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Structural Basis of Pore Formation by Mosquito-larvicidal Proteins from *Bacillus thuringiensis*

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Abstract: The insecticidal character of the three-domain Cry δ-endotoxins produced by *Bacillus thuringiensis* during sporulation is believed to be caused by their capability to generate lytic pores in the target larval midgut cell membranes. This review describes toxic mechanisms with emphasis on the structural basis of pore formation by two closely related dipteran-specific toxins, Cry4Aa and Cry4Ba, which are highly toxic to mosquito larvae. One proposed toxic mechanism *via* an "umbrella-like" structure involves membrane penetration and pore formation by the α4-α5 transmembrane hairpin. The lipid-induced β-conformation of α7 could possibly serve as a lipid anchor required for an efficient insertion of the pore-forming hairpin into the bilayer membrane. Though current electron crystallographic data are still inadequate to provide such critical insights into the structural details of the Cry toxin-induced pore architecture, this pivotal evidence clearly reveals that the 65-kDa active toxin in association with the lipid membrane could exist in at least two different trimeric conformations, implying the closed and open states of a functional pore.

Keywords: Cry δ-endotoxins, membrane-associated toxin complex, oligomericity, transmembrane helical hairpin, trimeric pore structure, umbrella-like model.

INTRODUCTION: PROTEIN-BASED BIOINSECTICIDES

In many parts of the world, particularly the developing countries, various insect species cause certain serious problems for human beings by acting as disease vectors. Dengue and malaria, transmitted respectively by mosquitoes of the genus *Aedes* (usually *Ae. aegypti* and *Ae. albopitus*) and *Anopheles*, are important vector-borne diseases which have a significant social and economic impact in many tropical countries. In the past, control of such disease vectors relied mainly on the intensive exploitation of chemical insecticides. Despite the success of this tactic, growing public concerns regarding the environmental incompatibility and insect resistance of many chemical insecticides have encouraged a greater interest in environment-friendly alternatives. A number of microorganisms that are pathogenic to insect larvae have become a preferred choice over chemical agents because they have higher specificity and often have lower rates of resistance development (Chandler TFST 2008) [1] (Thomas NRM 2007) [2]. One of the most promising candidates is *Bacillus thuringiensis* (*Bt*), a family of Gram-positive sporulating soil bacteria that synthesise cytoplasmic crystalline toxin inclusions with specific larvicidal activity (Schnepf MMRV 1998) [3]. The larvicidal properties of *Bt* toxin inclusions have already been exploited commercially as safe protein-based bioinsecticides for over two decades in agricultural and forestry pest management, as well as in disease-carrying vector control (Schnepf MMRV 1998) [3] (Federici JIP 2005) [4].

Bt cells synthesise highly specific larvicidal proteins in large quantities as different forms of parasporal crystalline inclusions which are released together with the spore upon completion of sporulation (Schnepf MMRV 1998) [3]. These cytoplasmic inclusions are composed of one or several insecticidal proteins known as "δ-endotoxins" which have been classified into two main families: the Cry (for Crystal) and Cyt (for Cytolytic) toxins based on the similarity of their deduced amino acid sequences (Höfte Microbiol Rev 1989) [5] (Crickmore MMRV 1998) [6]. The Cry toxins, which generally have two different sizes (~70 kDa or ~135 kDa), display amino acid identities varying between 20% and 90% whilst the ~27-kDa Cyt toxins show no sequence similarity to the Cry toxins (Höfte Microbiol Rev 1989) [5] (Crickmore MMRV 1998) [6]. Unlike the Cyt toxins which possess a broad-spectrum cytolytic activity *in vitro* but appear to be active only against mosquito larvae, the Cry toxins have been shown to be toxic to a wide variety of insect larvae in the orders Coleoptera (beetles and weevils), Diptera (mosquitoes and flies), Hymenoptera (wasps and bees) and Lepidoptera (moths and butterflies) (Schnepf MMRV 1998) [3] (De Maagd Trends Genet 2003) [7], and even to some non-insect species such as nematodes (Wei PNAS 2003) [8]. This suggests that many other important targets for the Cry toxins remain to be discovered. Nevertheless, the variation in molecular masses of the Cry toxins and their different insecticidal spectra raises a question as to whether they all share the same toxic mode of action.

Biochemically, *Bt* Cry toxins are sequestered as insoluble inactive protoxins which require dissolution in the larval midgut lumen (generally alkaline pH for dipteran and

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lepidopteran larvae), and subsequently processed by gut proteases to yield ~65-kDa toxic fragments (Schnepf MMRV 1998) [3]. For several Cry toxins, it has been shown that the activated toxins bind to specific receptors lining the apical brush-border membranes of midgut epithelial cells and possibly undergo conformational changes and oligomerisation (Pigott MMBR 2007) [9] (Gómez Peptides 2007) [10]. One widely accepted hypothesis is that this is followed by membrane insertion and pore formation, causing a net influx of ions and water that leads to osmotic cell lysis, resulting in severe damage of the midgut and eventual death of the insect larvae (Knowles AIP 1994) [11] (Whalon AIBP 2003) [12]. Even so, recent studies have proposed a dissimilar toxic mechanism by which the toxin-receptor interaction induces death of the target cells *via* certain signaling pathways (Zhang PNAS 2006) [13]. Despite the fact that knowledge of the molecular mechanisms of Cry toxin function has increased substantially over the last decade (Schnepf MMRV 1998) [3] (Pigott MMBR 2007) [9] (Whalon AIBP 2003) [12] (Bravo Trends Biotechnol 2009) [14], very little is known about the detailed structures of the toxin-induced pores. Herein, the review is primarily concerned with a toxic mechanism of two closely related mosquito-larvicidal proteins − Cry4Aa (134 kDa) and Cry4Ba (128 kDa) − which are highly toxic to the larvae of *Aedes* and *Anopheles* mosquitoes, major vectors for the life-threatening human diseases of dengue hemorrhagic fever and malaria (Schnepf MMRV 1998) [3] (Federici JIP 2005) [4]. As such, our group is devoted to delineating the structural basis of membrane-pore formation by these mosquito-larvicidal proteins.

STRUCTURAL DESCRIPTION OF THE THREE-DOMAIN TOXINS

Thus far, the three-dimensional structures of *Bt* Cry toxins have been determined by X-ray crystallography in almost all the major specificity classes, including the lepidoteran-specific Cry1Aa (PDB code: 1CIY) (Grochulski JMB 1995) [15], the lepidopteran/dipteran-dual specific Cry2Aa (PDB code: 1I5P) (Morse Structure 2001) [16], the coleopteran-specific Cry3Aa (PDB code: 1DLC) (Li Nature 1991) [17] and Cry3Bb (PDB code: 1JI6) (Galitsky ACD 2001) [18], the dipteran-specific Cry4Aa (PDB code: 2C9K) (Boonserm J Bacteriol 2006) [19] and Cry4Ba (PDB code: 1W99) (Boonserm JMB 2005) [20], and more recently another coleopteran-specific Cry8Ea (PDB code: 3EB7) (Guo JSB 2009) [21]. Undoubtedly, all these known structures have been a valuable contribution to the *Bt* research area since they have been providing a greater understanding for the structural basis of their insect specificity and gut epithelial cell lysis. Even if these Cry toxins exert their insecticidal activity against different target insect larvae, they all show a wedge-shaped appearance (approximate dimensions: 55×65×75 Å) and are composed of three structurally distinct domains: from the N- to C-terminus, an α-helical bundle (domain I), a β-sheet prism (domain II), and a β-sheet sandwich (domain III). As illustrated in Fig. (**1**), both Cry4Aa and Cry4Ba structures clearly display the three-domain topology characteristic of a ~65-kDa active form of the Cry toxin family believed to have a common evolutionary origin (Höfte Microbiol Rev 1989) [5] (Crickmore MMRV 1998) [6] (Li Nature 1991) [17] (De Maagd ARG 2003 [22].

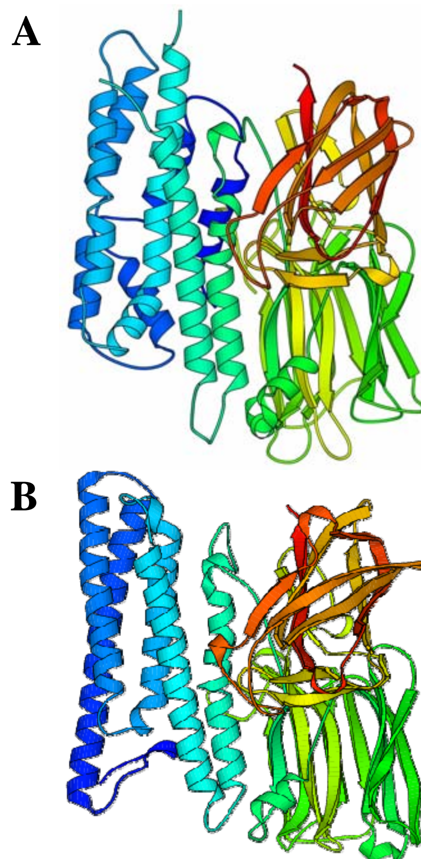

Fig. (1). Crystal structures of a 65-kDa activated form of (**A**) Cry4Aa (Boonserm J Bacteriol 2006) [19] and (**B**) Cry4Ba (Boonserm JMB 2005) [20] showing the three-domain organisation. Both ribbon representations are coloured in the rainbow order, from red at the N-terminus to blue at the C-terminus. The three domains are: I, a helical bundle (upper left); II, a three-sheet assembly (bottom); III, a β-sandwich (upper right). The structure is made with the program MOLSCRIPT.

The N-terminal domain is a group of eight helices − α1, α2a, α2b, α3, α4, α5, α6 and α7, initially assigned by Ellar group (Li Nature 1991) [17] − in which the most hydrophobic helix (α5) is surrounded by seven outer helices. The central helix is in fact not entirely hydrophobic, but rather exhibits an amphipathic character, as all of its polar or charged side-chains in the interhelical space are engaged in hydrogen bonds or salt bridges (Grochulski JMB 1995) [15] (Li Nature 1991) [17] (Boonserm J Bacteriol 2006) [19] (Boonserm JMB 2005) [20]. This is also the case for all the outer helices which are oriented with their polar or charged residues forming the outer surface of the helical bundle (Grochulski JMB 1995) [15] (Li Nature 1991) [17] (Boonserm J Bacteriol 2006) [19] (Boonserm JMB 2005) [20]. It has been experimentally evident that this domain, unaccompanied by the two other domains, is able to form functional pores in artificial lipid bilayers (Walters BBRC 1993) [23] (Puntheeranurak MMB 2004) [24]. The middle domain (α8-β11) is a three-fold symmetric assembly of anti-

parallel β-sheets, each forming a Greek key-like motif, that are packed around a hydrophobic core. From earlier studies of a functional role of this domain, it seems that most researchers have confined themselves to investigate a possible involvement in receptor binding of only three surface-exposed loops formed at the apex of this domain, *i.e.* loops β2-β3, β6-β7 and β8-β9 (Pigott MMBR 2007) [9] (Gómez Peptides 2007) [10] (Bravo Trends Biotechnol 2009) [14] (assigned as loops 1, 2 and 3, respectively (Li Nature 1991) [17]). Presently, loops connecting α8-β1 for Cry11Aa (Fernández FEBS Lett 2005) [25] or β8-β9 for Cry4Ba (Tuntitippawan FML 2005) [26] (Khaokhiew FML 2009 [27] have also been shown to be involved in receptor binding. The C-terminal domain (β12-β23) consists of two twisted anti-parallel β-sheets that are arranged in a jelly-roll-like topology or a face-to-face sandwich. Although an overall topology of this domain is rather similar to some carbohydrate-binding protein domains such as the cellulose-binding domain of a 1,4-β-glucanase enzyme (Johnson Biochemistry 1999) [28], its functional role is still not clearly elucidated. Nevertheless, it has been implicated in membrane permeabilisation (Masson AEM 2002) [29] or receptor recognition and specificity determination (Burton JMB 1999) [30] (De Maagd AEM 2000) [31] (Chayaratanasin JBMB 2007) [32]. This domain could be also critical for the structural integrity of the toxin molecule as the position of the C-terminus (*i.e.* β23) within the core structure may account for the resistance of the toxin to complete proteolysis (Li Nature 1991) [17].

Of particular interest, the helical domain is structurally equipped to be a transmembrane pore-forming unit, seeing as at least five helices, *i.e.* α3, α4, α5, α6 and α7, are long enough (>30Å) to span the lipid membrane. However, the hydrophobic faces of the outer-amphipathic helices of this pore-forming domain face inwards (Grochulski JMB 1995) [15] (Li Nature 1991) [17] (Boonserm J Bacteriol 2006) [19]. Therefore, these three-domain Cry toxins must undergo a major conformational change to convert the pore-forming apparatus into an aqueous transmembrane pore in which the hydrophobic surfaces would be in intimate contact with the membrane lipids. The trigger for the change has been thought to be provided by receptor binding and also the consequent interaction of the toxin with the membrane (Pigott MMBR 2007) [9] (Bravo Trends Biotechnol 2009) [14] (Li Nature 1991) [17]. Another trigger could be proteolytic cleavage in the solvent-exposed loops connecting helices in the bundle that could confer greater flexibility on the tertiary structure of the toxin molecule. Indeed, there is additional *in vitro* proteolysis occurring in the exposed loop linking α5 and α6 of both the 65-kDa activated Cry4Aa and Cry4Ba toxins, producing two non-covalently associated fragments of ~20 kDa and ~47 kDa which are mapped to the first five helices (α1-α5) and α6-α7-linked domains II-III, respectively (Angsuthanasombat JBMB 2004) [33]. However, a discrepancy was observed between *in vitro* and *in vivo* toxicity results when a tryptic cleavage site in this loop of Cry4Ba was eliminated (Angsuthanasombat FML 1993) [34]. This suggests that certain factors in the gut environment *in vivo* also have a major influence on toxicity and these still remain to be identified.

INSIGHTS INTO THE MECHANISM OF MEMBRANE PORE FORMATION

Up to now, membrane insertion behavior, oligomerisation and structural information of the functional state of the membrane-associated Cry larvicidal proteins are still not fully elucidated. As with any water-soluble pore-forming proteins, their toxic mechanism would involve protein-protein interactions, protein-membrane interactions, and a particular protein folding trail underlying the conformational transition from a stable water-soluble monomer to a membrane-inserted oligomeric form (Bayley Nature 2009) [35]. Among the proposed models for depicting the membrane-insertion and pore-formation stages (Knowles AIP 1994) [11] (Gazit JBC 1995) [36] (Loseva Biochemistry 2001) [37] (Alzate Biochemistry 2006) [38] (Tomimoto CBPB 2006) [39], the “umbrella-like” model seems now to be generally accepted as the best description of the membrane-bound state of the three-domain Cry toxins. This model involves an insertion of helices 4 and 5 into the lipid bilayers as a helical hairpin structure, and in so doing the remaining helices spread apart on the membrane surface like the opening of an umbrella (Knowles AIP 1994) [11] (Gazit JBC 1995) [36].

Several reports supporting the umbrella concept have further suggested that helix 4 is aligned to face the pore lumen and possibly participates in ion conduction (Masson JBC 1999) [40] (Sramala JBMBB 2001) [41] (Angsuthanasombat JBMB 2001) [42], whilst helix 5, which is relatively hydrophobic, would interact with the lipid membrane and is perhaps involved in toxin-pore oligomerisation (Nuñez-Valdez BBA 2001) [43] (Likitvivatanavong ABB 2006) [44]. Our group has also presented direct proof that the Cry4Ba α4-loop-α5 hairpin is capable of perturbing the membrane integrity of lipid vesicles, supporting its role as a fundamental membrane-inserted pore-forming determinant that could be separated as an isolated helical hairpin retaining at least its functionality (Leetachewa JBMB 2006) [45]. Other membrane permeation studies with synthetic peptides corresponding to Cry1Ac-domain I helices have also demonstrated that the loop connecting α4 and α5 is needed for efficient penetration of these two transmembrane helices into the lipid bilayers to form lytic pores (Gerber JBC 2000) [46]. This idea has been strengthened by our findings that an aromatic structure of one highly conserved tyrosine residue in this critical α4-α5 loop of the two closely related mosquito-specific toxins (Cry4Aa: Tyr^{202}; Cry4Ba: Tyr^{170}; *see* Fig. **2**) is an important determinant for toxicity, conceivably being involved in an interaction with lipid head groups for stabilising the oligomeric pore structure (Pornwiroon JBMB 2004) [47] (Kanintronkul Mol Biotechnol 2003) [48]. We have also provided biologically relevant evidence for a structural requirement of both the disulphide bridge (Cys^{192}-Cys^{199}) and the proline-rich motif ($Pro^{193}Pro^{194}$-Pro^{196}; especially for Pro^{193}) which are exclusively found within the α4-α5 loop of the Cry4Aa toxin (*see* Fig. **2**, left view) (Tapaneeyakorn BBRC 2005) [49]. Possibly, structural integrity of the α4-α5 loop may indeed play an important role in the membrane insertion step.

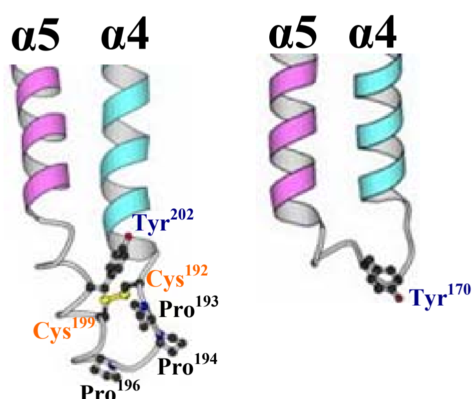

Fig. (2). Ribbon representations of the loop connecting α4 and α5 of two known structures: Cry4Aa (left) and Cry4Ba (right). The highly conserved aromatic residue (Tyr^{202} for Cry4Aa, Tyr^{170} for Cry4Ba) is shown in ball-and-stick in both interhelical loops. Three critical proline residues (Pro^{193}, Pro^{194} and Pro^{196}) and the unique disulphide bond between Cys^{192} and Cys^{199} are illustrated as ball-and-stick in the Cry4Aa α4-α5 loop.

A refined umbrella model further suggested that α7 may serve as a binding sensor that could initiate the binding of the pore-forming domain to the lipid membrane, facilitating the bilayer penetration of the α4-α5 hairpin (Gazit PNAS 1998) [50]. While helix 7 has also been implicated in ion-channel activity and receptor-binding affinity of Cry1A toxins (Chandra FEBS Lett 1999) [51] (Alcantara Biochemistry 2001) [52], or structural stability and crystallisation of Cry3Aa (Park Mol Biotechnol 2004) [53], the functional importance for toxicity of two highly conserved aromatic residues (Tyr^{249} and Phe^{264}) which are oriented on the same side of this helix has also been clearly highlighted for the Cry4Ba toxin (Tiewsiri JBMB 2007) [54]. In spite of the lack of membrane interaction and insertion studies, these two critical aromatic residues may in reality be the essential functional elements of helix 7, which could serve as a membrane-binding sensor to trigger the structural rearrangement of the pore-forming domain prior to membrane insertion. More recently, we have demonstrated that the Cry4Ba-α7 peptide when reconstituted into zwitterionic phospholipids could adopt either an α-helical conformation that prefers a membrane surface location or a β-structure with a membrane-inserted orientation (*see* Fig. **3**) (Tiewsiri ABB 2009) [55]. Other studies with the bacterial pneumolysin toxin have shown that there is indeed a conformational transition from the membrane-bound pre-pore to the transmembrane functional pore by the substantial refolding of α-helical regions into membrane-inserted β-hairpins (Tilley Cell 2005) [56]. Very recently, the structural transformations of β-sheets and loop regions to α-helices have also been observed during pore formation of the *Escherichia coli* cytotoxin (ClyA) (Mueller Nature 2009) [57]. This may conceivably reflect that α-helix 7 in the Cry4Ba pore-forming domain might be able to refold into an extended β-hairpin to insert into the lipid membrane, though the hairpin contains several charged and polar-uncharged residues that are energetically unfavourable for membrane insertion. It was thus proposed that this β-conformation induced by an interaction of helix 7 with lipid membranes would possibly impart greater ability to the Cry toxin molecule to protrude the transmembrane helical hairpin, *i.e.* α4-loop-α5 from the bundle to act as an initiator of bilayer penetration (Tiewsiri ABB 2009) [55].

In keeping with the pore size estimated, ranging from 10-26 Å (Soberón FML 2000) [58] (Peyronnet BBA 2002) [59], a single molecule of the three-domain Cry proteins would be inadequate to make up the pore. Accordingly, toxin oligomerisation would certainly have to happen. Indeed, various approaches have been used to inform the oligomerization process of the Cry toxins (Loseva Biochemistry 2001) [37] (Gómez FEBS Lett 2002) [60] (Puntheeranurak Ultramicroscopy 2005) [61]. Even though a

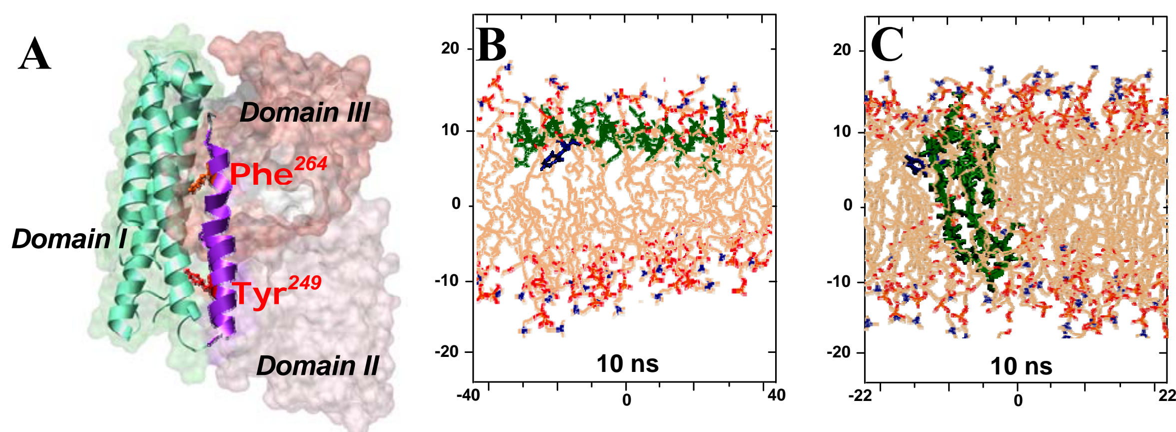

Fig. (3). (**A**) Ribbon and surface representation of the Cry4Ba structure with domain I (schematic ribbon) and domains II-III (surface model). Helix 7 (highlighted) within domain I illustrates the locations of the two critical aromatic side-chains, Tyr^{249} and Phe^{264} (ball and stick model). Snapshots of molecular dynamic trajectories of the Cry4Ba-α7 peptide in a fully hydrated DMPC (1,2 dimyristoyl-sn-glycero-3-phosphocholine) system at 10-ns simulation for the membrane-associated helical model (**B**), and for the membrane-inserted β-hairpin model (**C**) (Tiewsiri ABB 2009) [55].

range of approaches have been employed to portray a membrane-pore architecture formed by the Cry toxins, different points of view can still be made for their oligomeriscity (*see* Table **1**). In other words, the structural information obtained from the soluble monomeric Cry toxins has not been able to give comprehensive insights into the structural basis of the toxin-pore formation. To deal with this important issue, envisaging the pore structure and oligomeric state of the membrane-associated form would certainly be obligatory. Recently, as revealed by electron crystallography at 17 Å resolution, we have provided pivotal evidence for the first time that the 65-kDa activated Cry4Ba toxin in association withlipid membranes could exist in at least two different trimeric conformations, conceivably implying the closed and open states of the Cry toxin-induced pore (*see* Fig. **4**) (Ounjai BBRC 2007) [62]. As can be inferred from (Fig. **4A**), the projection map of the propeller-like structure appears to have an aperture in the middle, which could perhaps reflect the cavity of the open-state Cry4Ba complex whilst this feature does not appear in the pinwheel-like structure (Fig. **4B**). However, this perception of two different trimeric conformations remains to be evidently verified by performing more detailed structural and electrophysiological characterisation.

Table 1. Proposed Oligomeric Membrane-Bound State of a 65-kDa Active form of *Bt* Cry Toxins

Oligomericity	Toxin	Oligomer Size	Experimental Approach
Dimer	Cry1Ab Cry1Ac	~130 kDa	Western blotting of BBMV[1]-treated toxins after analysis by modified SDS-PAGE (Tigue AEM 2001) [63].
Trimer	Cry1Aa Cry1Ab Cry1Ac	~200 kDa	Western blotting of BBMV[1]-treated toxins after analysis by modified SDS-PAGE (Aronson AEM 1999) [64] (Kumar J Bacteriol 1999) [65].
Trimer	Cry1Ab	Diameter ~10 nm	Electron crystallography of 2D crystals of lipid-bound toxins (Muñóz-Garay BBA 2009) [66].
Trimer	Cry4Ba	~200 kDa	Western blotting of liposome-treated toxins after analysis by modified SDS-PAGE (Likitvivatanavong ABB 2006) [44].
Trimer	Cry4Ba	Diameter ~10 nm	Electron crystallography of 2D crystals of lipid-bound toxins (Ounjai BBRC 2007) [62].
Tetramer	Cry1Aa	Diameter ~5 nm	AFM[2] of toxins inserted in bilayers in liquid cell (Vie JMB 2001) [67].
Tetramer	Cry1Ab	~250 kDa	SDS-PAGE of ScFV73[3] cross-linked toxins (Gómez FEBS Lett 2002) [60].
Tetramer	Cry4Ba	Diameter 20-30 nm	AFM[2] of toxin inserted in bilayers in liquid cell (Puntheeranurak Ultramicroscopy 2005) [61].
Multimer	Cry11Aa	~250 kDa	Western blotting of BBMV-associated toxins after analysis by modified SDS-PAGE (Fernandez Biochemistry 2009) [68].

[1]BBMVs = Brush-border membrane vesicles prepared from susceptible insect larval midgets.
[2]AFM = Atomic force microscopy.
[3]ScFV73 = A single chain antibody mimicking Bt-R1 receptor.

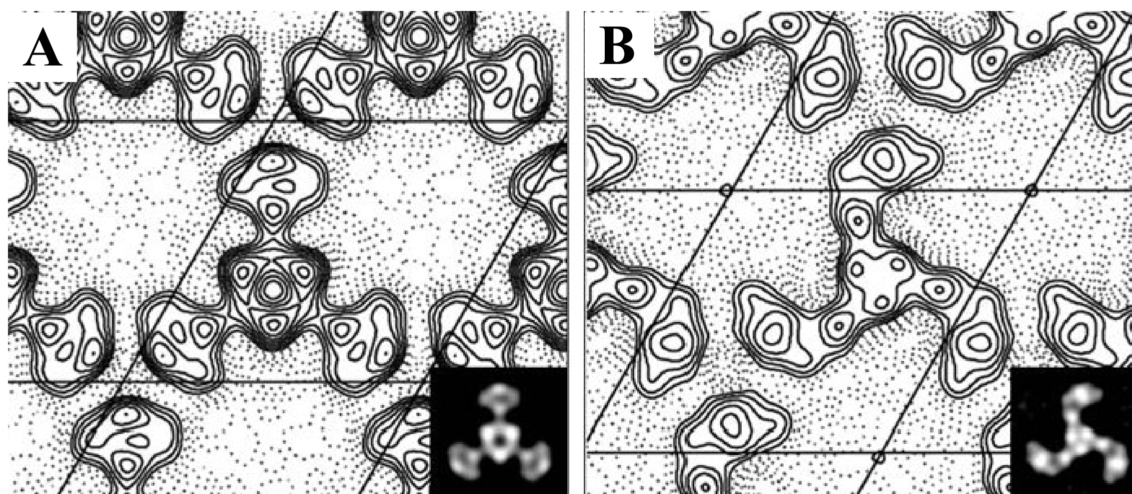

Fig. (4). The p3 symmetry-imposed maps of (**A**) propeller- and (**B**) pinwheel-like crystal forms of Cry4Ba toxins which were crystallised in the presence of DMPC *via* detergent dialysis (Ounjai BBRC 2007) [62]. The contour lines were plotted at 17 Å resolution. Insets show projection density maps of the propeller-like and pinwheel-like trimeric structures.

CONCLUDING REMARKS AND PERSPECTIVES

During the past several years, our research has focused on the molecular mechanism of toxicity of the two closely related mosquito-larvicidal proteins, Cry4Aa and Cry4Ba (Angsuthanasombat JBMB 2004) [33]. We now feel able to tackle some of the key steps *viz* the killing mechanism of these insecticidal proteins, particularly on the events following insertion of the α4-α5 hairpin of the three-domain activated toxin into the lipid membrane, resulting in the formation of ion-leakage pores. Conceivably, the lipid-induced β-conformation of α7 might serve as a lipid anchor needed for an efficient membrane penetration of the transmembrane helical hairpin. Nevertheless, it remains a challenge for experimental approaches to provide more structural and functional details of such a lipid-induced β-structure. Further studies for a higher-resolution model of the toxin-induced pore complex within the lipid membrane are also of great interest since these would shed light on a more defined structural basis of the pore-forming mechanism of the insecticidal proteins in the Cry family. Detailed understanding of this insecticidal mechanism is important because it would pave the way for the future development of better protein-based bioinsecticides.

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