Evidence for intermolecular interaction as a necessary step for pore-formation activity and toxicity of \textit{Bacillus thuringiensis} Cry1Ab toxin

Mario Soberón a,*, Rigoberto V. Pérez a, María E. Núñez-Valdés a, Argelia Lorence b, Isabel Gómez a, Jorge Sánchez a, Alejandra Bravo a

\textsuperscript{a} Instituto de Biotecnología, U.N.A.M., Apdo Postal 510-3, Cuernavaca, Morelos 62271, Mexico
\textsuperscript{b} Centro de Investigación en Biotecnología, U.A.E.M., Av. Universidad 1000, Col. Chamilpa, Cuernavaca, Morelos 62210, Mexico

Received 20 May 2000; received in revised form 21 July 2000; accepted 15 August 2000

Abstract

Based on the observation of large conductance states formed by \textit{Bacillus thuringiensis} Cry toxins in synthetic planar lipid bilayers and the estimation of a pore size of 10–20 Å, it has been proposed that the pore could be formed by an oligomer containing four to six Cry toxin monomers. However, there is a lack of information regarding the insertion of Cry toxins into the membrane and oligomer formation. Here we provide direct evidence showing that the intermolecular interaction between Cry1Ab toxin monomers is a necessary step for pore formation and toxicity. Two Cry1Ab mutant proteins affected in different steps of their mode of action (F371A in receptor binding and H168F in pore formation) were affected in toxicity against \textit{Manduca sexta} larvae. Binding analysis showed that F371A protein bound more efficiently to \textit{M. sexta} brush border membrane vesicles when mixed with H168F in a one to one ratio. These mutant proteins also recovered pore-formation activity, measured with a fluorescent dye with isolated brush border membrane vesicles, and toxicity against \textit{M. sexta} larvae when mixed, showing that monomers affected in different steps of their mode of action can form functional hetero-oligomers. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: δ-Endotoxin oligomerization; Pore formation; \textit{Bacillus thuringiensis}; \textit{Manduca sexta}

1. Introduction

\textit{Bacillus thuringiensis} (Bt) is an aerobic, spore-forming soil bacterium that produces crystalline inclusions during the sporulation phase of growth. Crystal inclusions are composed of proteins known as insecticidal crystal proteins (ICP) or δ-endotoxins, which are toxic to larval forms of several insects of different orders as well as to other invertebrates [1]. The ICPs form two very different multigenic families, \textit{cry} and \textit{cyt} [2–4], which have been classified into 28 different Cry subgroups and two different Cyt subgroups [5], based on amino acid identity.

In the case of the lepidopteran-specific Cry1 toxins, after ingestion by susceptible larvae, the ICPs are solubilized in the alkaline and reducing conditions of the midgut lumen, releasing the protoxin from the crystal. The protoxin is further processed by the insect gut proteases into a protease-resistant active toxin fragment. The activated toxin binds to specific receptors located on the apical membrane of the midgut epithelial cells [6–8] and inserts irreversibly into the membrane, forming pores that allow a net uptake of ions and water that causes the swelling and osmotic lysis of the cells [9–11]. These events lead to a breakdown in the integrity of the midgut cells and to the insect’s death [11].

The three-dimensional structures of the Cry3A and Cry1Aa ICPs have been resolved by X-ray diffraction crystallography [2,3]. They share many similar features and are comprised of three domains. Domain I, extending from the N-terminus, is a seven-\(\alpha\)-helix bundle with helix \(\alpha\)-5 in the center encircled by the other helices. This domain has been implicated in the channel formation in the membrane. Domain II consists of three anti-parallel \(\beta\)-sheets and domain III is a \(\beta\)-sandwich of two anti-parallel \(\beta\)-sheets [2,3]. Domains II and III are involved in receptor
binding [2,3]. Domain III also protects the toxin from further proteolysis [2].

Based on the observation of large conductance states formed by Cry1Ac, Cry3A, Cry3B and Cry1C toxins in synthetic planar lipid bilayers [12–14] and the estimation of a pore size of 10–20 Å [15], it has been proposed that the pore could be formed by an oligomer of Cry toxins containing four to six toxin monomers [16,17]. The oligomeric state of some Cry toxins in solution has been analyzed. A recent report showed that Cry proteins in solution do not form oligomers of a defined subunit number suggesting that oligomers form after the toxin is inserted into the membrane [18]. Also, Cry1Ac oligomeric state was analyzed after binding to brush border membrane vesicles where multimers were identified [19]. In the binary toxin, produced by Bacillus sphericus, evidence for oligomer formation was obtained by recovering toxicity of point mutant toxins after mixing them, showing that altered binary toxins can functionally complement each other by forming oligomers [20].

In this study we provide direct evidence showing that the intermolecular interaction between Cry1Ab monomers is a necessary step for pore formation and toxicity. Two Cry1Ab mutant proteins affected in different steps of their mode of action (receptor binding and pore formation) recovered binding, pore-formation activity and toxicity against Manduca sexta larvae when mixed, showing that monomers affected in different steps of their mode of action can form functional hetero-oligomers.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Plasmids pC34 [21], pBlueScript-SK (Stratagene, La Jolla, CA) and pTH315 [22] were used for cloning. An Escherichia coli strain harboring cry1Ab F371A was kindly supplied by Dr. Dean (Ohio State University, USA). Acrysomaliferous Bt strain 407 [23] was provided by Dr. Lereclus (Institut Pasteur, France). E. coli strains were grown in Luria broth at 37°C and Bt in nutrient broth sporulation medium (NB) [24] at 30°C. Antibiotics were as follows: ampicillin 100 μg ml⁻¹ (for E. coli); erythromycin 250 μg ml⁻¹ for E. coli and 7.5 μg ml⁻¹ for Bt.

2.2. Construction of the H168F mutant

The H168F mutant was constructed by overlap-extention PCR site-directed mutagenesis as described [25]. Plasmid pC375 containing a 375-bp XhoI fragment from the cry1Ab gene (480–813 bp, α-helix 5) was used as template, in two sequential PCRs. The first round of PCR involved two separate PCRs, using T3 primer and the mutagenic primer RM11 (5′-TCT AAA AAC TGA TAA AAA TAA ATT TGC AGC TTG) (residues 631–663), to amplify the first portion of the fragment and mutagenic primer RM7 (5′-CTT TCA TGA NNS GTT CAA GCT GCA AAT TTA C) (residues 610–643), and T7 to amplify the second part of the fragment. PCRs were done with 3 U Pfu (Stratagene) with the program: one cycle of 94°C, 5 min; 53°C, 2 min and 72°C, 5 min; followed by 23 cycles of 94°C, 1 min; 53°C, 1 min and 72°C, 1 min. The resulting fragments overlap by 16 bp.

The second round of PCR involved the purification of both PCRs and treatment with Klenow. 50 ng of each PCR product was mixed, boiled for 10 min, and cooled on ice for 5 min. Extension of the overlapping products was allowed by adding PCR buffer and 3 U Pfu: 72°C for 3 min, followed by 10 cycles of 94°C, 1 min; 53°C, 1 min and 72°C, 1 min. Then, 20 pmol of T3 and T7 primers were added in addition of 3 U Pfu and allowed to amplify for 25 cycles. The final PCR product was 567 bp. This fragment was digested with XhoI and cloned into pBlueScript-SK. E. coli DH5α was transformed and plasmid from the resulting colonies was sequenced. The 375-bp XhoI H168F mutated fragment was subcloned into the cry1Ab protoxin gene, cloned in pHT315 [22] and transformed into Bt strain 407.

2.3. Purification of wild-type Cry1Ab, and Cry1Ab F371A and H168F mutant toxins

Bt strains containing cry1Ab or cry1Ab H168F were grown for 3 days in NB until complete sporulation. Crystals were purified by sucrrose gradients as in [26]. Purified crystals were solubilized and activated by trypsin 1:10 w/w for 2 h and purified by anion exchange chromatography (Q-Sepharose) as described [27]. The purified toxins were dialyzed against 1000 volumes of 150 mM N-methylglucamine chloride (MeGluCl), 10 mM HEPES pH 8 and stored at 4°C until used. Toxin purity was examined by SDS–PAGE. Mutant F371A was expressed in E. coli and purified as previously described [28]. Protein was measured by a protein dye method [29].

2.4. Toxicity assay of Manduca sexta

M. sexta larvae used in this study were supplied by Dr. J. Ibarra (CINVESTAV, Irapuato, Mexico). Toxicity assays were performed with first-instar larvae. Twenty-four larvae were fed with 20 ng cm⁻² of toxin on an artificial diet (Bio-Serv). The mortality was recorded after 5 days.

2.5. Preparation of brush border membrane vesicles (BBMV)

BBMV from fifth-instar M. sexta larvae were prepared and analyzed as previously reported [30]. BBMV were dialyzed overnight against 500 volumes of 150 mM KCl, 2 mM EDTA, 10 mM HEPES-HCl pH 7.6 and stored at −70°C until used.
2.6. Binding assay

Homologous competition of biotinylated Cry1Ab, H168F and F371A toxins to *M. sexta* BBMV was performed as previously described [31].

2.7. Fluorescence measurements

Membrane potential was monitored with the positively charged fluorescent dye, 3,3′-dipropyl-thiodiacyrbocyanine (dis-C3-(5) ( Molecular Probes, Eugene, OR, USA), as previously described [10]. Fluorescence was recorded at the 620/670 nm excitation/emission wavelength pair using a Hansatech system (Norfolk, UK). Hyperpolarization causes dye internalization into the BBMV and fluorescence decrease. Dye calibration and determination of resting membrane potential were performed in the presence of valinomycin (2 mM) by successive additions of KCl to BBMV (20 μg) in 140 mM MeGluCl, 10 mM HEPES–HCl pH 8.0. Resting membrane potential was determined from a ΔF (%) vs. K+ equilibrium potential (E_{K+}) (mV) curve (n=4). E_{K+} was calculated with the Nernst equation.

3. Results and discussion

In order to obtain evidence for the interaction of different Cry1Ab monomers during pore-formation activity and toxicity, we decided to explore if two independent mutants affected in different regions could recover toxicity and pore formation when assayed as protein mixtures. Mutant F371A of Cry1Ab protein was chosen since previous reports showed that this mutant was affected in receptor binding and toxicity [32]. The mutation F371A maps in loop 2 of domain II and it is affected primarily in the irreversible binding step of the toxin. Also, no effects on stability to trypsin treatment were observed in this mutant and it presented a 40-fold reduction in toxicity against *M. sexta* larvae [32]. In this study we show that mutant F371A could not form pores on BBMV prepared from *M. sexta* larvae (see below).

3.1. Isolation of Cry1Ab H168F

In order to have a different mutant affected exclusively in pore-formation activity and not in binding to the receptor, we decided to mutagenize His 168 localized within helix α-5 of domain I. In the closely related Cry1Ac toxin, several mutations in the conserved residue H168 showed altered toxicity to *M. sexta* larvae [33]. The positive charge seemed to be important for toxicity since a mutant with a change to negative charge (H168D) or a change to no charge (H168N) showed reduced toxicity in contrast to a conservative change (H168R) that showed three-fold increased toxicity against *M. sexta* larvae [33]. Mutations that reduced toxicity (H168D, H168N) did not affect binding while toxicity was diminished [33]. We decided to change H168 for a non-charged amino acid. H168 was mutated and changed to phenylalanine. The H168F crystals were purified, solubilized and trypsinized. A 55-kDa trypsin-resistant fragment was produced in low yield. Bioassays done with the H168F mutant protein showed that this protein had reduced toxicity to *M. sexta* larvae in contrast to Cry1Ab toxin (Table 1).

3.2. Binding analysis of H168F and F371A to *M. sexta* BBMV

Analysis of binding of biotinylated H168F protein to BBMV prepared from *M. sexta* showed that this protein was able to bind to BBMV (Fig. 1A). This interaction is specific since heterologous competition using 100-fold excess of unlabeled Cry1Ab protein competed for binding with H168F to *M. sexta* BBMV. Also, we analyzed the binding of biotinylated F371A to *M. sexta* BBMV. In our binding conditions, F371A did not bind to BBMV (Fig. 1A); previously it was shown that although F371A is affected in receptor binding it was not affected in initial reversible binding using a similar protocol for binding [32]. This difference could be due to the fact that in our assay toxin was labeled with biotin, while 125I-labeled toxin was used previously [32]. Nevertheless, binding of F371A was evident when a 100-fold excess of unlabeled Cry1Ab protein was incubated along with F371A (Fig. 1A). We also analyzed if mutant F371A could bind to BBMV when mixed with H168F. Fig. 1B shows that F371A bound efficiently to BBMV when mixed with H168F in a one to one ratio. F371A also bound to BBMV when mixed with 100-fold excess of H168F although less efficiently (Fig. 1B). These results showed that mutant F371A binds efficiently to BBMV when mixed with toxin proteins that are not affected in receptor binding, suggesting that intermolecular interaction between monomers could occur after receptor binding.

3.3. Pore-formation activity and toxicity of F371A and H168F protein mixtures

In order to determine if proteins F371A and H168F

<table>
<thead>
<tr>
<th>Mutant*</th>
<th>Mortality (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ab</td>
<td>100</td>
</tr>
<tr>
<td>F371A</td>
<td>0</td>
</tr>
<tr>
<td>H168F</td>
<td>4</td>
</tr>
<tr>
<td>F371A+H168F*</td>
<td>25</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

*All proteins were tested as pure trypsin-activated toxins.

*Mortality as percent of 24 larvae treated with 20 ng cm$^{-2}$ after 5 days.

*1:1 protein mixture.
could form functional hetero-oligomers, the pore-formation activity of wild-type Cry1Ab and F371A–H168F mixtures was analyzed in BBMV from *M. sexta* larvae. BBMV were loaded with 150 mM KCl and assayed with the membrane potential-sensitive fluorescent dye dis-C3-(5) as previously reported [10]. The resting membrane potential was $-42.3 \pm 3$ mV ($n = 4$) and the potassium equilibrium potential ($E_K$) calculated with the Nernst equation was $-115.8$ mV. Addition of 100 nM of Cry1Ab toxin to BBMV suspended in 150 mM MeGluCl produced a fast hyperpolarization ($-66.7 \pm 5$ mV, $n = 4$). After toxin exposure, the vesicles also increased their response to KCl additions ($m = 0.19$), when compared to the control ($m = 0.017$) to which the same amount of buffer was added (Fig. 2). The slope of the trace after KCl addition reflects the K$^+$ permeability of BBMV. In contrast to Cry1Ab toxin, proteins F371A ($m = 0.02$) or H168F ($m = 0.035$) did not induce an increased K$^+$ permeability when compared to control ($m = 0.017$) (Fig. 2), showing that both mutants are affected in pore-formation activity. Addition of 100 nM of a 1:1 mixture of F371A–H168F to BBMV showed an increased K$^+$ permeability ($m = 0.174$) very similar to that induced by wild-type Cry1Ab toxin, and also produced a fast hyperpolarization of $-80.4 \pm 3$ mV, $n = 3$. These data showed that the protein mixture of F371A and H168F recovered their capacity to form ionic channels in vitro.

Finally the toxicity against *M. sexta* larvae was analyzed. *M. sexta* larvae were fed with a 1:1 mixture of F371A–H168F proteins as described in Section 2. Table 1 shows that the mixture of both proteins recovered toxicity to some extent since mortality was 25% in contrast to larvae fed with F371A or H168F mutants, which showed no mortality, or with Cry1Ab protein, which showed 100% mortality (Table 1).

The pore-formation activity by the F371A–H168F protein mixture was very similar to the pore-formation activity of Cry1Ab. However, toxicity was only partially recovered by the F371A–H168F protein mixture (Fig. 2, Table 1). This result could be explained by a reduced stability of H168F mutant toxin in the midgut. Nevertheless, the fact that the F371A–H168F protein mixture presented increased mortality in comparison with toxicity displayed by individual mutant proteins clearly shows that functional hetero-oligomers could also be formed in vivo. These data also suggest that residue H168 is not involved in oligomer formation since mutant H168F is capable of interacting with F371A protein. Finally, these data are the first to show that the interaction of more than one monomer is fundamental for pore formation and toxicity of Cry proteins. Also, assays of protein mixtures to recover pore formation and toxicity could be useful to identify
specific regions that might be involved in Cry1 protein–protein interactions for oligomer formation.

Acknowledgements

We thank Dr. Donald Dean for kindly providing us with the F317A mutant and Dr. Didier Lereclus for Bt strain 407 Cry3 and plasmid pTH315. We thank Laura Lina and Leopoldo Güereca for technical assistance. This work was partially supported by CONACyT Contracts 27637-N and 25248-N, DGAPA-UNAM IN217597 and UC MEXUS-CONACYT.

References