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Asn^{183} in α5 is essential for oligomerisation and toxicity of the *Bacillus thuringiensis* Cry4Ba toxin

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Abstract

The proposed toxicity mechanism of the *Bacillus thuringiensis* Cry insecticidal proteins involves membrane penetration and lytic pore formation of the α4–α5 hairpins in the target larval midgut cell membranes. In this study, alanine substitutions of selected polar residues (Tyr^{178}, Gln^{180}, Asn^{183}, Asn^{185}, and Asn^{195}) in the hydrophobic helix-α5 of the Cry4Ba mosquito-larvicidal protein were initially conducted via PCR-based directed mutagenesis. Upon IPTG induction, all the 130-kDa mutant protoxins were highly expressed in *Escherichia coli* as cytoplasmic inclusions, with yields similar to the wild-type protoxin. When *E. coli* cells expressing each mutant toxin were tested against *Stegomyia aegypti* mosquito larvae, the larvicidal activity of the N183A mutant was almost completely abolished whereas the four other mutants showed only a small reduction in toxicity. Additionally, replacements of this critical residue with various amino acids revealed that the uncharged polar residue at position 183 in α5 is crucial for larvicidal activity. Further characterisation of the N183K bio-inactive mutant revealed that the 65-kDa activated toxin was unable to form oligomers in lipid vesicles and its ability to induce the release of entrapped calcein from liposomes was much weaker than that of the wild-type toxin. These results suggest that the highly conserved Asn^{183} located in the middle of the transmembrane α5 of Cry4Ba plays a crucial role in toxicity and toxin oligomerisation in the lipid membranes.

Keywords: *Bacillus thuringiensis*; Calcein release; δ-Endotoxin; Liposome; Mosquito-larvicidal activity; Toxin oligomerisation

Bacillus thuringiensis (*Bt*)[1] insecticidal proteins have been classified into two main families of Cry (crystal) and Cyt (cytolytic) δ-endotoxins, based on the similarity of their deduced amino acid sequences [1]. The Cry toxins, highly produced in the form of crystalline inclusions during sporulation, have been shown to be toxic towards a wide variety of insect larvae in the orders Diptera (mosquitoes and flies), Lepidoptera (moths and butterflies), Coleoptera (beetles and weevils), and Hymenoptera (wasps and bees) [2,3]. When ingested by susceptible insect larvae, the *Bt* protoxin inclusions are solubilised in the alkaline contents of the larval midgut lumen, and are proteolytically activated by the gut proteases. The activated toxins afterwards bind to specific receptors lining the apical brush-border membranes of the midgut epithelium. A subsequent conformational change of the toxin molecules allows the insertion of their pore-forming portion into the target cell membrane to form ion-leakage pores that would cause a net influx of ions and water, leading to osmotic cell lysis and eventual death of the larvae (for reviews, see [4,5]). However, the underlying molecular basis of this toxicity process, especially the steps of membrane insertion and lytic pore formation, is not yet completely described.

To date, the X-ray crystal structures of several Cry toxins, including Cry4Ba [6], Cry1Aa [7], Cry2Aa [8], Cry3Aa

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[1] *Abbreviations used: Bt*, *Bacillus thuringiensis*; CD, circular dichroism; Ch, cholesterol; FPLC, fast-performance liquid chromatography; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl) piperazine-*N*′-2 ethanesulfonic acid; LUV, large unilamellar vesicle; PC, L-α-phosphatidylcholine; PE, L-α phosphatidylethanolamine.

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[9], and Cry3Bb [10], have been elucidated, leading to a better understanding of the molecular basis of insect specificity and gut epithelial cell lysis. Although these Cry toxins show different insect specificities, they all share a high degree of overall structural similarity with a three-distinct domain organisation [11]. Of particular interest, the N-terminal seven-helix domain in which the relatively hydrophobic helix-α5 is encircled by six other amphipathic helices has been proved to be responsible for membrane insertion, leading to formation of the ion-leakage pores [12,13].

The "umbrella-like" model seems now to be widely accepted as the best description for the membrane-bound state of the *Bt* Cry toxins, which involves an insertion of α4 and α5 into the lipid bilayers as a helical hairpin structure, with the remaining helices spread over the membrane surface [14]. Subsequent oligomerisation of the transmembrane hairpins occurs to form an oligomeric pore [15]. Several reports supporting this model have suggested that α4 arranges in the aqueous interface of the pore and participates in ion conduction [16,17], whilst the more hydrophobic α5 interacts with the lipid bilayers and is involved in toxin oligomerisation [18,19]. Furthermore, it has been demonstrated that the loop connecting α4 and α5 of the lepidopteran-active Cry1Ac toxin is needed for efficient penetration of these two transmembrane helices into the lipid bilayers to form lytic pores and subsequently causes toxicity [20]. This notion has been strengthened by our findings that one highly conserved tyrosine residue in this critical loop of the two closely related dipteran-specific toxins (Cry4Aa: Tyr^{202}; Cry4Ba: Tyr^{170}) is an important determinant for larvicidal activity, conceivably being involved in an interaction with lipid head groups for stabilising the oligomeric pore structure [21,22]. In addition, we have recently provided biologically relevant evidence for a structural requirement of both the disulphide bridge (C_{192}–C_{199}) and the proline-rich motif ($P_{193}PNP_{196}$), which are exclusively found within the α4–α5 loop of the Cry4Aa toxin [23]. Very recently, we have also demonstrated via Langmuir–Blodgett technique that the 65-kDa trypsin-activated Cry4Ba toxin is capable of inserting itself into the lipid monolayers [24]. By using atomic force microscopy, the activated Cry4Ba toxin was also found to form a symmetric pore-like structure in receptor-free lipid bilayers [25].

A number of reports suggested a role of polar residues in driving and stabilising the association of the transmembrane helices within the lipid bilayers by providing interhelical interactions, mainly via hydrogen bonding [26,27]. In the present report, five polar uncharged residues (Tyr^{178}, Gln^{180}, Asn^{183}, Asn^{185}, and Asn^{195}) within helix 5 which is likely to be part of the central transmembrane pore-forming element of the Cry4Ba toxin were investigated for their functional significance. The results revealed that the highly conserved residue, Asn^{183}, is an essential determinant for larvicidal activity of the Cry4Ba toxin, conceivably involved in toxin oligomerisation to form a lytic pore in the lipid membranes.

Materials and methods

Plasmid and site-directed mutagenesis

The recombinant plasmid, pMU388, encoding the 130-kDa Cry4Ba toxin, which has been cloned from *Bt* subsp. *israelensis* into the pUC12 vector [28], was used as a template for site-directed mutagenesis. Complementary pairs of mutagenic oligonucleotide primers were designed and purchased from Proligo (Singapore). All mutations were generated by a polymerase chain reaction (PCR) using a high fidelity *Pfu* DNA polymerase, following the procedure of the QuickChange Mutagenesis Kit (Stratagene, La Jolla, USA). All mutant plasmids were verified by DNA sequencing, using a BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, USA).

Toxin expression, inclusion solubilisation, and proteolytic activation

Escherichia coli JM109 clones harbouring the wild-type plasmid or mutants were grown at 37 °C in Luria–Bertani medium containing 100 μg/ml ampicillin until OD_{600} of the culture reached 0.3–0.5. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM for 4 h and subsequently analysed by sodium dodecyl sulphate-(10% w/v) polyacrylamide gel electrophoresis (SDS–PAGE).

Escherichia coli cultures overexpressing the toxins as cytoplasmic inclusions were harvested by centrifugation and resuspended in cold distilled water. Cells were then disrupted by using a French Pressure Cell at 12,000 psi. After centrifugation at 10,000*g*, 4 °C for 10 min, the pellets were washed three times in cold distilled water and suspended by sonication. Protein concentrations of the partially purified inclusions were determined by using a protein microassay reagent (Bio-Rad, Hercules, USA), with bovine serum albumin fraction V (Sigma, St. Louis, USA) as a standard. Inclusions (1–2 mg/ml) were solubilised by incubation at 37 °C for 1 h in 50 mM Na_2CO_3, pH 9.0. The solubilised protoxins were assessed for their proteolytic stability by digestion with trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone treated) at a ratio of 1:20, (w/w) enzyme/toxin in 50 mM Na_2CO_3, pH 9.0, for 16 h. The 130-kDa protoxins would be cleaved into two trypsin-resistant fragments of ca. 20 and 47 kDa, in addition to the removal of the C-terminal half of the toxin molecules [11].

Mosquito-larvicidal activity assays

Bioassays for mosquito-larvicidal activity were performed as previously described [13], using 2-day-old *Stegomyia (Aedes) aegypti* larvae (hatched from eggs supplied by the mosquito rearing facility of the Institute of Molecular Biology and Genetics, Mahidol University, Thailand). Both rearing and bioassays were carried out at room temperature (25 °C). The assays were done in 1 ml of *E. coli* suspension