Domains II and III of *Bacillus thuringiensis* Cry1Ab Toxin Remain Exposed to the Solvent after Insertion of Part of Domain I into the Membrane* S

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Luís Enrique Zavala, Liliana Pardo-López, Pablo Emiliano Cantón, Isabel Gómez, Mario Soberón, and Alejandra Bravo

From the Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca 62250, Morelos, Mexico

*Bacillus thuringiensis* produces insecticidal proteins named Cry toxins, that are used commercially for the control of economical important insect pests. These are pore-forming toxins that interact with different receptors in the insect gut, forming pores in the apical membrane causing cell burst and insect death. Elucidation of the structure of the membrane-inserted toxin is important to fully understand its mechanism of action. One hypothesis proposed that the hairpin of α-helices 4–5 of domain I inserts into the phospholipid bilayer, whereas the rest of helices of domain I are spread on the membrane surface in an umbrella-like conformation. However, a second hypothesis proposed that the three domains of the Cry toxin insert into the bilayer without major conformational changes. In this work we constructed single Cys Cry1Ab mutants that remain active against *Manduca sexta* larvae and labeled them with different fluorescent probes that have different responses to solvent polarity. Different soluble quenchers as well as a membrane-bound quencher were used to compare the properties of the soluble and brush border membrane-inserted forms of Cry1Ab toxin. The fluorescence and quenching analysis presented here, revealed that domains II and III of the toxin remain in the surface of the membrane and only a discrete region of domain I is inserted into the lipid bilayer, supporting the umbrella model of toxin insertion.

Most of the Cry proteins are produced as crystalline inclusions of 130 or 70 kDa protoxins. Protoxins are solubilized in the larval gut lumen and activated by midgut proteases in both terminal ends to yield activated monomeric 60-kDa toxins with a three domain structure (1–3). Domain I is a seven α-helix bundle implicated in oligomerization and in membrane channel formation. Domain II consists of a beta-prism of three anti-parallel β-sheets packed around a hydrophobic core and domain III is a β-sandwich of two antiparallel β-sheets. Domains II and III are important for interaction with toxin receptors and thus have important roles in insect specificity (1–3).

The mechanism of action of Cry toxins involves several steps and interactions with different receptors that depend on the oligomeric state of the toxin in a ping-pong binding mechanism (3–5). The activated monomeric toxins bind to highly abundant low affinity receptors, glycosylphosphatidylinositol (GPI)-anchored proteins, such as aminopeptidase N (APN) and alkaline phosphatase (ALP), localizing the toxin in the brush border microvilli. Specifically, loop 3 of domain II and β-16 of domain III are involved in this first interaction (4, 5). After this, the toxin binds to low abundant cadherin receptor, in a high affinity and complex interaction involving participation of loop 2, loop 3, and loop α-8 of domain II the toxin. Binding with cadherin facilitates additional protease cleavage of the N-terminal end of the toxin eliminating helix α-1 of domain I (6). This cleavage induces assembly of an oligomeric form of the toxin. The conformational changes in toxin oligomers results in 100-fold increased binding affinity to APN and ALP receptors, through loop 2 (3–6). After the oligomers bind to these receptors they insert into membrane microdomains, creating pores in the apical membrane of midgut cells causing osmotic shock, bursting the midgut cells and finally ending with the death of the insect (7, 8).

The insertion of the toxin into the membrane is one of the less characterized steps in the mechanism of action of Cry toxins and elucidation of the structure of the membrane-inserted toxin is important to fully understand its mechanism of action. The hypothesis is that interaction of toxin oligomer with the APN and ALP receptor proteins triggers a conformational change that is necessary for the insertion of the toxin into the membrane (9). Previous studies demonstrated that separation of domains I and II is a necessary step for pore formation in black lipid bilayers (10). In addition, several studies performed with single point mutations in the different helices of domain I...
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of Cry1A toxins, supported that helices α-3, α-4, and α-5 are important for toxicity and pore formation, without affecting their binding interaction with toxin receptors (11–15). The helix α-3 participates in the oligomerization process (13), and it is proposed that the hydrophobic hairpin formed by helices α-4 and α-5 inserts into the phospholipid bilayer whereas the rest of amphipathic helices of domain I, excluding helix α-1, are spread on the membrane surface in an umbrella-like conformation (10, 16–19). It was shown that the hydrophilic face of helix α-4 is facing the lumen of the pore, by in situ restoration of a negative charge of mutant D136C, that resulted in restitution of ionic conductance to the levels of the wild-type Cry1Aa toxin (16).

In 2001, an alternative model was proposed based in data of the activation energy of denaturation of Cry3Aa toxin when it is bound to the membrane (20). In this model the authors proposed that the conformation of the toxin do not change upon insertion into the membrane and that the three domains of Cry3Aa inserted into the bilayer without further conformational changes, with the exception of the first three α-helices (20). In 2008, a study that involved the analysis of several single cysteine (Cys) Cry1A mutants labeled with fluorescent dyes, such as 1,5-IAEDANS, or acrylodan, also concluded that all domains of the toxin insert into the membrane (21). These data contradicted previous studies that had shown that some regions of domains II and III of Cry1A toxins remain in the surface of the membrane (9, 19, 22). Quenching analysis of tryptophan (Trp) fluorescence of the membrane-inserted Cry1Ac oligomer, showed that Trp-545, located in domain III, remains exposed to the solvent (9). In addition, fluorescence resonance energy transfer (FRET) analysis of Trp residues to 1-anilinonaphthalene-8-sulfonic acid dye (ANS) in membrane-bound Cry1Ab oligomer, indicated that domains II and III were located in the surface of the membrane, in contrast to domain I that was inserted into the membrane (22). It is important to mention that most of the pore forming toxins, such as anthrax toxin, aerolysin, α-hemolysin, and CDC toxins, that kill different cell types, behave in a similar way, since only a small part of these proteins inserts into the lipid bilayer, while the rest of the protein remains outside of the membrane (23). Recently, a three-dimensional structure model of the Cry4Aa pre-pore, and the pore oligomer was presented (24). In this model the authors suggested that domains II and III of Cry4Aa toxin remain in the surface in the membrane bound oligomer and only the hairpin formed by helices α-4 and α-5 of domain I is involved in the insertion into the lipid bilayer (24).

The aim of this work was to understand how Cry toxins insert into the membrane. We isolated several mutants with single Cys residues in the three domains of Cry1Ab toxin and analyzed their insertion into the membrane using fluorescence spectroscopy and quenching analysis with different soluble quenchers as well as a membrane-bound quencher to compare the properties of the soluble and membrane-inserted forms of Cry1Ab toxin. Three mutants are located in domain I, two in domain II, and two in domain III. Among the mutants located in domain I, one mutant (V171C) is located in helix α-5, that corresponds to the region involved in membrane insertion according to Taveecharoenkool et al. (24). Our results indicate that domains II and III of the toxin remained exposed to the solvent in the surface of the membrane and only a discrete region of domain I was inserted into the lipid bilayer.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Plasmid pHT315 (25) containing cry1Ab gene was used as template for site-directed mutagenesis with QuickChange mutagenesis kit from Stratagene (La Jolla, CA), following the manufacturer’s instructions. Mutagenic oligonucleotides used to construct all mutants were: T122C, GAGTGGGAAGCAGATCCTTGATACTCAGATTAGAACGAG; V171C, GCTGCAAATTATCATTCATGTGGAGATGTTCAGT; G183C, GTGTTTGGGACGATTGGGAGTTGTTTTGTGCGCGACTATC; S324C, CATCCAAA TAATGCGCTGCCCTGAGGTTGCTCG; S364C, GTGTATAGACATTAATCGTCACCTTTATATAGAAGACC; K490C, TGGAACTTCGTCCTTGGCGCGACCAGTTTACAG; and N547C, ATTAGCGGAAAGACCCTATTGTGACCGGAAATTTTCAG.

Purification and Activation of Cry1Ab Toxins—The acrylamidoliferous Bt strain 407– (25) was transformed with wild-type pHT315-cry1Ab or the same plasmid containing cry1Ab mutant genes. Bt transformant strains were grown for 3 days at 30°C in HCT sporulation medium (26) supplemented with 10 μg/ml erythromycin.

After sporulation, crystals were purified by sucrose gradients as described (26). Proteoxins were solubilized 2 h at 37°C in alkaline buffer: 100 mM Na₂CO₃, 0.2% of β-mercaptoethanol, pH 10.5 and activated with trypsin (1:50 trypsin/protoxin, w/w) for 2 h at 37°C, after this incubation 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to stop proteolysis and the pH was equilibrated by adding same volume of 1 M Tris-HCl, pH 8.5. Activated proteins were then purified by anion exchange chromatography Mono Q-Sepharose fast flow from GE Healthcare (Little Chalfont, UK) in an AKTA FPLC System from Amsterdam Biosciences, using a 50 mM Tris-HCl, 50 mM NaCl, pH 8.5 buffer, and a linear NaCl concentration gradient from 50 to 300 mM. The samples were reduced by adding 1 mM EDTA and 3 mM dithiothreitol to minimize the oxidation of the thiol group of Cys residues, concentrated up to 1 μg per μl and buffer was changed to PBS by using Amicon 30,000 MWCO centrifuge filters from Millipore (Hayward, CA). Protein concentration was determined by the Bradford assay using bovine serum albumin as standard.

Toxicity Bioassays—Bioassays were performed with Manduca sexta neonate larvae by the surface contamination method. Different doses of spore/crystal suspensions (from 0.1 to 200 ng/cm²) were applied onto the diet surface contained in 24-well polystyrene plates (Cell Wells; corning Glass Works, Corning, New York). A total of 24 larvae per plaque, were fed with the different toxin doses in quadruplicate. The plates were incubated at 28°C with 65 ± 5% relative humidity and a light: dark photoperiod of 16:8 h. Mortality was recorded after 7 days and the 50% lethal concentration (LC₅₀) was analyzed with Probit LeOra software.

Labeling Mutant Proteins with Fluorescent Dyes—To reduce the disulfide bonds and improve the labeling of the Cys residues introduced into the mutant toxins, the pure protein samples
(400 – 600 μg), were incubated in PBS containing 3 mM dithiothreitol, 1 mM EDTA for 10 min at 37°C. The dithiothreitol was then removed by filtration centrifugation (27) in Sephadex G-25 columns (1 cm²), equilibrated with PBS pH 7, and proteins were incubated 2 h in dark at 37°C with 10-fold molar excess of the probes at 600 rpm agitation. Two different probes were used to label the Cry1Ab mutants, the 5-(2-((2-iodo-1-oxoethyl) amino) ethylamino)-1-naphthalenesulfonic acid (1,5-IAEANDS) or Alexa Fluor-350 from Molecular Probes. Unbound label was removed by filtration centrifugation, using Sephadex G-50 columns equilibrated with PBS pH 7. Finally, the pH of labeled proteins was changed to pH 9 by adding 1/10 of the volume of 500 mM Na₂CO₃, pH 9. The efficiency of labeling was measured using the molar extinction coefficient of each probe and Equation 1,

$$\frac{A_x}{\epsilon} \times \frac{MW \text{ protein}}{mg \text{ protein/ml}} = \frac{mol \text{ dye}}{mol \text{ protein}} \quad (\text{Eq. 1})$$

where Aₓ corresponds to the maximum wavelength of dye absorbance that in the case of Alexa Fluor-350 corresponds to 346 nm, and in the case of 1,5-IAEANDS corresponds to 336 nm and ϵ, corresponds to the molar extinction coefficient of the dye, that for Alexa Fluor-350 is 19,000 M⁻¹ cm⁻¹ at 346 nm, and for 1,5-IAEANDS is 5700 M⁻¹ cm⁻¹ at 336 nm.

Purity of the proteins was analyzed on SDS-PAGE 12% acrylamide. The labeling of the proteins was visualized by excitation of the SDS-PAGE gel with UV light transilluminator and analyzed in a Gel Doc XR System (Bio-Rad) as reported (28).

Preparation of Brush Border Membrane Vesicles (BBMV) and BBMV Containing Membrane-bound Quencher 5-Doxyl-PC—Insect midgut tissue of third instar M. sexta larvae were dissected and used to prepare BBMV by differential precipitation using MgCl₂ in absence if protease inhibitors (29). The BBMV were finally suspended in 50 mM Na₂CO₃, pH 9 and stored at –70°C until use. Concentration of protein in the BBMV was analyzed by using the Lowry DC protein assay (Bio-Rad). For preparation of BBMV containing the membrane-bound quencher, 1 mg of 1-palmitoyl-2-stearoyl-(5-doxyl)-sn-glycero-3-phosphocholine (5-doxyl-PC) lipid (Avanti Polar Lipids, Alabaster, AL) was dried by argon flow evaporation followed by overnight storage under vacuum to remove residual chloroform. The lipid was hydrated in 1.16 ml of 50 mM Na₂CO₃, pH 9 buffer to have a final concentration of 1 mM, and subjected to sonication five times for 1 min each time in a Branson-1200 bath sonicator (Danbury, CT). Finally, for each 30 μg of BBMV we added 20 μl of 5-doxyl-PC lipid, and the mixture was sonicated four times as described above.

Steady State Fluorescence Quenching Measurements—Experiments were carried out in an Aminco Bowman Luminescence Spectrometer (Urbana, IL). All measurements were made in a (4 × 10 mm) quartz cuvette at 22°C. The excitation and emission slits were 4 nm. The excitation wavelength was 346 nm for Alexa Fluor-350 and 336 nm for 1,5-IAEANDS, and the emission spectrum was recorded from 420 to 540 nm for 1,5-IAEANDS or from 400 to 500 nm for Alexa Fluor 350. The spectra were an average of three to four scans and were also corrected for background and dilution.

Fluorescent quenching experiments were performed with potassium iodide (KI) for Alexa Fluor-350-labeled proteins or with acrylamide for 1,5-IAEANDS-labeled proteins. Small-volume aliquots of 4 mM KI or 4 mM acrylamide stock solutions, prepared in the protein buffer were added to the samples and gently stirred. The emission spectra were recorded to check the effect on fluorescence intensity and λₒ, as control in the KI quenching assays, KCl was added so that the total salt (KI + KCl) concentration remained constant and equal to the highest concentration of KI used. Sodium thiosulfate (800 mM) was added to KI solutions to avoid the formation of I₃⁻. Effective Stern-Volmer constants (KSV) were obtained from the fluorescent data according to the Stern-Volmer equation for dynamic quenching (30),

$$\frac{F_o}{F} = 1 + K_{SV}[Q] \quad (\text{Eq. 2})$$

where Fo and F are the fluorescence intensities in absence and presence of the quencher, respectively. The value for KSV was obtained from the Stern-Volmer plots, calculating the slope of Fo/F versus concentration of quencher. The value for KSV can be considered to be a reliable reflection of the bimolecular collisional constant for collisional quenching, since KSV = k_o τ_o, where k_o is the bimolecular collisional constant and τ_o is the lifetime constant in the absence of quencher.

The quenching data with 5-doxyl-PC are presented as % quenching efficiency defined as the ratio of the fluorescence in the presence of the quencher to that without (Fₒ) quencher as follows, [(DFₒ – DF) × 100]/DFₒ, where DFₒ and DF are the changes in fluorescence intensity upon binding of 1,5-IAEANDS-labeled Cry1Ab proteins to BBMV and to BBMV-5-doxyl-PC containing the membrane bound quencher, respectively.

Insertion of Cry1Ab Mutant Toxin into the BBMV—We incubated 30 μg of each labeled protein with 150 μg of BBMV isolated from M. sexta larvae in 50 mM Na₂CO₃, pH 9 for 1 h at 25°C, the reaction was stopped with 1 mM PMSF and the inserted toxin into the BBMV was recovered by centrifugation 30 min at 50,000 rpm at 4°C. The pellet was suspended in 1.1 ml of 50 mM Na₂CO₃ buffer pH 9. We used 1 ml of inserted toxin for the quenching assays, using 200 μl of this sample per quencher concentration as explained above and the rest of the sample was used for Western blot analysis. Laemmli sample buffer 4X (0.125 M Tris/HCl, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromphenol blue) was added to the samples, boiled 5 min, loaded into 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membrane (PVDF) (Amersham Biosciences). The mutant proteins were revealed by Western blot assay using rabbit anti-Cry1Ab polyclonal antibody (1:30,000) and secondary anti-rabbit antibody (1:30,000) conjugated with horseradish peroxidase. The final blots were visualized using luminol (Amersham Biosciences).
RESULTS

Expression and Purification of Cry1Ab Mutants—To analyze the insertion of Cry1Ab toxin into *M. sexta* larvae BBMV, we introduced single cysteine substitutions in several regions of the three domains of this protein. We took advantage that Cry1Ab toxin does not contain any Cys residue in its structure to produce these single Cys toxins, with the aim of labeling these residues with different fluorescent probes, that either have a strong response to solvent polarity or that could be quenched with negative charged water soluble quenchers as iodide (I⁻). Three domain I mutants (T122C, V171C, and G183C), two domain II mutants (S324C and S364C), and two domain III mutants (K490C and N547C) were constructed. Fig. 1 shows the location of these residues in the three-dimensional Cry1Ab structure model based in the coordinates of the closely related Cry1Aa toxin. In this figure we show an analysis of the exposition of these residues to the solvent. All residues were located in exposed regions of the protein with exception of Val-171 that is located in helix /H9251–5 at the center of Domain I and is buried in the three-dimensional structure (green color in Fig. 1, which is not visible in panels A, B, and C). The other mutants were located in surface-exposed locations: T122C is located in the loop between helices /α3–α4; G183C in the loop between helices /α5–α6; S324C in loop of strands β3–β4 of domain II; S364C in strand β6 of domain II; K490C is located in loop between strands β14–β15 of domain III and finally, N547C is found in middle of strand β19 of domain III. The location of all of these residues in the three-dimensional structure of Cry1Ab, suggests that all residues, with exception of V171C, would be accessible for the labeling reaction with the fluorescent dyes. After mutagenesis, plasmids were sequenced and transformed into an acrystalliferous Bt strain. The crystalline inclusions produced by all these Bt strain were bipyramidal, similar to the crystal inclusions produced in the wild-type strain. The crystal inclusions were solubilized, activated with trypsin and purified as described under “Experimental Procedures.” The trypsin activated proteins of all mutants produced a 65 kDa protein similar to the Cry1Ab toxin, which were recognized by several monoclonal and polyclonal Cry1Ab specific antibodies (data not shown), suggesting that these mutations did not cause major structural disturbance in the Cry1Ab toxin.

Toxicity Effects of Single Cys Mutants Isolated in Cry1Ab Toxin—We determined the effect of the different Cys substitutions in the toxicity of Cry1Ab against *M. sexta* larvae. Supplemental Table S1 shows the LC₅₀ lethal values obtained with the different mutants. All mutants were active against *M. sexta* larvae showing similar toxicity as the wild type Cry1Ab protein, because 95% confidential limits overlapped. These data confirmed that the single Cys mutants isolated in this work did not cause significantly the three-dimensional structure of the toxin.

Characterization of Fluorescent Probes—The fluorescent probes used in this study were 1,5-IAEDANS and Alexa Fluor-350. The 1,5-IAEDANS was previously used in a related study where the insertion of Cry1Aa and Cry1Ab toxins into the membrane, was analyzed (21). Nair and Dean (21) argued that 1,5-IAEDANS fluorophore has a high dipole moment and
hence increased quantum yield of emission in an aqueous environments, that gets quenched inside the membrane vesicles (21). However, we found that the properties of 1,5-IAEDANS were mistaken since, in the contrary, 1,5-IAEDANS is a naphthalene derivative that is quite dependent upon environment and severe changes in fluorescence intensity are determined by degree of aqueous solvation, showing low quantum yield in water and polar solvents, and much higher quantum yield in apolar or hydrophobic environments (31, 32). Thus, the fluorescence intensity of 1,5-IAEDANS should increase when inserted into in hydrophobic environment of the membrane.

To confirm this, we analyzed the fluorescence of 1,5-IAEDANS in different solvents with different polarity. Fig. 2A shows the emission spectra of 1,5-IAEDANS in different solvents, showing much higher fluorescence intensity in methanol and ethanol than in water soluble phosphate buffer. Fig. 2B shows the SternVolmer plots of 1,5-IAEDANS and Alexa Fluor 350 (panel D) after quenching with KI (dotted line, C) or by acrylamide (solid line, D).

FIGURE 2. Characterization of 1, 5-IAEDANS and Alexa Fluor-350 fluorescent dyes. Panels A and C show the emission spectra of 1,5-IAEDANS (panel A) and Alexa Fluor 350 (panel C) in different solvents. Solid line, ethanol; dotted line, methanol; and gray line, water-soluble phosphate buffer. Panels B and D show the SternVolmer plots of 1,5-IAEDANS (panel B) and Alexa Fluor 350 (panel D) after quenching with KI (dotted line, C) or by acrylamide (solid line, D).

In this work, we also used Alexa Fluor-350 maleimide probe that is a sulfonate coumarin derivative that reacts with thiol groups of proteins (33–35), showing an optimal excitation at 346 nm and a bright blue fluorescent emission. The fluorescent emission of this dye is independent of pH, and is not sensitive to changes in solvent polarity (33–35). Fig. 2C show that fluorescence intensity did not change in the different solvents and the maximum wavelength of emission showed a shift of only 7 nm between ethanol and water. In contrast to 1,5-IAEDANS, the Alexa Fluor 350 was efficiently quenched by KI with an effective $K_{SV}$ of 29.0 M$^{-1}$, while acrylamide showed no effect (Fig. 2D).

Labeling Cry1Ab Mutants with Fluorescent Probes—Cry1Ab mutants were independently labeled with 1,5-IAEDANS or with Alexa Fluor-350 and the efficiency of labeling was measured using the molar extinction coefficient of each probe as described under “Experimental Procedures.” In general we found a lower stoichiometry of labeling when proteins were labeled with 1,5-IAEDANS probe, showing values of 0.6–0.8 mol of 1,5-IAEDANS per mol of toxin. Only mutant V171C located in domain I showed less efficiency of labeling (0.24 mol per mol of toxin). Because V171C may be less accessible to the solvent we opened the structure of Cry1Ab with 6 M urea as was previously described (22, 36) to facilitate the access of the reagent to this partially buried cysteine. We then labeled this mutant with Alexa Fluor-350. The efficiency of labeling with Alexa Fluor-350 was 0.8 to 1.0 mol of Alexa Fluor-350 per mol of monomeric toxins (supplemental Table S2). Fig. 3, A and B shows the SDS-PAGE of labeled proteins with 1,5-IAEDANS or Alexa Fluor-350, respectively, stained with Coomassie Brillant Blue dye. All proteins showed similar size to the unlabeled wild-type Cry1Ab toxin. The labeling of the toxins was also analyzed directly on the SDS-PAGE, visualizing the labeled protein by excitation with UV light transilluminator (Fig. 3, C and D).

In the case of V171C this could be due to the low efficiency of labeling with the dye as shown in supplemental Table S2). In the case of mutants S364C and S324C, both of them showed a similar ratio of labeling (0.7 mol of 1,5-IAEDANS per mol of toxin), but their difference in fluorescence intensity observed in the denaturant SDS-PAGE, was quite significant (Fig. 3C). This
could be explained by the highly sensitive response to the environment of the 1,5-IAEDANS in a polar environment where this dye is severely quenched (Fig. 2A), suggesting that residue S364C is highly exposed to a polar environment after SDS-PAGE electrophoresis.

Analysis of the Insertion of Cry1Ab-labeled Mutants into Brush Border Membrane Vesicles (BBMV)—We isolated BBMV from 3rd instar M. sexta larvae and analyzed the insertion of toxin mutants. The interaction of Cry1Ab proteins with the membrane was first assayed by Western blot, showing that labeled mutants have a monomeric structure in solution, but after interaction with BBMV, all of them formed oligomeric structures that were resolved as protein bands of 250 kDa molecular size in SDS-PAGE as previously described (3) (supplemental Fig. S1).

To determine the degree of solvent exposure of each labeled residue, we used I⁻ or acrylamide, as quenchers for Alexa Fluor 350 or 1,5-IAEDANS fluorescence, respectively. Both quenchers yielded linear Stern-Volmer plots (Fig. 4). The apparent dynamic quenching constants $K_{SV}$ derived from the slopes of these plots are presented in Table 1.

In the case of quenching with I⁻ of Alexa Fluor-350-labeled mutant toxins, we found that V171C and T122C mutants, located in domain I, showed a lower susceptibility to be quenched by I⁻ when they were inserted into the membrane than in solution, suggesting that these residues were buried into the membrane environment (Fig. 4A). As expected, the effective $K_{SV}$ for I⁻ quenching of these toxins was much lower in the membrane inserted state than in solution, showing a blue shift in $\lambda_{max}$ of 3 nm (Table 1), supporting a more hydrophobic environment for these residues when the toxins are inserted into the membrane. In contrast, residue G183C also located in domain I, showed similar Stern-Volmer plots in solution and in the membrane-inserted state, suggesting that this residue remains exposed to the solvent even in the membrane associated protein (Fig. 4B).

To analyze the interaction of Cry1Ab with the M. sexta BBMV vesicles, the fluorescence of the labeled single Cys proteins with the two different probes was analyzed, first in the soluble state and then compared after membrane association. To determine the degree of solvent exposure of each labeled residue, we used I⁻ or acrylamide, as quenchers for Alexa Fluor 350 or 1,5-IAEDANS fluorescence, respectively. Both quenchers yielded linear Stern-Volmer plots (Fig. 4). The apparent dynamic quenching constants $K_{SV}$ derived from the slopes of these plots are presented in Table 1.

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TABLE 1

Fluorescent properties of single cysteine mutants labeled with Alexa Fluor 350 probe quenched with KI or labeled with 1,5-IAEDANS probe quenched with acrylamide.

| Toxin     | Probe-Quencher          | $\lambda_{\text{max}}$ of emission | $K_{SV}$ |
|-----------|--------------------------|-------------------------------------|---------|
|           |                          | Solution | BBMV   | Solution | BBMV   | Change after BBMV insertion |
| T122C     | Alexa Fluor 350-KI       | 439 ± 1  | 437 ± 2 | 11.2 ± 0.3 | 6.0 ± 0.6 | Buried |
| V171C     | Alexa Fluor 350-KI       | 433 ± 2  | 430 ± 2 | 3.5 ± 0.3  | 2.0 ± 0.1 | Buried |
| G183C     | Alexa Fluor 350-KI       | 438 ± 1  | 437 ± 2 | 10.0 ± 0.3 | 9.8 ± 0.3 | No change |
| S324C     | Alexa Fluor 350-KI       | 438 ± 1  | 437 ± 2 | 9.1 ± 0.2  | 9.7 ± 0.3 | No change |
| S364C     | Alexa Fluor 350-KI       | 433 ± 2  | 433 ± 2 | 4.8 ± 0.6  | 2.0 ± 0.1 | Buried |
| K490C     | Alexa Fluor 350-KI       | 438 ± 1  | 439 ± 2 | 5.0 ± 0.5  | 5.3 ± 0.4 | No change |
| N547C     | Alexa Fluor 350-KI       | 438 ± 1  | 441 ± 2 | 7 ± 0.6    | 8.1 ± 0.7 | No change |
| T122C     | 1,5-IAEDANS-acrylamide   | 493 ± 1  | 488 ± 2 | 6.4 ± 0.4  | 4.8 ± 0.3 | Buried |
| S324C     | 1,5-IAEDANS-acrylamide   | 490 ± 1  | 490 ± 2 | 4.2 ± 0.2  | 5.7 ± 0.3 | No change |
| K490C     | 1,5-IAEDANS-acrylamide   | 490 ± 1  | 492 ± 2 | 3.8 ± 0.4  | 8.9 ± 0.5 | More exposed |
| N547C     | 1,5-IAEDANS-acrylamide   | 488 ± 3  | 475 ± 4 | 2.7 ± 0.5  | 2.4 ± 0.5 | No change |

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Mutant N547C located in domain III, showed a slightly higher value in the dynamic quenching constant $K_{SV}$, suggesting that this residue increased its exposure to the solvent when it is inserted into the membrane.

Regarding to the single Cys mutants that were labeled with 1,5-IAEDANS, we only analyzed those that showed high fluorescent emission. We used the neutral acrylamide quencher, that is able to penetrate into the interior of the protein but not into the hydrophobic core of membrane bilayer (37). The acrylamide quenching analysis of 1,5-IAEDANS, confirmed that the T122C residue has a buried position when the toxin is interacting with the membrane (Fig. 4B and Table 1). In contrast domain II and III residues S324C, K490C, and N547C, remained exposed to the solvent. In fact residues S324C and K490C showed higher quenching when the toxin is inserted into the membrane, showing higher values of the $K_{SV}$ dynamic quenching constant (Table 1), suggesting that these residues are more accessible to the solvent when the toxin is inserted into the membrane.

Mutant S364C is located at the end of strand β-6 of domain II and Fig. 4a shows that this residue showed lower quenching when the toxin is inserted into the membrane, suggesting that this residue may be also buried into the lipid bilayer when the protein is inserted into the membrane. Nevertheless, it is important to mention, that this residue is close to loop II, that plays an important role on protein contacts with cadherin, APN, and ALP receptors (4–7). We incubated S364C mutant labeled with Alexa Fluor-350, with 10-fold excess of a purified cadherin fragment or 20-fold pure M. sexta ALP, and analyzed its quenching with KI in the presence and absence of these receptor molecules. As control, we made same incubations with Alexa Fluor-350 labeled S324C mutant. We found that when mutant S364C was bound to cadherin fragment or to ALP, the quenching with KI was reduced, in contrast with the other mutant located in domain II, mutant S324C that was quenched similarly, independently if it is bound to the cadherin receptor molecule (Fig. 4C), and showed a slight change after interaction with ALP (Fig. 4D).

Additional support for the close proximity of the Val-171 and Thr-122 residues to the membrane lipids when the toxin is inserted into the membrane, come from data on quenching with ALP (Fig. 4D).
Insertion of Cry1Ab into the Membrane

since it is resistant to SDS treatment and boiling suggesting a strong interaction between monomers (39). It was previously shown that the Cry1A oligomeric structure is a functional structure that is able to interact with the membrane lipid bilayer, forming stable pores, in contrast to the monomer that has marginal interaction with the liposomes (39). In this work, we showed that binding of monomeric Cry1Ab mutant toxins to BBMV resulted in the formation of a 250 kDa structure, that remained associated with BBMV (supplemental Fig. S1), supporting that oligomer formation is important for membrane insertion of Cry1Ab toxin.

In the case of other pore-forming toxins (PFT) produced by other bacteria, an oligomeric pre-pore structure is also produced after receptor binding and the oligomeric structures of these toxins represents a toxin intermediate before insertion into the membrane (23). Regarding membrane insertion of these PFT, it has been documented that insertion into the membrane involves only small regions of these proteins (23). There are several examples of PFT that span the membrane bilayer by forming β-barrel structure, named β-PFT. Other PFT insert clusters of α-helices, and are named α-PFT. In most of the β-PFT, the insertion into the membrane, involves a subtle conformational change, where only a small part of the protein is located in the lipid environment, while the rest of the protein remain outside of the membrane (23). In several β-PFT, the structural region that inserts into the membrane, represents a small-unstructured loop motive in the soluble protein that refoards to form a transmembrane β-strand, that will be part of the β-barrel pore (23). In other cases such as the CDC toxin-family, important conformational changes from α-helix to β-strand, have been documented (23, 36). In contrast, α-PFTs, like colicins, have a dynamic structure that involved the insertion of preformed α-helices into the membrane (23). Also, in the case of α-PFT cytolsin A from Staphylococcus aureus, a conformational change must undergo to form the transmembrane pore, which is composed of 12 monomers, where only amphipathic α-helix A1 and part α-helix F1 penetrate the lipid bilayer (40).

Nair and Dean (21) reported that all domains of Cry1A toxin insert into the membrane, based in fluorescent studies of single Cys mutants labeled with 1,5-IAEDANS and acrylodan (21). However, their conclusions reached by the analysis of fluorescent changes of Cry1Ab mutants labeled with 1,5-IAEDANS were incorrect, because 1,5-IAEDANS is highly sensitive to polar environment, showing much higher fluorescence in hydrophobic environments, and this dye is not quenched by KI (Fig. 2) (31, 32). The misinterpretations of their data drove these authors to incorrect conclusions. In fact, the analysis of the data of Nair and Dean (21), in view of our observations on the quenching of 1,5-IAEDANS upon exposure to the solvent (Fig. 2), indicates that most of domain II and domain III residues analyzed by them, remained exposed to the solvent, since most of the Cys mutants analyzed by them showed reduced fluorescence intensities when inserted into the membrane, indicating higher exposure to the solvent (21). In addition, we show that 1,5-IAEDANS fluorescence is not susceptible to be quenched by KI, indicating that their KI quenching analysis of 1,5-IAEDANS-labeled mutants were not conclusive.

The most reliable way to determine changes on the exposure to the solvent, of a particular residue labeled with a fluorescent probe, is the use of soluble hydrophilic collisional quenchers (30). In this work, we incubated Cry1Ab toxin with BBMV at pH 9 since we have previously reported that alkaline pH enhanced Cry1Ab membrane insertion and pore formation, by facilitating a molten globe intermediate which is consistent with the alkaline pH found in lepidopteran midgut lumen (26, 39). Here we demonstrate that interaction of all Cys mutant proteins of Cry1Ab with BBMV resulted in the formation of the 250 kDa oligomeric structure (supplemental Fig. S1).

Our data indicate that only a small region of domain I is involved in membrane insertion and that domain II and III remain in the surface during pore formation. We report here that residue Gly-183, located in the loop between α5 and α6 of domain I, remains exposed to the solvent upon membrane insertion. These data indicated that not all domain I inserts into the membrane, supporting the umbrella insertion model, which proposed that only the hairpin conformed by helices α-4 and α-5 is inserted into the lipid bilayer (10, 16–19). Other reports have also supported the umbrella model of toxin insertion. Residue Trp-545 of Cry1Ac toxin, that is located in domain III, remained exposed into the solvent after membrane insertion as shown by the analysis of W545A mutant (9). FRET analysis between tryptophan residues of domain I and ANS, showed that domain I moves apart from domain II and III upon membrane insertion (22). Finally, pore formation activity of biotin labeled single Cys Cry1Aa mutants, showed that binding of streptavidin protein to biotin-labeled residues located in the loop between helices α-4 and α-5, affected pore formation activity, confirming that this hairpin is important for pore formation (19).

Our analysis indicated that three residues, two in domain I (Val-171 and Thr-122) and one in domain II (Ser-364) were less exposed to the solvent upon toxin membrane insertion. To further analyze if the change in solvent exposure of these residues was due to membrane insertion, quenching by 5-doxyl-PC lipid incorporated to BBMV was analyzed. The 5-doxyl-PC phospholipid used as quencher of fluorescence is a good ruler for probing membrane insertion of peptides labeled with 1,5-IAEDANS, because it acts over a short distance, without drastically perturbing the membrane. Experiments with 5-doxyl-PC-labeled phospholipids further demonstrated that Val-171 and Thr-122 residues interact with the lipid bilayer in contrast to Ser-364 residue that was not quenched in BBMV containing this quencher lipid suggesting that it remains in the surface, out side of the lipid core. Furthermore, we show here that interaction with cadherin and ALP receptors that are present in the BBMV affected the interaction of S364C with the KI collisional quencher indicating that this residue is less exposed to the solvent upon membrane insertion due to protein-protein interaction with BBMV receptor molecules.

Our results support the umbrella model of toxin insertion and are in agreement with a recently three-dimensional structure model of the Cry4Aa pre-pore oligomer that shows that the loop formed by helices α-4 and α-5 of domain I is the region that inserts into the lipid bilayer (24). We agree that the precise structure of the toxin in its membrane-inserted state still...
remains to be solved; nevertheless, it is clear that only discrete regions of domain I insert into the membrane and that domains II and III remain exposed to the solvent.

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