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Insertion behavior of the *Bacillus thuringiensis* Cry4Ba insecticidal protein into lipid monolayers

Yodsoi Kanintronkul [a], Toemsak Srikhirin [a,*], Chanan Angsuthanasombat [b], Teerakiat Kerdcharoen [a,*]

[a] *Department of Physics, Center of Nanoscience and Nanotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[b] *Laboratory of Molecular Biophysics and Structural Biochemistry, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakornpathom 73170, Thailand*

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Abstract

Toxicity mechanisms of *Bacillus thuringiensis* Cry insecticidal proteins involve membrane insertion and lytic pore formation in lipid bilayers of the target larval midgut cell membranes. The *B. thuringiensis* Cry4Ba mosquito-larvicidal protein has been shown to be capable of permeabilizing liposome vesicles and of forming ion channels in planar lipid bilayers. Here, the membrane interaction of the 65-kDa activated Cry4Ba protein with the lipid monolayers, comprising dipalmitoyl phosphatidylcholine, dioleoyl phosphatidylethanolamine, and cholesterol (Chol), was studied using Langmuir–Blodgett technique. The interactions of the Cry4Ba protein with the lipid monolayers were measured from the surface pressure versus area isotherms of the protein–lipid monolayers. The increase in the mean molecular area was demonstrated as an incorporation of the protein into lipid monolayers. The insertion of the Cry4Ba protein was monitored by measuring as an increase of the surface pressure at constant molecular area. For a given monolayer, the membrane insertion of the Cry4Ba reduced as the initial surface pressure increased. The Cry4Ba protein showed a strong preference of an insertion towards a Chol monolayer. In addition, the mixed monolayers of Chol showed an enhanced effect on the insertion kinetics of Cry4Ba into lipid films, suggesting its involvement in the modulation of the protein insertion. These findings provide the first evidence that the Cry4Ba protein is capable of inserting itself into lipid monolayers, depending on the packing density of the monolayers. Our results also indicate that only a limited part of the protein is likely to be involved in the insertion.

Keywords: *Bacillus thuringiensis*; Insecticidal protein; Langmuir–Blodgett; Membrane insertion; Protein–lipid interaction

Bacillus thuringiensis (*Bt*) is a Gram-positive bacterium which produces intracellular crystalline inclusions during sporulation. These inclusions consist of one or more insecticidal proteins known as Cry and/or Cyt δ-endotoxins which have been used as potential biocontrol agents, an alternative to chemical insecticides [1]. For instance, *B. thuringiensis* subsp. *israelensis* produces 130-kDa Cry4Ba δ-endotoxins, which are highly toxic to the larvae of disease-carrying mosquitoes, such as the *Aedes* and *Anopheles* species [2].

To date, the tertiary structures of five different Cry proteins, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Bb, and Cry4Ba have been determined by X-ray crystallography [3–7]. All the known structures display a high degree of overall similarity with a three-distinct domain organization. The N-terminal domain (domain I) is a bundle of seven α-helices in which the central helix (α5) is relatively hydrophobic and is encircled by six other amphipathic helices. Of particular interest, this helical domain has been proved to be responsible for membrane insertion, leading to formation of the ion-leakage [8–13]. Domain II consists of

* Corresponding authors. Fax: +662 2015843.
E-mail addresses: sctsk@mahidol.ac.th, sctkc@mahidol.ac.th (T. Srikhirin).

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three anti-parallel β-sheets. This domain is similar to antigen-binding regions of immunoglobulins, and has been shown to be responsible for receptor binding [14,15]. Domain III contains two anti-parallel β-strands. The function of this domain is still not clear [6,16].

The *Bt* Cry proteins are presented as inactive protoxin inclusions. Upon ingestion by susceptible larvae, the protoxin inclusions are solubilized in the larval midgut and activated by midgut proteases. It is believed that the activated proteins first bind to a specific receptor located on apical membranes of midgut epithelial cells. Subsequently, the proteins insert into the lipid membrane and disrupt the permeability of the midgut cell membranes, resulting in a net influx of ions and water that lead to osmotic lysis of the cell [17,18]. However, the underlying molecular basis of this toxicity process, especially in the step of membrane insertion and lytic pore formation, is not yet clearly understood.

Currently, an umbrella model best describes molecular mechanisms of membrane insertion and pore formation of the Cry δ-endotoxins [9]. There is a lot of evidence supporting this model in which the α4 and α5 helices form a helical hairpin to initiate membrane penetration upon specific receptor binding [12,13,19]. Recently, atomic force microscopy (AFM) measurements suggested that the Cry1Aa and Cry4Ba pores are tetramer-like structures [20]. Although a number of studies on the protein–lipid interaction at the air–water interface have been published for several proteins [21–23], the research work concerning the nature of interaction between Cry proteins and the lipid membrane has not been clearly elucidated.

Protein–lipid monolayers have been widely used for investigating mechanisms occurring in biological membranes. Langmuir–Blodgett (LB) technique is an ideal method to mimic the behavior of biological membranes. Insecticidal properties of the Cry4Ba protein are primarily related to its interaction with lipid membranes. In the present study, we therefore employed the LB approach to understand the interaction of the Cry4Ba protein with various lipid monolayers at different given pressures. The results clearly showed that the Cry4Ba protein is able to penetrate into lipid monolayers, with distinctive insertion behavior depending on the nature of lipid compositions.

Materials and methods

Chemicals and solutions

Lipids used were 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC),[1] 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and cholesterol (Chol) purchased from Avanti Polar Lipids, USA. DPPC, DOPE, and Chol stock solutions (20 mg/ml) were prepared in HPLC grade chloroform and stored at −20 °C under nitrogen. The freshly made solution was spread using a microsyringe on carbonate buffer prepared from MiliQ Millipore water (resistivity ⩾18 MΩ cm) in a LB trough (K.S.V. Instrument, Finland).

Cry4Ba protein preparation

The *Bt* Cry4Ba protein was expressed as cytoplasmic inclusions in the *Escherichia coli* strain JM109 under control of the *LacZ* promoter. Cells were grown in a Luria–Bertani medium containing 100 μg/mL ampicillin until OD_{600} reached 0.3–0.5. After addition of isopropyl-β-D-thiogalacto pyranoside (IPTG) to a final concentration of 0.1 mM, incubation was continued for another 4 h. *E. coli* cells expressing Cry4Ba were harvested by centrifugation, resuspended in distilled water, and finally disrupted in a French Pressure Cell at 10,000 psi. Protein concentrations of the partially purified protein inclusions were determined by using the Bradford-based protein microassay (Bio-Rad), with bovine serum albumin fraction V (Sigma) as a standard. Protoxin inclusions were solubilized in 50 mM Na_2CO_3, pH 9.0, at 37 °C for 1 h.

The stability of protein was tested by digesting with trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated, Sigma) at enzyme/toxin ratio of 1:20 (w/w) in 50 mM Na_2CO_3, pH 9.0, at 37 °C for 16 h. Purification of the 65-kDa trypsin-treated Cry4Ba protein was accomplished using a size-exclusion FPLC system (Superose 12/HR10 column, Amersham Pharmacia Biotech) eluted with carbonate buffer (50 mM Na_2CO_3, pH 10.5).

Monolayer and Langmuir–Blodgett experiments

The experiment was performed by using KSV2000 (KSV, Finland). The surface pressure–area isotherms were obtained by monitoring a surface pressure as a function of a reduction in molecular area. The surface pressure was measured by Wilhelmy plate method using a platinum plate connected to a microbalance as described previously [21]. Before starting the experiment, the trough was cleaned successively with ethanol and followed by rinsing with deionized water (resistivity >18.0 MΩ cm). Carbonate buffer (pH 9.0) was used as the subphase throughout this study. For monolayer preparation, the synthetic lipids (DPPC, DOPE, and Chol) were spread on the aqueous subphase using micropipette. The temperature of the subphase was kept constant at 25 °C. Each experiment was repeated at least three times to ensure reproducibility of the data.

For characterizing the isotherms of mixed protein–lipid monolayers, the protein was injected into subphase

[1] *Abbreviations used:* DPPC, 1,2-dipalmitoylphosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; LB, Langmuir–Blodgett; π–A, surface pressure–molecular area.