

Mode of Action of *Bacillus thuringiensis* Cry1Ab Toxin: Role of Domain II Residue in Insertion into Insect Brush Border Membranes

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ABSTRACT

Cry1Ab is a member of the family of crystal (Cry) toxins, produced by a soil bacterium *Bacillus thuringiensis*, and used globally as a commercial pesticide. A thorough knowledge of the mechanism by which the toxin acts is essential for preventing possible pest resistance to the toxin. While the upstream steps of the mechanism, including activation of the toxin in the alkaline midgut of the target lepidopteran insect followed by binding to its receptors are well understood, the final step of insertion of the toxin into insect brush border membranes remains controversial. The Umbrella and Penknife models hypothesize that the toxin partitions into the apical membrane of the insect midgut by insertion of only two α -helices from domain I of the protein, α -helices 4 and 5 in the case of the Umbrella Model and α -helices 5 and 6 in the case of the Penknife Model. Neither model envisages membrane partitioning by domains II and III. In this study, we present data suggesting that mutations in domain II residue, F371 affect insertion of the whole toxin into *Manduca sexta* brush border membrane vesicles (BBMV).

INTRODUCTION

Insecticidal crystal proteins (Cry toxins) produced by the soil bacterium *Bacillus thuringiensis* are a large family of toxins that target a wide range of insects and nematodes but are harmless to mammals (1). The crystal toxins belonging to the Cry1A series target the insect order Lepidoptera. They are produced as inactive protoxins and are activated inside the alkaline

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lepidopteran gut by proteases. Crystal structures of the active toxin in solution (2, 3) have shown that the toxin has 3 structural domains. Domain I is an α -helical bundle made of 7 α -helices. Domain II is predominantly composed of anti-parallel beta sheets, with predominant loops at the apex. Domain III is a β - sandwich. The active form binds to one or more receptors on the brush border membrane vesicles (BBMV) of the insect, including cadherins, alkaline phosphatases and/or one or more forms of the aminopeptidases (4-6). The receptor bound toxin then is proposed to undergo several conformational changes such as aggregation and oligomer formation (7, 8) before inserting into the membrane.

Initial models of insertion of toxin into the membrane, the umbrella and penknife models, suggest that only α -helix 4 and 5 of domain I, in the case of the umbrella model, and α -helices 5 and 6 in the case of the penknife model, insert to form an ion channel (9-11). However there is no conclusive evidence presented on the fate of domain II and domain III, which account for 60% of the bulk of the toxin, upon insertion. Studies using non-specific proteases on Cry toxins that have been inserted into the BBMV show that almost the entire toxin of 60 kDa is protected inside the membrane (12-15); only α -helix 1 is lost from the active toxin in the membrane-bound state.

Site-directed mutagenesis studies involving replacement of single amino acids in domain II of Cry1Ab toxin (16, 17) show that mutations in phenylalanine 371 residue to several residues including cysteine, serine, alanine, valine, tyrosine and phenylalanine do not affect the competition binding of the toxin to BBMV, but there was a significant loss of toxicity that is inversely correlated to the hydrophobicity of the replaced residues. This could have been due to the hydrophobic residue playing a role in tighter binding of the toxin to BBMV or due to the regions of domain II including loop 2 being inserted into the apical membrane of the gut. The

present study presents evidence supporting the latter model of domain II inserting into the membrane.

RESULTS

Production of stable toxins: The desired cysteine and alanine mutations were obtained as described in methods and were verified using DNA sequencing. The wild type and mutant proteins were expressed in *E. coli* DH5 α as 130 kDa protoxin molecules. All four proteins used in this study were digested with trypsin to yield 65 kDa toxin molecules. The protoxin and toxin molecule sizes were verified on an 8% SDS –PAGE gel (data not shown). The trypsin activated mutant toxins displayed the same stability as the wild type activated toxin. All activated toxins were purified through an ion exchange and 2 gel filtration columns as a monomer of 65 kDa. The secondary structures of the purified mutants were compared to that of the wild type Cry1Ab using CD spectrometry (data not shown).

Toxicity Bioassays: The biological activity of each toxin was measured using surface contamination method against *M. sexta* larvae and the results reported as LC₅₀ (concentration required to kill 50% of the larva tested) as shown in Table 1.

Table 1: Bioassay measurements of Cry1Ab and its mutants on 1st instar larvae of *M. sexta* (tobacco hornworms) using surface contamination method. Eight larvae were used per concentration of toxin. The results were measured after 5 days of incubation and calculated as LC₅₀ using Probit analysis (Softtox).

Sample	LC ₅₀ <i>M. sexta</i> (ng/cm ²)
Cry1Ab	20.00 [7.5-31.7]
1AbV171C	40.3 [26.6-53.4]
1AbF371C	>2000
1AbV171C/F371A	>2000

Surface plasmon resonance analysis: The binding of the mutant toxins to the toxin binding cadherin repeats 11 and 12 in the BT-R₁ sequence was compared to that of Cry1Ab wild type toxin. Table 2 shows that compared to the wild type ($K_D \sim 18$ nM), the 1AbF371C ($K_D \sim 48$ nM) and the double mutant 1AbF371A/V171C ($K_D \sim 66$ nM) have only a minor decrease in binding.

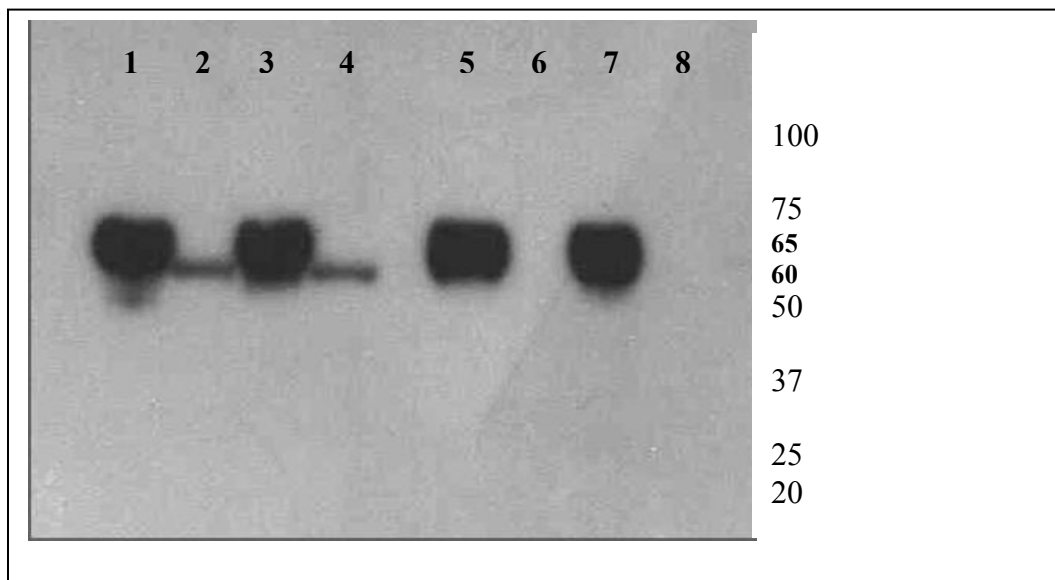
Table 2: Binding measurements of the toxin 1Abwt and its mutants to cadherin repeats 11 and 12 using surface plasmon resonance analysis (Biacore). The results are expressed as K_D values (ratio of the $k_d:k_a$) and are expressed in Molar units. Sets of measurements for each sample were performed twice, as shown

Sample		k_a (1/Ms)	k_d (1/s)	K_D (M)	χ^2
1Abwt:	exp1	6.51E+04	1.17E-03	1.79E-08	1.08
	exp 2	6.42E+04	1.21E-03	1.89E-08	0.859
1AbV171C:	exp1	7.64E+04	1.84E-03	2.41E-08	6.02
	exp 2	7.04E+04	1.55E-03	2.21E-08	1.1
1AbF371C:	exp1	3.66E+04	1.79E-03	4.89E-08	5.38
	exp 2	3.83E+04	1.83E-03	4.78E-08	6.32
1AbF371A/V171C:	exp 1	1.83E+04	1.25E-03	6.80E-08	6.58
	exp 2	1.83E+04	1.17E-03	6.41E-08	2.15

Proteinase K protection assays: Protection assays were carried out on toxin bound BBMV to determine if each of our toxins inserted into the BBMV. Western blot analysis show that while the wild type and the toxic mutant 1AbV171C were protected from 10 fold excess of proteinase K even after 30 min of incubation at 37° C, seen as a 60 kDa band on the gel, the domain II mutant 1AbF371C and the double mutant 1AbV171C/F371A were completely digested by the non-specific protease in the same conditions (Figure 1). The results suggest that the change in

residue F371 to cysteine has an affect on the insertion process. We have observed that F371A alone has the same affect (not shown).

Figure 1: Proteinase K protection assay of Cry1Ab wt and its mutants. Reaction was run on 4-20% SDS-PAGE gels and the membranes were blotted using anti-1A polyclonal antibody and HRP tagged anti rabbit secondary antibody. Lane1: Pure Cry1Abwt (10 μ g). Lane 2: proteinase K treated BBMV bound to Cry1Ab wt. Lane 3: Pure 1AbV171C (10 μ g). Lane 4: proteinase K treated BBMV bound to 1AbV171C. Lane 5: Pure 1AbF371C (10 μ g). Lane 6: proteinase K treated BBMV bound to 1AbF371C. Lane 7: Pure 1AbF371A/V171C (10 μ g). Lane 8: proteinase K treated BBMV bound to 1AbF371A/V171C.



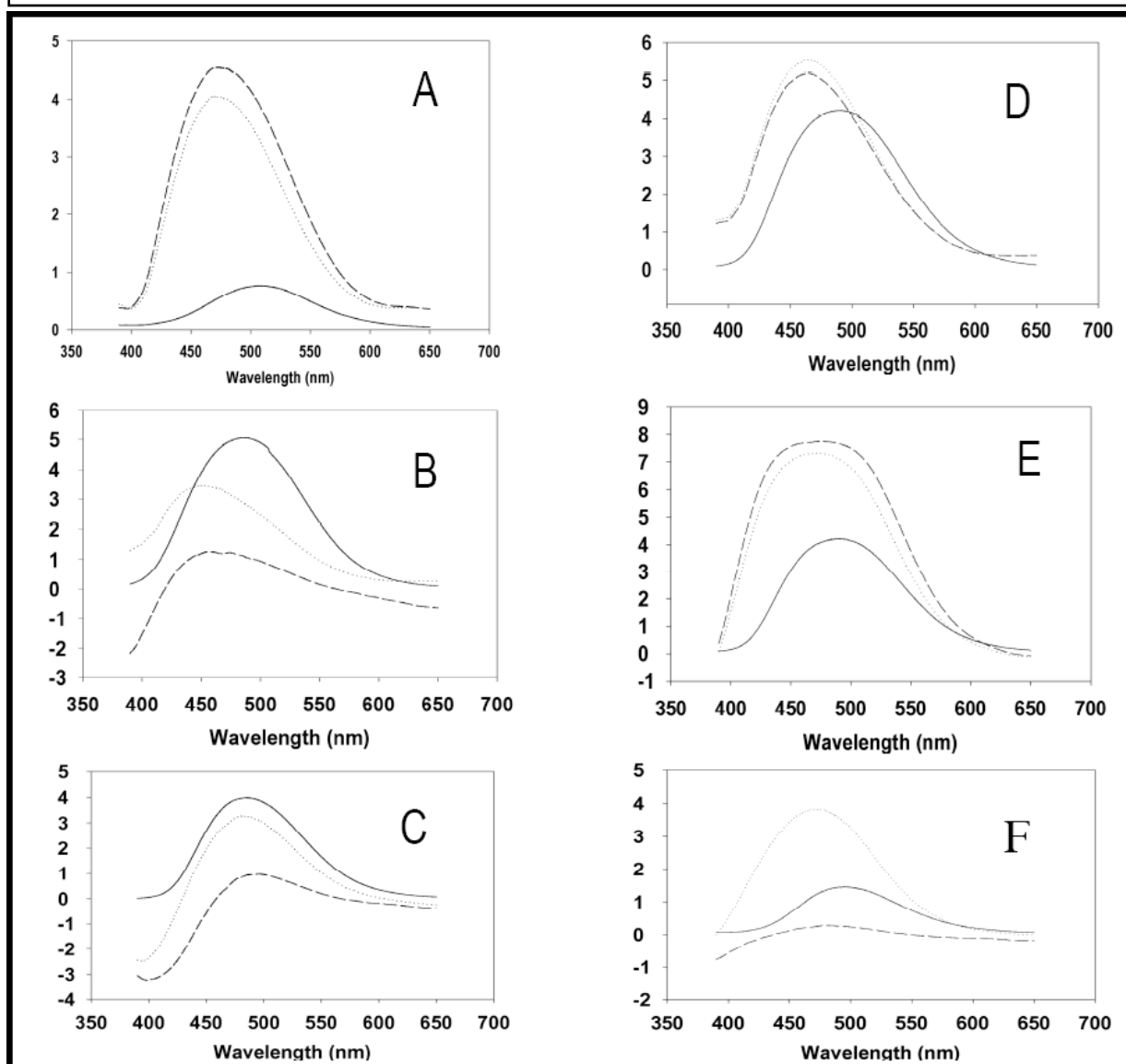
Fluorescence measurements: The fluorescence emission of acrylodan is highly sensitive to the environment of the fluorophore, with the fluorescence maxima of ≥ 480 nm in a hydrophilic environment and < 460 nm in a hydrophobic environment (24, 25). Our studies with the label show that while the excited state emission of the acrylodan bound to the protein is very low in solution there is a dramatic blue shift in fluorescence emission when the labeled protein is in either artificial SUVs or in BBMVs. The dipole moment of the label being highly sensitive to the environment, the emission of each labeled mutant is different in the free form itself depending on the location of the mutation (24). While a blue shift is the predominant indication of the change

in the environment, the intensity of the emission is also a representation of the environment of the label (25).

The acrylodan-labeled toxic mutant protein, 1AbV171C shows a blue shift in maximal emission wavelength from 500 ± 10 nm in free solution to 462 ± 9 nm in BBMV and to about the same value (462 ± 11 nm) in BBMV treated with proteinase K (Figure 2A). Table 3 records the maximal wavelengths for each mutant bound to either SUV or BBMV before and after proteinase K treatment. That the shift was not from hydrophobic effects of the receptors outside the vesicles was ensured by the emission spectra from the proteinase K treated BBMV or SUV bearing the labeled toxin, since receptors are not present in the case of SUV, and are removed from BBMV by proteinase K. The intensity of emission of the spectra both before and after proteinase K treatment was similar for this mutant suggesting that the particular region of the toxin was embedded into the vesicles. The acrylodan-labeled domain II mutant, 1AbF371C mixed with vesicles (both BBMV and SUV) showed blue shift in the spectra, but upon proteinase K treatment, showed a significant drop in fluorescence intensity (Figure 2C and 2F).

<u>Table 3A</u>	Maximal emission wavelength of acrylodan labeled protein (nm)		
	In carbonate buffer	Bound to BBMV	Bound to BBMV+PK
1AbV171C (Fig 2A)	500 ± 10	462 ± 9	462 ± 11
1AbV171C/F371A (Fig 2B)	486 ± 0.5	465 ± 15	458 ± 5
1AbF371C (Fig 2C)	484 ± 1	482 ± 2	497 ± 2

Figure 2: Steady state fluorescence spectra of Cry1Ab mutants labeled with acrylodan. The samples were excited at 360 nm and the resultant emission recorded from 390 nm to 650 nm. The relative fluorescence (Y-axis) is expressed in arbitrary units. Correction of the spectra was made against either a buffer blank for the free protein in solution or against SUV or BBMV for the protein bound to the respective vesicles. (—) represents the spectra of the purified labeled protein in solution. (·····) represents the spectra of the pure labeled protein bound to SUV or BBMV before proteinase K treatment. (-----) represents the spectra of labeled protein bound to BBMV or SUV after proteinase K treatment. Figure 2A: Cry1AbV171C treated with BBMV. Figure 2B: Cry1AbF371A/V171C treated with BBMV. Figure 2C: Cry1AbF371C treated with BBMV. Figure 2D: Cry1AbV171C treated with SUV. Figure 2E: Cry1AbF371A/V171C treated with SUV. Figure 2F: Cry1AbF371C treated with SUV

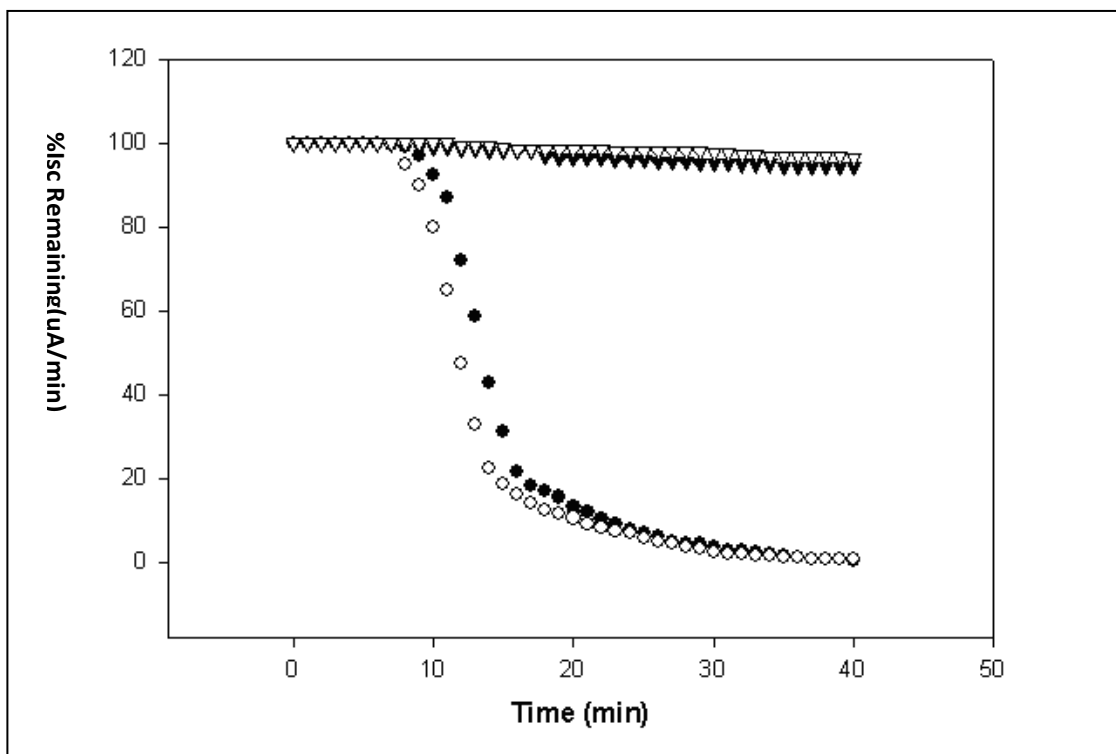


<u>Table 3B</u>	Maximal emission wavelength of acrylodan labeled protein (nm)		
	In carbonate buffer	Bound to SUV	Bound to SUV+PK
1AbV171C (Fig 2D)	493± 2	475± 3	470± 6
1AbV171C/F371A (Fig 2E)	492± 1	472± 0	480± 3
1AbF371C (Fig 2F)	492± 0	466± 5	474± 1

However for the double mutant (Figure 2E), where the protein was labeled with acrylodan in domain I (position 171), the protein was able to insert into the SUVs and was also protected from proteinase K in these artificial vesicles as seen by protection of the label in the protease treated vesicles. However, upon proteinase K treatment of the toxin bound to BBMV (Figure 2B), there was a loss of fluorescence intensity.

Voltage clamping analysis: To confirm the lack of pore formation of the non –inserting mutants, we carried out voltage clamping of *M. sexta* guts and measured the percentage of remaining short circuit current in the gut upon addition of Cry1Ab and its mutant proteins used in this study. The results as shown in Figure 3 demonstrate that while the domain I mutant V171C can form ion channels better than Cry1Ab wild type, the domain II mutant and the double mutant proteins have completely lost their ability to form pores in the midgut membrane.

Figure 3: Voltage clamp response of Cry1Ab wt (●) compared to that of BBMV- inserting mutant V171C (○) and BBMV non-inserting mutants F371C (▼) and V171C/F371A (▽)



DISCUSSION

Rajamohan, et.al (17) reported that mutating residue F371 to a number of amino acids did not affect its competition binding to *M. sexta* BBMV, but affected toxicity and “irreversible binding” in a manner inversely related to the hydrophobicity of the replacement amino acid. At the time of that study, it was unclear whether “irreversible binding” was due to tightness of binding to the receptor or proficiency of insertion into the membrane. Our binding studies of the mutant protein 1Ab-F371C and the double mutant protein 1Ab-F371A/V171C to cadherin receptor (Dorsch TBR sequence of repeats 11 and 12 (26)) using surface plasmon resonance support the view that these mutants do not suffer significant loss in binding to *M. sexta* cadherin when compared to the wild type protein. However, the data from toxicity bioassays and the voltage clamping studies reflect the inability of the mutant forms to retain their toxicity or ion

channel activity. This indicates a role for the residue F371 in associations of the toxin with the BBMV post receptor-binding in membrane insertion. Steady state fluorescence measurements of the bound and protected toxin show a blue shift in the maximal wavelength of the domain I mutant protein, suggesting the displacement of the label to a hydrophobic (membrane) environment (24). The hydrophobicity could hypothetically be due to binding of toxin to receptor (assuming the receptor is sufficiently hydrophobic), however, since binding is not greatly affected, this seems unlikely. In addition, one would expect proteinase K treatment would be able to access and digest the region of the receptor exposed outside the membrane and thereby also digest the toxin, given the incubation conditions of 30 min. Furthermore, when V171C, a domain I residue that is not believed to make contact with the receptor is labeled with acrylodan, we observe that the label migrates to a more hydrophobic environment in BBMV (Figure 2A). Our data from the proteinase K for the domain I mutant protein 1AbV171C and other mutants (manuscript in progress) dispersed across the toxin confirm that the label is in an environment not accessible by the protease suggesting that it is embedded into the bilayer. However, when the label is attached to the cysteine on position 371, the toxin was unable to enter the vesicles, leaving the protein exposed to proteinase K even after binding to the receptors on BBMV. This is also the case for the double mutant where the fluorescent label is on domain I residue but also incorporates a mutation in position 371 in domain II. Non receptor-mediated residual partitioning of the regions of domain I (around position 171) into the vesicles occurs in BBMV explaining the residual fluorescence associated with the proteinase K treated BBMV, in the case of double mutant 1Ab-F371A/V171C. SDS PAGE gels show a 60 kDa protected form of toxin for the wild type and 1AbV171C (Figure 1), which is also seen in case of several other mutations in 1Ab toxin (Figure 4). But an absence of that form for the F371C or F371A double mutant

corroborates the pattern of fluorescence data.

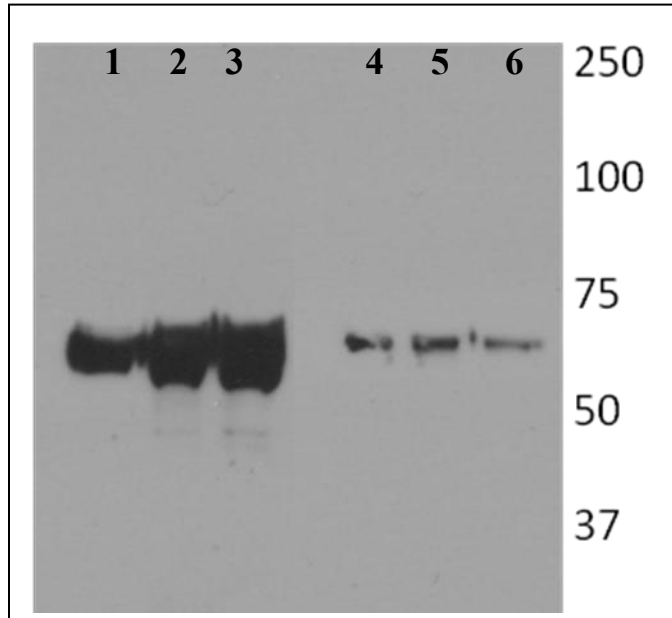


Figure 4: Proteinase K protection assay of mutants in 3 domains of Cry toxin. Mutations 1Ab S176C (Domain I), 1Ab S443C (Domain II) and 1Ab F461C (Domain III) were expressed and purified (Lanes 1, 2 and 3) respectively. Proteinase K protection assay was performed and SDS-PAGE showing a 60 kDa protected form is observed for 1Ab S176C (Lane 4), 1Ab S443C (Lane 5) and 1Ab F461C (lane 6).

An interesting observation in these studies is that Cry1Ab membrane partitioning into BBMV is different than its partitioning into SUV. Labeling the toxin on α -helix 5 of domain I (residue 171) with acrylodan showed a blue shift in both BBMV and SUV when domain II residue 371 is wild-type (Phe). When residue 371 is mutated to alanine the acrylodan-labeled domain I undergoes a blue shift in SUV and is protected from proteinase K, but not in BBMV. This indicates that domain I, at least residue 171 or α -helix 5, is able to enter the artificial SUV membrane but not the BBMV membrane, even if residue 371 is alanine or cysteine.

The larger goal of this study is to determine the mechanism of insertion of the toxin. The ability of domain II mutant to partition into the membranes of these vesicles is in agreement with the hypothesis proposed in our recent paper (27) that while α -helices from domain I are able to

insert, the mechanism of insertion is not based solely on individual helices entering the membrane. Other studies (13) suggest a buried “unchanged structure” model for the toxin where almost all of the toxin is buried into the membrane. Our data support the view that the mode of entry of the toxin into the membrane is in the form of an intact 60 kDa monomer or oligomeric toxin that lacks α -helix 1 at its N-terminus.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis. Uracil-containing template of Cry1Ab was obtained as described (17). Site-directed mutagenesis was carried out using the MutaGene M13 *In Vitro* Mutagenesis kit as described in the manufacturer’s manual (BioRad). The presence of the expected mutations in Cry1Ab (V171C, F371C and the double mutant: V171C/F371A) were confirmed by sequencing double-stranded DNA at the Plant Microbe Genomics Facility, Ohio State University, Columbus, Ohio. Expression and purification of the Cry1Ab wild-type and mutant toxins were carried out as described elsewhere (18). Secondary structure of each mutant was confirmed using circular dichroism spectrophotometry.

Preparation of small unilamellar vesicles. 1-Palmitoyl-2-oleyl-sn-glycerol-3-phosphatidylcholine, 1-Palmitoyl-2-oleyl-sn-glycerol-3-phosphatidylethanolamine and cholesterol (Avanti Polar lipids Inc.) were used in the ratio of 7:2:1 to prepare small unilamellar vesicles (SUV) using a Branson Sonifier water bath. The protocol is described elsewhere (19).

Preparation of BBMVs. Fifth instar larvae of *M.sexta* were dissected as described elsewhere (20). BBMVs were prepared by modified differential magnesium precipitation method (21). The final BBMV pellet was resuspended in binding buffer (10 mM HEPES, 150 mM NaCl pH 7.4). Protein concentration was estimated using Coomassie Protein Assay reagent (Pierce Biotechnologies, Inc.)

Proteinase K protection assays. Protection assays were performed as described using a protocol described elsewhere (12). Western blots of the protected sample were done using anti-Cry1A polyclonal antibody (1:5000)

Labeling of purified cysteine mutant toxins. Purified cysteine mutants were mixed with 10-fold molar excess of 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan) (Invitrogen Inc.) and incubated in the dark overnight. The labeled protein was purified off the free label using desalting Sephadex G25 column (GE Healthcare). Purity of the labeled protein was checked on an 8% SDS-PAGE gel and the degree of labeling was estimated using the molar extinction coefficient of acrylodan. 50 μ g of the labeled protein was mixed with 500 μ g BBMV or 5 mgs of SUV and incubated for 60 min. Bound forms of the labeled protein were separated from the unbound labeled proteins by either centrifuging the BBMV pellet down at 15000 g or by passing the SUVs through a Sephadex G100 column (GE Healthcare). The BBMV or SUV was treated with 500 μ g of proteinase K and incubated for 30 min at 37° C. 1 mM PMSF was added to stop the reaction. The reaction was then spun down at 15000 g to recover the BBMV or passed through Sephadex G 100 column (GE Healthcare) to recover the SUV.

Fluorescence measurements. Steady state fluorescence measurements were carried out on Fluoromax 3 fluorimeter (JY Horiba Instruments). The labeled proteins were excited at 360 nm and emission intensity was measured from 390- 600 nm. The labeled protein in solution, bound to BBMV and proteinase K treated were measured simultaneously to avoid any instrumentation error. Spectra were corrected for background from buffer and /or the vesicles. Each experiment was performed three times. Emission spectra were plotted using relative fluorescence of the mutants in buffer to that in the membrane before and after proteinase K treatment.

Surface plasmon resonance analysis. Surface plasmon resonance experiments on BIAcore 3000 were performed for kinetic analysis. BIAcore's carboxymethylated dextran matrix (CM5) sensor chip was used. The analysis temperature was set to 25° C. Around 15000 RU of anti-MBP IgG was immobilized on the surface of flow cell 2 on the CM5 sensor chip using an EDC/NHS-mediated amine coupling procedure. A freshly prepared solution of 50 mM NHS (N-hydroxysuccinimide) and 0.2M EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] was injected for 7 min to activate the flow cell. Anti-MBP IgG was reconstituted in 10 mM NaCOOH, pH 5.0 and injected at a flow rate of 10 μ l/min. Excess activated

ester groups on the surface were deactivated using a 7 minute injection of 1 M ethanolamine-HCl, pH 8.5. Flow cell 1 was activated with 50 mM NHS and 0.2 M EDC and blocked with 1M ethanolamine-HCl, pH 8.5 without immobilization of anti-MBP IgG, serving as the reference surface.

Maltose binding protein-receptor fusion protein, MBP-CAD-D was constructed as a subclone of the *M. sexta* CAD gene provided to us in pMECA vector (22) including CAD regions 11 and 12. MBP-CAD-D at 50 µg/ml was injected at a flow rate of 5µl/min to interact with IgG on the chip. Over a 3 min period of time, approximately 100 RU of MBP-receptor was captured. The (anti-MBP IgG)-(MBP-CAD-D) surface was allowed to stabilize for 1 min, before wild type toxin at various concentrations were injected. Buffer only was included as a blank. The flow rate for toxin injections was at 30 µl/min. The association phase was 3 min and the dissociation phase was 10 min. Regeneration was achieved by two 30-second injections of 10 mM glycine, pH 1.8 at 100 µl/min. The control flow cell that was activated and blocked without immobilization of the antibody had both MBP-receptor fusion protein and toxin flowing through in each cycle. Running buffer in all experiments was HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% surfactant). MBP-CAD-D fusion protein was purified in the same buffer, while toxin was prepared in sodium carbonate buffer and filter-dialyzed into the HBS-P buffer.

Toxicity Bioassays. Toxicity levels were determined on first instar *M. sexta* larvae. The median lethal concentration (LC₅₀) was estimated by diet surface contamination assays as described (18).

Voltage clamp analysis. Voltage clamp analysis was performed as described earlier (20). After stabilization of the midguts in the buffer (23), 100 ng of each toxin was added to the lumen side of the chamber. The volume of the lumen chamber is 3.75ml. Recorded data was normalized to the percentage of I_{sc} remaining. Each experiment was repeated at least 3 times.

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