion efficiency and homogeneous ion conductance when compared to the monomeric form [18,19], and has been proposed to be the pore-forming species.

* Corresponding author. Tel.: +65 6316 2857; fax: +65 6791 3856.
E-mail address: jtorres@ntu.edu.sg (J. Torres).

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The precise nature of the putative pore or channel observed in vitro, however, is not known [4,8,21]. In the so-called "umbrella" model [22], only helices α4 and α5 of domain I are inserted into the membrane as an α-helical hairpin [23,24], with the remaining α-helices in domain I parallel to the membrane surface.

Mutagenesis and biophysical data indicate that helix α5 is involved in oligomerization, whereas helix α4 is lining the lumen of a putative channel or pore [24]. Indeed, α5 mutations that affect toxicity generally also affect oligomerization [11,12]. Further, synthetic α5 peptides, but not α4, oligomerize in solution as well as in membranes. Also, mutations at α4 which render the protein non-toxic, or reduce conductance in planar lipid bilayers, do not affect either oligomerization [12,14] or binding to BBM vesicles [25,26]. However, some role of α5 in ion transport is likely, because α5 can form ion channels in planar lipid bilayers [27] and a charge dependency has been observed on ion conductance for residue H168 of α5 in Cry1Ac [28,29].

In the present work, we have performed a computational study to detect possible homo-oligomeric interactions in either α4 or α5. We have explored all possible conformational space [30] for a given transmembrane α-helical bundle. In our approach [31], low energy models are filtered by performing the same simulation for many homologous sequences that are likely to share the same structure. The rationale is that the mutations present in these sequences will destabilize only incorrect models, but not the correct native one. A stable structure which is present simultaneously in the simulations of all sequences tested, hereafter a 'complete set', is considered to represent the native interaction. This method is now established, and we have used it over the last few years to predict a number of transmembrane α-helical bundles [32–36].

Herein, sixteen homologous sequences of Bt toxins were used (Fig. 1), obtained from the National Center for Biotechnology Information (NCBI). Simulations were performed using a Compaq Alpha Cluster SC45 which contains 47 nodes, and calculations were carried out using the parallel version of the Crystallography and NMR System (CNS Version 0.3, PCNS) [37], as described elsewhere [30]. The protocol for the global search and clustering of structures has been described previously [33,34]. For each sequence, three trials were carried out for each starting configuration using different initial random velocities, testing right and left-handed configurations, from dimers to hexamers, and producing a total of 84,000 structures.

We found no 'complete set' for α4 homo-oligomers, for any tilt, handedness or oligomeric size. When simulations were performed for α5 sequences, however, one backbone structure was shared by all homologues: a trimer in a right handed crossing angle with an average helix tilt of 30°. This trimer is shown in Fig. 2A for the sequence Cry1Ab. The Cα RMSD (root mean square deviation) between any pair of structures within this 'complete set' was never higher than 1.3 Å. No other 'complete sets' were found for other tilts or oligomeric sizes (results not shown).

The orientation of residue N166 (Fig. 2A, upper panel) is consistent with mutagenesis results in Cry4Ba, where alanine

Cry1Ab	126	LREEMRIQFNDMNSALTTAIPLF	148	AAW31761
Cry1Ba2	145	TRSVLHTQYIALELDFLNAMPLF	167	CAA65003
δ-Endotoxin	170	SQGRIRELFSQAESHFRNSMPSF	192	AAA22336
Cry3Aa	170	SQGRIRELFSQAESHFRNSMPSF	192	AAU29411
Cry3Ca	160	SQGRIRELFSQAESHFRRSMPSF	182	Q45744
Cry3Bb2	163	SQDRIRELFSQAESHFRNSMPSF	185	AA74198
Cry9Ed1	166	—NLAFQRFENLHTAFVTRMPSF	186	AAX78440
Cry9Db1	166	—SLVSQRFNILDSLFTQFMPSF	186	AAX78439
Cry9Bb	163	—NLVLQRFENLHALFVSSMPSF	183	AAV28716
Insecticidal CP	157	TRSVLYTQYIALELDFLNAMPLF	179	AAO39720
Cry8	164	ARSVVVTQYIALELDFVAKIPSF	186	BAC07226
Cry8Ca	165	SAALVKERFGNAEAILRTNMGSF	187	Q45706
Cry1Cb	125	TRTRVVDRFRILDGLLERDIPSF	147	P56953
δ-Endotoxin CryIII	164	TRSVVSNQFIALDLNFVSSIPSF	186	AAA21117
Cry032	124	LKEEMRTQFNDMNSILVTAIPLF	146	AAL50330
Cry4Ba	142	YRTAVITQFNLTSAKLRETAVYF	164	P05519

Cry1Ab	157	LLSVYVQAANLHLSVLRDVSVFG	179
Cry1Ba2	176	LLMVYAQAANLHLLLLRDASLFG	198
δ-Endotoxin	201	FLTTYAQAANTHLFLLKDAQIYG	223
Cry3Aa	201	FLTTYAQAANIHLFLLKDAQIYG	223
Cry3Ca	191	FLPTYAQAANTHLLLLKDAQIYG	213
Cry3Bb2	194	FLPTYAQAANTHLLLLKDAQVFG	216
Cry9Ed1	199	LLTVYAQAANLHLLLLKDAEIYG	221
Cry9Db1	199	LLPVYAQAANLHLLLLKDADIYG	221
Cry9Bb	196	LLLVYAQAANLHLLLLRDAEIYG	218
Insecticidal CP	188	LLMVYAQAANLHLLLLRDASLYG	200
Cry8	195	LLSVYAQAANLHLLLLRDASIFG	217
Cry8Ca	196	LLPTYAQAASLHLLVMRDVQIYG	218
Cry1Cb	156	LLSVYAQAANLHLAILRDSSIFG	178
δ-Endotoxin CryIII	195	LLAVYAQAVNLHLLLLRDASIFG	217
Cry032	155	FLSVYVQAANLHLSVLRDVSVFG	177
Cry4Ba	174	LLPIYAQVANFNLLLIRDGLINA	196

Fig. 1. Alignments of all α4 (upper panel) and α5 (lower panel) sequences used in this work, with specific numbering indicated. The column on the right (top panel) shows the NCBI accession numbers. The conserved Asn residue in α5 is highlighted in red and a conserved pair of charged residues is indicated in blue.

mutation at the equivalent residue N183 (see Fig. 1) showed complete loss of both trimerization and toxicity [11], whereas similar mutations at other α5 polar residues did not affect either. Also, substitutions with polar residues, N183Q and N183T, retained high toxicity in contrast to changes to Arg, Lys, or Phe. This is consistent with the stabilizing effect of asparagine and other polar residues in transmembrane α-helix–helix interactions [38,39].

The short distance, less than 2.0 Å, between the amine hydrogen (HH) in R173 and the carboxylic oxygen atom (OD) in D174, (Fig. 2A, lower panel) suggests that further stabilization of this trimer may be provided by an inter-helical salt bridge. Indeed, these two residues are well conserved (Fig. 1, lower panel, blue). Evidently, this salt bridge had a strong stabilizing effect during our simulations (see Fig. 2B, red rhombes), and could have produced a biased result.

However, we repeated each simulation after changing the consecutive residues RD/KD to AA, and we still found the same structure as a 'complete set', despite the energies of the clusters being similar in magnitude (see Fig. 2B). This suggests that Van der Waals and polar residue interactions are sufficient to make this interaction specific, and that the putative salt bridge merely stabilizes the complex.

The rotational orientation of this trimer (see legend of Fig. 2B) could not be confirmed using site specific infrared dichroism

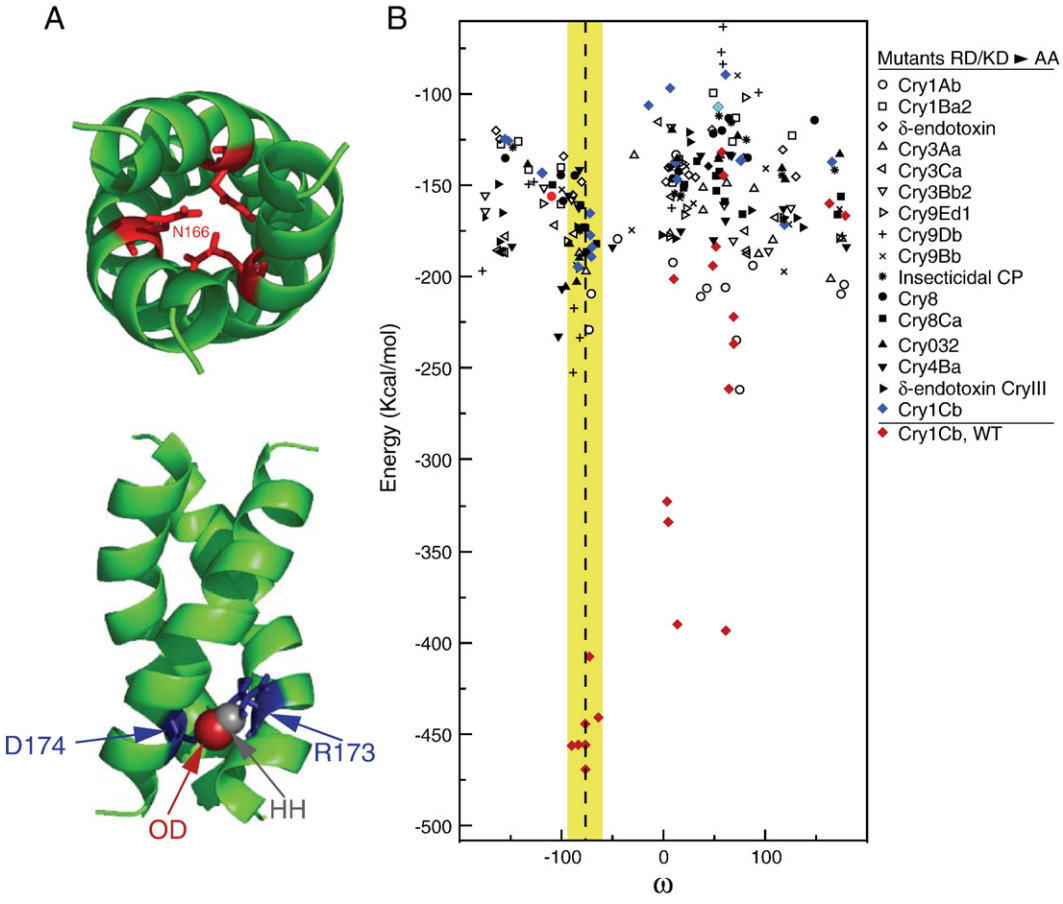

Fig. 2. A, representative model of evolutionarily conserved α5 homotrimer, for sequence Cry1Ab. Upper panel: top view, with residue N166 (red) indicated. Lower panel: side view, with amine hydrogen HH [50] atom in R173 and carboxylic oxygen OD [50] atom in D174 shown as spheres. B, energy plot versus helix rotational orientation (ω defined in [31]) of the low energy clusters found after a global search of α5 homo-trimeric bundles when RD, or KD, were mutated to AA (see symbols on the right). The parameter ω was calculated for N166, or equivalent residues (highlighted red in Fig. 1). The ω corresponding to the conserved trimer (shown in Fig. 2A), both for native sequences and for mutated to AA sequences, is indicated by a broken line, centered at $-78° \pm 15°$ (yellow column).

(SSID [40,41]) because synthetic α5 shows both α-helical and β-structure conformation when incorporated into DMPC bilayers (data not shown), suggesting that the correct insertion of α5 requires the presence of α4.

In an α-helical hairpin, the orientation between the helices is antiparallel, therefore the N-terminus in α4 and the C-terminus in α5 should be in close apposition. Although the nature of the helix contacts in the α4–α5 interaction are not known, a hint to the solution may be found in the simultaneous mutations, potentially involved in inter-helix salt bridges, observed in certain sequences (Fig. 3). For example, in Cry3 the third N-terminal residue of α4 is positively charged (R), and the third C-terminal residue in α5 is negatively charged (E or D), whereas in Cry1 sequences, the charge of these residues is changed (E in α4, R in α5).

We have also observed the presence of possible opposite-charge pairs in other sequences, although the picture looks more uncertain due to the lack of structural information and imprecise alignment of α4 and α5 in the membrane. Thus, we propose that a trimer of α4–α5 hairpins is stabilized by α5–α5 and α4–α5 salt bridges, which is reminiscent of the transmembrane interactions described in the T-cell receptor [42].

A central α5 trimer with adjacent α4 helices (Fig. 4, black dotted line), however, is unlikely to represent a pore or a channel; first, because three α-helices are not sufficient to form a channel, and second, because experimental data point to predominantly

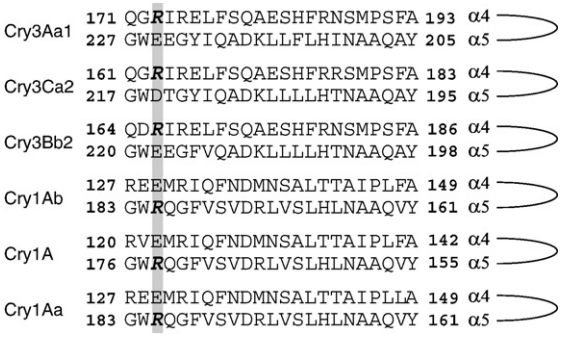

Fig. 3. Hairpins formed by helices α4 and α5 in several Cry3 and Cry1 toxins. The residues involved in a putative α4–α5 salt bridge are highlighted in gray.

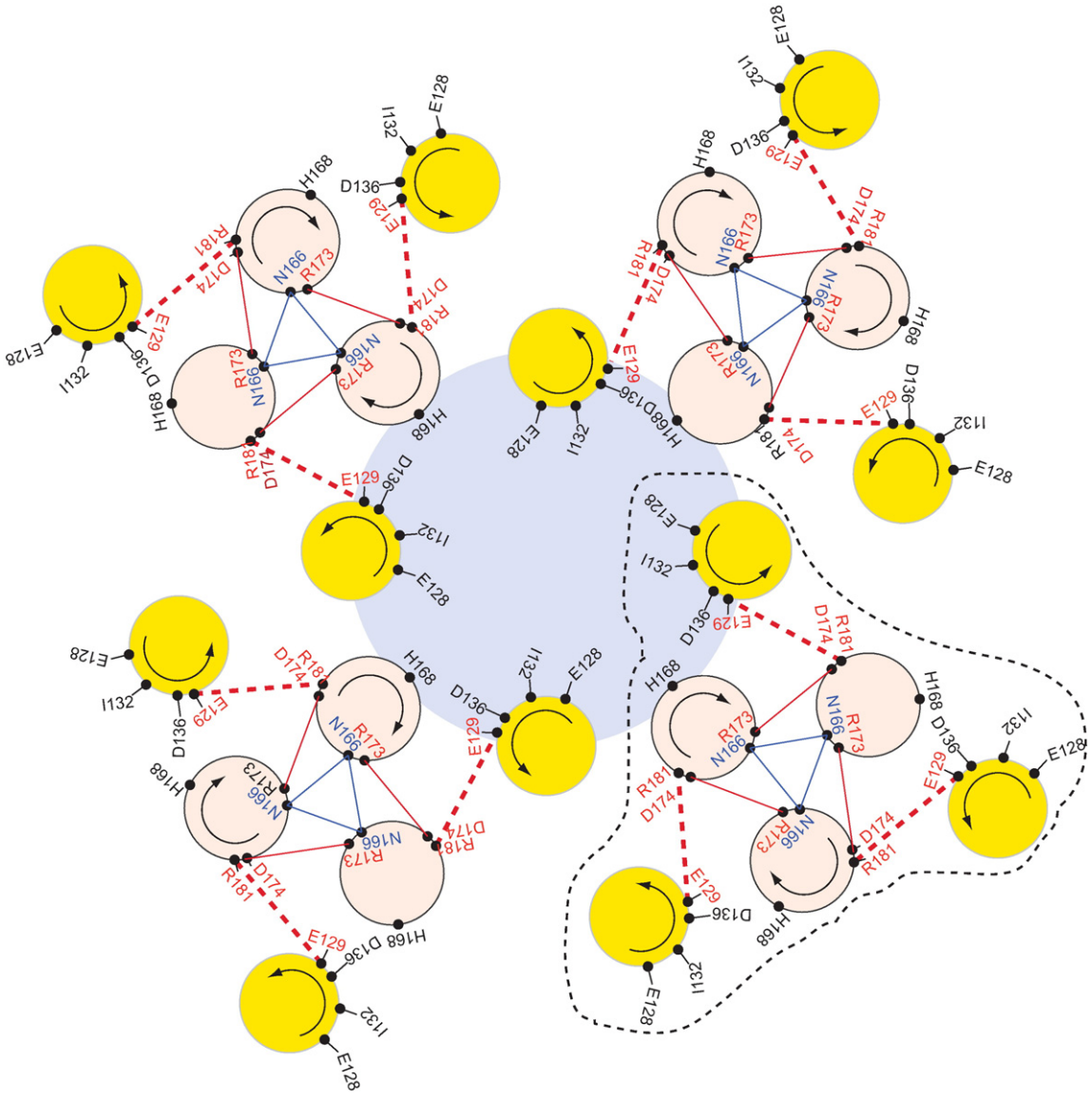

Fig. 4. Symmetric oligomeric arrangement using stable trimers (black broken line) of helices α4 (yellow) and α5 (pink). Residue numbers correspond to Cry1Ab. α5–α5 interactions between N166 (blue broken lines), and putative α4–α5 (red broken thick line) or α5–α5 salt bridges (red broken thin line) are indicated. The curved arrow indicates the direction from N- to C-terminus. For α5, the α carbon atoms in the conserved trimer (Fig. 2A) have been projected on a plane parallel to the lipid bilayer. For α4, we have used a canonical representation of a helical wheel. Residues H168 in α5, and D136, I132, and E128 in α4, face the lumen of the putative pore, in agreement with previous mutagenesis data (see text and references). A tetramer of trimers is represented, but a trimer or a pentamer of trimers is equally possible.

α4, not α5, lining its lumen. Therefore, we propose that further aggregation between these trimers is necessary where both α4, and partially α5, line the lumen of the pore (see considerations above).

A symmetric arrangement can be obtained if each trimer, $[\alpha4–\alpha5]_3$, contributes only one α4 and one α5, each from a different monomer, to the lumen of the pore (Fig. 4). This model is compatible with mutagenesis data, with residues H168 in α5 [28,29], and D136 [25,26], E128 [26], and I132 [14] in α4 exposed to the lumen, and is also compatible with the salt bridge interactions proposed here (see Fig. 4, legend).

Thus, we predict that membrane association of Cry toxins, either as a monomer or as a pre-pore tetramer [10,43], leads to a conformational change with concomitant membrane insertion of an α4–α5 helical hairpin. Homo-trimerization is then driven by the contacts between the lipid-exposed α5 and α4 helices. We propose that weak interactions between these stable trimeric building blocks are required for the lytic pore observed in vitro, and that these interactions may be optimal when a tetrameric pre-pore intermediate has been formed ‘upstream’.

The latter could explain the multiple, non-specific, conductance states observed when toxin monomers are incubated with receptor-free membranes, and the elusive character of the Cry toxin pore, which herein we assume is held by comparatively weak interactions between stable homotrimers. This model is also consistent with the observation of large conductance states

in Cry1Ac, Cry3A, Cry3B, and Cry1C in synthetic planar lipid bilayers [44] and the estimate of a pore size of 1–2 nm [8,45], with a pore formed by 4 to 6 toxin monomers.

Finally, we stress that the oligomeric size of the toxin after membrane insertion has been usually studied in SDS, which is not its native environment; samples analyzed in less stringent conditions (not boiled, 0.1% SDS) showed other intermediate oligomers [46]. Other factors however, are likely to contribute to toxicity because it has been shown that tetrameric oligomers can form in the absence of receptors, and are not toxic to insect cells [47]. The conditions under which each mode of Cry toxin action, pore lytic model [10,20,22,48] and modification of signal transduction pathways [7,47] is predominant, the precise role of lipid rafts, or if a combination of mechanisms is possible [49], are not yet elucidated.

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