Role of Domain II, Loop 2 Residues of Bacillus thuringiensis CryIAb δ-Endotoxin in Reversible and Irreversible Binding to Manduca sexta and Heliothis virescens*

Site-directed mutagenesis was used to examine the role of domain II, loop 2 residues, 368RRPFNIGI375, of Bacillus thuringiensis insecticidal protein CryIAb. Alanine substitution of residues 368RRP370, called B4, abolished potency toward Manduca sexta and Heliothis virescens, and the loss of toxicity was correlated directly to substantially reduced binding affinity to brush-border membrane vesicles (BBMV) prepared from the target insect midguts. These results indicated that these positive charges might be essential to orient the toxin to midgut receptor molecule(s). The role of residue Phe371 of CryIAb toxin to M. sexta was investigated by substituting a series of residues at this position. Irreversible binding and toxicity were affected significantly by hydrophobic, aliphatic, and smaller side-chain residues such as Cys, Val, Leu, and Ser but not by Tyr or Trp. A hydrophobic aromatic side-chain residue at position 371 was therefore essential for irreversible binding of CryIAb toxin in M. sexta. The role of residues 370PFNIGI375 of CryIAb toxin on H. virescens was also examined. Mutants D2 (deletion of residues 370–375), G374A (alanine substitution of Gly374), and I375A had reduced toxicity to H. virescens. In contrast to our findings with M. sexta, the reduction in toxicity of these mutants was correlated directly with loss of initial binding to H. virescens BBMV, indicating that these residues perform functionally distinct roles in binding and toxicity to different insects. In ligand blots, CryIAb recognized a major 210-kDa peptide in M. sexta BBMV and a 170-kDa peptide in H. virescens BBMV.

Bacillus thuringiensis is a Gram-positive soil bacterium that produces one or more insecticidal crystal proteins (δ-endotoxins) which are largely responsible for its pathogenicity to agriculturally important insect pests and vectors of human diseases. In recent years, the mechanism of action of δ-endotoxins to lepidopteran insects has been studied intensively, but several factors still need to be examined. Upon ingestion, the δ-endotoxin (120 to 140 kDa) is solubilized and activated into a toxic form by removal of 28–30 residues from the N terminus and approximately 500 residues from the C terminus by proteolytic enzymes present in the susceptible larval midgut (1). Following enzymatic activation, the toxic protease-resistant core (60 to 65 kDa) binds to specific receptor(s) (toxin-binding proteins) located in the midgut apical brush-border membrane of the columnar cells. Binding of toxin to a receptor molecule is thought to trigger a conformational change in the toxin and enable the toxin to insert into the plasma membrane, generating pores or ion channels which lead to cellular swelling and lysis (2–6). Intoxicated insects stop feeding and eventually die.

Binding of the activated toxin to specific gut receptor(s) is considered one of the key factors for insect toxicity for several reasons. Firstly, ion and water leakage induced by toxin in phosphatidylcholine vesicles is enhanced by the addition of brush-border membrane proteins or purified receptors (7–9). Secondly, insect resistance to Cry toxins is often correlated with reduced binding affinity or binding site concentrations for specific receptors (10–12). Thirdly, to date there have been no reports of insecticidal activity of Cry toxins that do not have measurable membrane binding to insect brush-border membrane vesicles (BBMV).1 For some toxins, however, the opposite is true, i.e. more membrane binding but relatively less insect toxicity (13). Also, resistance to Cry toxins in a strain of tobacco budworm, Heliothis virescens, is not caused by altered receptor binding (14). Factors such as imperfect activation of the toxin by insect gut juice (15), defective insertion of the toxin into the membrane, and failure to make sufficient toxin dimers to form a functional pore might account for a lack of toxicity in the above-mentioned exceptions.

Recently, a two-step receptor binding process for CryIAb toxin to several lepidopteran insects has been proposed (16, 17). Interestingly, the sum of irreversibly bound toxins to midgut BBMV is correlated directly to insect toxicity (16). Other factors, including the aggregation of toxin monomers to form oligomers either in solution or in the membrane might aid to enhance toxicity. Recently, Walters et al. (18) have shown that CryI1A molecules exist as dimers in solution. However, the precise role of oligomerization in toxicity needs to be investigated.

The recently solved structure of a coleopteran active δ-endotoxin, CryI1A (19), reveals a three-domain structure. This structure is believed to be common for other Cry toxins since the interfaces holding the domains are highly conserved in most of the Cry toxins. Based on the crystal structure, domain I consists of six antiparallel sheets that include the hypervariable region of Cry toxins. The three major surface-exposed loops of this domain have been suggested to be involved in insect specificity and membrane binding (19). Moreover, the transfer of Bombyx mori activity from a highly active toxin (CryIAt) to a relatively non-active toxin (CryIAt) by switching residues 330 to 450 has led to the proposal that insect specific-
ity and receptor binding of δ-endotoxins might be confined to the hypervariable region of domain II, at least for this insect (20, 21).

In our effort to identify the amino acid residues of Cry toxins that participate in receptor binding, we have previously shown that residues 365 to 370 of CryAA toxin are involved in initial membrane binding on B. mori (22) and that residues 370 to 375 are important for the irreversible association of CryAb toxin to M. sexta BBMV, although this region does not directly participate in initial binding on M. sexta (16). In this article we report the functional role of another putative loop 2 segment, 368RRP370, of CryAb δ-endotoxin in toxicity and initial binding toward H. virescens and M. sexta larvae. We substituted residue Phe371 of CryAb toxin with amino acids of diverse physical and chemical properties to analyze their membrane-association function on M. sexta. Membrane binding properties of single alanine substitution mutants among residues 370–375 of CryAb toxin on H. virescens were also examined. These mutational analyses, together with voltage-clamping and ligand-blotting experiments, provided valuable information on the functional role of these predicted loop 2 residues in membrane binding and toxicity to the target insects, M. sexta and H. virescens.

MATERIALS AND METHODS

Construction of Mutants—Construction of single alanine substitution mutants from residues 371 to 375 (F371G) and deletion of residues 368–370 (F371A) (D2) were described in our previous article (16). The mutant B4 was constructed by substituting the putative loop 2 residues 368RRP370 with alanine (368AAA370).

Toxicity Assays of Manduca sexta—M. sexta eggs used in this study were supplied by D. L. Dahlman (Dept. of Entomology, University of Kentucky, Lexington). The eggs were hatched and raised on an artificial diet. Mortality toxicity assays were performed with neonate hatched larvae. A total of 100 μl (per well) of toxin dilutions (diluted in phosphate-buffered saline, 8 mM NaH₂PO₄, 2 mM KH₂PO₄, 150 mM NaCl, pH 7.4) were layered on an artificial diet in a 24-well Falcon microtiter plate. Two larvae were placed per well (2 cm²) to a total of at least 15 larvae per toxin concentration. The mortality rates were recorded after 5 days and the effective dose estimates (50% lethal concentration of toxin [LC₅₀], and 95% fiducial limits) were calculated using probit analysis (23). At least five concentrations per toxin were used to estimate the LC₅₀ value. Each bioassay was repeated at least three times.

Bioassays of H. virescens—Bioassays were conducted with neonate larvae placed on artificial diet (24). Six concentrations of each of the toxins were added to the diet just before the diet harden, and the toxin was mixed thoroughly into the diet using a 1-liter blender. For each toxin concentration, approximately 0.5 ml of the toxin-containing diet was placed in each of 20 ml sample vials and allowed to cool. One neonate larva was placed in each vial, and the vial was capped. Three days later, small holes were made in the cap to allow air circulation. After 7 days, the larvae were scored for survival. Survival data were analyzed by probit analysis with a correction for control mortality. The H. virescens strain used (YDK) is described in Gould et al. (25).

Site-directed Mutagenesis—The primers used for oligonucleotide-directed site-specific mutagenesis were provided by Sandez Agro Inc., Palo Alto. Uracil-containing template of the cryAB gene was obtained by transforming Escherichia coli with plasmid pSB033b (16). Site-directed mutagenesis procedure (Mutagene M13 in vitro mutagenesis kit, Bio-Rad) was as detailed in the manufacturer's manual. Single-strand DNA sequencing was carried out by the method of Sanger et al. (26) following the manufacturer's (United States Biochemical Corp.) instructions. All restriction enzymes were from Boehringer Mannheim.

Purification and Solubilization of Toxin—Mutant and wild-type δ-endotoxin crystals were solubilized in E. coli inclusion bodies and purified as described previously (21). The final pellet, referred to as the crystal protein, was solubilized in crystal solubilization buffer (50 mM NaCl, pH 9.5, 10 mM dithiothreitol) at 37 °C for 3 h. Final yield of the solubilized crystal protein (protoxin) was estimated using Coomassie protein assay reagent (Pierce). Activation of the solubilized protoxin was carried out by treating with 2% (by mass) trypsin at 37 °C for 5 h. Tryptsin-activated toxin was analyzed by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE).

Toxin Iodination—Iodination of trypsin-activated toxin was carried out with IODOBEADS (Pierce) using the manufacturer's directions. Iodination of wild-type and mutant proteins were performed as described previously (21). The specific activities of labeled CryAB, D2, F371A, F371C, F371I, F371V, F371L, F371Y, and B4 were 1.6, 1.7, 1.5, 1.4, 1.4, 1.6, 1.5, 1.6, and 1.7 μCi/mg, respectively.

Brush-Border Membrane Vesicle (BBMV) Preparation—Dissection of fifth instar M. sexta and H. virescens larvae was carried out as described previously (21). The purified midguts were stored at −70 °C until use. BBMV were prepared by the differential magnesium precipitation method as modified by Walfersberger et al. (27). The final BBMV pellet was resuspended in binding buffer (8 mM NaHPO₄, 2 mM KH₂PO₄, 150 mM NaCl, pH 7.4, containing 0.1% bovine serum albumin) to a final protein concentration of 1 mg/ml and stored at −70 °C until use.

Competition Binding Assay—Heterologous competition binding assay (competition between one labeled ligand and another nonlabeled ligand) procedure was as described before (16). A total of 100 μg of BBMV/ml was used in each competition experiment. The mean values of three individual experiments were plotted using the CA-CRICKET Graph III application program. Binding data were analyzed using the LIGAND program (28).

Dissociation Binding Assay—Dissociation of M. sexta BBMV bound 125I-labeled toxins by the addition of an excess amount of corresponding nonlabeled toxin was measured as described in Rajamohan et al. (16). The total counts of labeled toxin at 2 h of association reaction (no nonlabeled toxin) were considered to be 100% binding.

Iodination of trypsin-activated toxin with freshly prepared M. sexta or H. virescens gut enzymes was as described before (16). The final product was separated by SDS-10% PAGE, transferred onto a polyvinylidene difluoride membrane, and processed as described before (16).

Toxin Binding Autoradiography—125I-labeled toxin (5 μg) was incubated with M. sexta or H. virescens BBMV (300 μg/ml) in binding buffer for 1 h at room temperature. The samples were centrifuged for 10 min at 25,500 × g in a Hermle microcentrifuge (2255MK). The pellet was washed three times with binding buffer, and the final pellet was solubilized in Laemmli sample buffer (29), separated by SDS-8% PAGE, and transferred on to a polyvinylidene difluoride membrane using a Bio-Rad Trans-Blot apparatus. The blot was incubated with 125I-labeled (1 × 10⁶ cpm) toxin and processed as described before (16).

Immuno blotting—Gut enzyme digestion of mutant and wild-type proteins were performed by incubating the toxin with freshly prepared M. sexta or H. virescens gut enzymes as described before (16). The final product was separated by SDS-10% PAGE, transferred onto polyvinylidene difluoride membrane, and processed as described before (16).

Toxin binding autoradiography was performed as described by Chen et al. (30). The dissection and mounting of insect midgut followed the protocol by Harvey et al. (31). After 15–30 min of digestion, wild-type or mutant δ-endotoxins were injected into the lumen side of the chamber, and the change in short-circuit current (Iₛₙ) over time (minutes) was recorded. The volume of toxin added was always 10 μl. The total volume of the lumen chamber is 3.75 ml, and the bathing solution was used as described by Chamberlin (32). During the experiment, the bathing solution was bubbled continuously with O₂. The Iₛₙ was tracked with a Kipp and Zonen recorder, and data were collected with the MacLab data acquisition system.

RESULTS

Expression and Stability of Mutant Proteins—Crystal proteins from wild-type and mutants were solubilized and analyzed on SDS-10% PAGE. The mutant proteins (B4, F371C, F371I, F371V, F371L, F371Y, and F371W) were expressed in amounts comparable to the wild-type protein (Fig. 1A). The mutant protoxins yielded a stable 65-kDa trypsin-resistant toxin core upon activation with trypsin (Fig. 1B). Since the trypsin-activated toxins were used for toxicity and BBMV binding assays, these toxins were treated further with M. sexta gut juice enzymes to analyze their stability under insect gut conditions. The Western blot analysis showed that all the mutants yielded stable 60-kDa toxins similar to those of the wild-type (Fig. 1C).

Toxicity and Binding of B4 Mutant toward M. sexta—The toxicity of wild-type and B4 mutant toxin was analyzed and is
reported in Table I. The LC50 values showed a >600 times difference in potency between the wild-type and B4 toxin. Our competition binding studies showed that B4 toxin competes only marginally with the binding of the wild-type toxin to M. sexta BBMV (Fig. 2). Since the binding ability of B4 toxin to BBMV is negligible, we could not determine the Kd and Bmax values using the LIGAND program. Autoradiography binding studies revealed that less than 1% of the input labeled B4 toxin bound to the BBMV (Fig. 2). Since the binding ability of B4 toxin to BBMV (16). In this study we systematically replaced Phe371 with Cys (F371C), Ser (F371S), Val (F371V), Leu (F371L), Tyr (F371Y), and Trp (F371W) and analyzed the effect of amino acid side-chain size and chemical properties on toxicity and membrane binding to H. virescens—Our bioassay results showed that the B4 mutant was significantly less toxic than the wild-type toxin to M. sexta (Table II). The LC50 value of wild-type was 824 ng/ml diet, whereas B4 did not show any mortality up to a concentration of 30 μg/ml. The exact LC50 value for this mutant was not calculated since there was not enough mortality even at higher concentrations. The competition binding experiment showed that B4 toxin had lost its ability to compete for the binding of wild-type toxin on H. virescens BBMV (Fig. 5). The Kd and Bmax values for the wild-type toxin on H. virescens BBMV are given in Table II. Our autoradiography binding studies showed that only 2% of the input labeled B4 toxin bound to the BBMV, whereas 40% binding was observed for the wild-type toxin (Fig. 3A).

Effect of Side-chain Modification at Residue Phe371 of CryIAb on Toxicity and Binding to M. sexta—Our previous results showed that amino acid Phe371 of CryIAb is involved in the irreversible association of the toxin to M. sexta BBMV (16). In this study we systematically replaced Phe371 with Cys (F371C), Ser (F371S), Val (F371V), Leu (F371L), Tyr (F371Y), and Trp (F371W) and analyzed the effect of amino acid side-chain size and chemical properties on toxicity and membrane binding to H. virescens—Our bioassay results showed that the B4 mutant was significantly less toxic than the wild-type toxin to H. virescens (Table II). The LC50 value of wild-type was 824 ng/ml diet, whereas B4 did not show any mortality up to a concentration of 30 μg/ml. The exact LC50 value for this mutant was not calculated since there was not enough mortality even at higher concentrations. The competition binding experiment showed that B4 toxin had lost its ability to compete for the binding of wild-type toxin on H. virescens BBMV (Fig. 5). The Kd and Bmax values for the wild-type toxin on H. virescens BBMV are given in Table II. Our autoradiography binding studies showed that only 2% of the input labeled B4 toxin bound to the BBMV, whereas 40% binding was observed for the wild-type toxin (Fig. 3A).

Effect of Side-chain Modification at Residue Phe371 of CryIAb on Toxicity and Binding to M. sexta—Our previous results showed that amino acid Phe371 of CryIAb is involved in the irreversible association of the toxin to M. sexta BBMV (16). In this study we systematically replaced Phe371 with Cys (F371C), Ser (F371S), Val (F371V), Leu (F371L), Tyr (F371Y), and Trp (F371W) and analyzed the effect of amino acid side-chain size and chemical properties on toxicity and membrane binding to H. virescens—Our bioassay results showed that the B4 mutant was significantly less toxic than the wild-type toxin to H. virescens (Table II). The LC50 value of wild-type was 824 ng/ml diet, whereas B4 did not show any mortality up to a concentration of 30 μg/ml. The exact LC50 value for this mutant was not calculated since there was not enough mortality even at higher concentrations. The competition binding experiment showed that B4 toxin had lost its ability to compete for the binding of wild-type toxin on H. virescens BBMV (Fig. 5). The Kd and Bmax values for the wild-type toxin on H. virescens BBMV are given in Table II. Our autoradiography binding studies showed that only 2% of the input labeled B4 toxin bound to the BBMV, whereas 40% binding was observed for the wild-type toxin (Fig. 3A).
M. sexta

Our toxicity results showed that F371W was very similar to the wild-type toxin, whereas F371C, F371V, F371S, F371L, and F371Y were 600, 400, 40, 11, and 6 times less toxic than the wild-type toxin, respectively (Table I). Heterologous competition experiments were performed to evaluate whether the mutant proteins recognize the same binding site as that of the wild-type toxin. When 125I-labeled CryIAb was put into competition with nonlabeled mutant proteins, all the mutants competed for the CryIAb binding site(s) as efficiently as did nonlabeled CryIAb (Fig. 2). This observation was further supported by the autoradiography binding study which showed that 40% of labeled F371C was bound to the BBMV compared to 35% binding of the wild-type toxin (Fig. 3A).

Since all the mutant toxins showed similar binding affinities but differed in toxicity, we examined whether all the BBMV-bound toxins were irreversibly associated to the BBMV. The toxins were first allowed to bind saturably to the BBMV and were then chased with 1000-fold excess of corresponding nonlabeled toxins. Our results showed that 88% of the wild-type and mutant F371W toxins bound to the BBMV could not be displaced by the addition of nonlabeled ligands (i.e., they were irreversibly associated). In contrast, only 55%, 58%, 65%, 80%, and 82% of the mutants F371C, F371V, F371S, F371L, and F371Y, respectively, were associated irreversibly with M. sexta BBMV (Fig. 6). The continued decrease in binding in the case of some mutants (F371C and F371V) was not due to labeled toxin breakdown, since we could recover the intact labeled toxin after incubation with BBMV for more than 4 h (data not shown).

We also examined the inhibition of I sc in response to the addition of wild-type and mutant toxin to the lumen side of an isolated M. sexta midgut under voltage clamped conditions. I sc measures the active transport of ions from the hemolymph side of the midgut to the lumen side by the ion pumps present in the membrane. When we used a toxin concentration of 50 ng/ml, the slope of I sc inhibition of mutant F371W and wild-type was comparable (Fig. 4A), whereas the slope for mutant F371Y was about 4 times less than that of the wild-type (Fig. 4A). Mutants F371C, F371V, F371L, and F371S did not show any measurable inhibition of I sc at this toxin concentration (Fig. 4A). However, when the toxin concentration was increased to 500 ng/ml, a measurable difference in the slope of inhibition of I sc among the mutants F371C, F371V, F371L, and F371S was observed (Fig. 4B). The I sc slope values calculated with 500 ng of toxin/ml are given in Table I.

Effect of Loop-2 Residues 370PFNIGI 375 on Toxicity and Receptor Binding to H. virescens—Construction, expression, and stability of toxin upon trypsin digestion of single alanine substitution mutants (F371A, N372A, G374A, and I375A) and their toxicity and receptor binding to H. virescens were determined. Table II summarizes the results. The LC 50 values, dissociation constants (Kd), and maximum binding site concentrations (Bmax) for each mutant protein were calculated. The relative potency (wild-type LC50/mutant LC50) was also determined. The LC 50 values for each mutant are given in Table II. The Kd and Bmax values for each mutant are also given in Table II. The relative potency values are given in Table II. The 95% confidence limits are given in parentheses.

### Table II

| Toxins | LC50 μg/ml | Kd nM | Bmax pmol/mg | Relative potency |
|--------|------------|-------|--------------|-----------------|
| CryIAb | 0.824 (0.4–1.4) | 2.92 ± 0.31 | 30.1 ± 3.19 | 1.0 |
| B4 | >30 | UD | UD | >0.02 |
| F371A | 0.583 (0.3–0.9) | 2.09 ± 0.19 | 29.4 ± 2.98 | 1.6 |
| N372A | 1.4 (0.8–2.1) | 3.99 ± 0.73 | 28.9 ± 3.22 | 0.6 |
| G374A | 7.2 (3.8–14.8) | 16.55 ± 2.76 | 47.4 ± 5.13 | 0.1 |
| I375A | 4.07 (2.3–6.5) | 10.61 ± 2.07 | 32.1 ± 3.23 | 0.2 |
| D2 | >45 | UD | UD | >0.02 |

* 95% confidence limits are given in parentheses.
* Kd, dissociation constant (determined from homologous competition binding).
* Bmax, binding site concentration (determined from homologous competition binding).
* Relative potency (wild-type LC50/mutant LC50).
* Insufficient mortality to calculate the LC50.
* UD, undetectable.

**Fig. 3.** Determination of stability and binding of 125I-labeled CryIAb, B4, F371C, and D2 toxins after incubation with M. sexta (A) and H. virescens (B) BBMV. Lane 1, amount bound to the BBMV; lane 2, total input counts. See “Materials and Methods” for details.

**Fig. 4.** Inhibition of I sc across M. sexta midgut. A total of 50 (A) and 500 (B) ng of CryIAb, B4, F371C, F371S, F371V, F371L, F371Y, and F371W toxins per ml were injected in separate experiments into the lumen side of the chamber, and the drop in I sc was measured. The I sc measured before the addition of toxin is considered as 100%.

**Fig. 5.** Determination of stability and binding of 125I-labeled CryIAb, B4, F371C, and D2 toxins after incubation with M. sexta (A) and H. virescens (B) BBMV. Lane 1, amount bound to the BBMV; lane 2, total input counts. See “Materials and Methods” for details.

**Fig. 6.** Inhibition of I sc across M. sexta midgut. A total of 50 (A) and 500 (B) ng of CryIAb, B4, F371C, F371S, F371V, F371L, F371Y, and F371W toxins per ml were injected in separate experiments into the lumen side of the chamber, and the drop in I sc was measured. The I sc measured before the addition of toxin is considered as 100%.
deletion of residues 370–375 (D2) were described in our previous article (16). Stability of the mutant toxins (F371A, N372A, G374A, I375A, and D2) were analyzed by incubating the toxins with H. virescens gut juice enzymes. Our results showed that all the mutants were processed into a stable 60-kDa toxin similar to the wild-type (Fig. 1D). The biological activity of these mutants on H. virescens larvae were tested and reported in Table II. Mutants F371A and N372A showed similar toxicity compared to that of the wild-type toxin (LC50 values 0.58, 1.4, and 0.82 μg/ml, respectively). In contrast, mutants D2, G374A, and I375A showed >60, 9, and 5 times less toxicity, respectively, than the wild-type. In competition binding assays, the wild-type and toxic mutants (F371A and N372A) were able to bind to H. virescens BBMV with similar binding affinities and binding site concentrations (Fig. 5 and Table II). In contrast, mutants D2, G374A, and I375A competed to the binding of the wild-type toxin with reduced binding affinity (Fig. 5). The KD and Bmax values are reported in Table II. When labeled D2 toxin was incubated with H. virescens BBMV and autoradiographed, only 2% of the total input toxin bound to the BBMV in contrast to 35% binding of the wild-type toxin (Fig. 3B).

Identification of the Toxin-binding Polypeptide—In ligand blot experiments, CryIAb strongly recognized a 210-kDa peptide and also reacted to a 120-kDa peptide to some extent on M. sexta BBMV (Fig. 7A). On H. virescens BBMV, CryIAb reacted with a 170-kDa peptide (Fig. 7B). However, labeled mutant B4 toxin did not recognize any (210, 120, or 170 kDa) of the toxins and their interaction with susceptible insect gut cell surface exposed receptors. Cummings and Ellar (34) also observed the involvement of arginine residues in toxicity and binding against M. sexta when they chemically modified arginine residues of CryI Ac toxin, which has more than 90% amino acid homology to CryIAb.

Voltage clamping experiments on M. sexta midguts provided additional evidence that the insecticidal property, inhibition of
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short-circuit current across the insect midgut, did not occur with B4 toxin, which failed to bind to the receptor. Taking together all these results, it is reasonable to speculate that receptor binding is a two-step process, initial reversible binding and irreversible binding. The positively charged residues of the loop, 368RR389, establish the initial contact with the receptor (reversible binding). Since the deletion of residues 370PFNIGI375 did not inhibit the initial binding (16), Pro370 is not likely to be involved in the initial binding to M. sexta. Binding is further strengthened (irreversibly) by the interaction of residues Phe371 and Gly374 either with the receptor or with the membrane (16). However, this study does not exclude any possible involvement of these arginine residues (since arginine can form large number of hydrogen bonds) with neighborhood amino acids that participate in receptor binding.

Secondly, our previous study showed that alanine substitution mutants of CryIAb among residues 370PFNIGI375 had initial binding similar to CryIAb, as measured by competition binding, but significantly decreased irreversible binding on M. sexta BBMV. The 400-fold reduction in potency of the mutants, especially F371A, was correlated directly to the substantially reduced irreversible association of the toxin on M. sexta BBMV (16). To further substantiate the physical and chemical requirement for the amino acid at position 371, in this study we replaced Phe371 with residues such as Ser, Cys, Val, Leu, Tyr, and Trp. This set of residues contains side-chains that vary in volume, hydrogen bonding capability, and hydrophobicity. Hence, the influence of each of these properties on the irreversible membrane association of CryIAb toxin could be determined precisely. Our bioassay data showed four categories of mutants: 1) those >400 times less toxic (F371C, F371V); 2) 10–40 times less toxic (F371S and F371L), 3) 6 times less toxic (F371Y), and 4) comparably toxic as to wild-type (F371W).

These data are consistent with the dissociation binding data; that is, the greater the irreversible association of the toxin to the BBMV, the more toxic the toxin to the insect (Fig. 6). Thus, the aliphatic, small side-chain substitutions are considerably less toxic than the aromatic bulky side-chain substitutions. Interestingly, the loss of toxicity by the smaller side-chain substitution (F371C) could be regained consistently with the increase in side-chain volume. The residues which strengthen the membrane association (irreversible binding) of the toxin ranks in the order of Trp > Phe > Tyr > Leu > Ser > Val > Cys. The inhibition of short-circuit current of these mutant proteins to M. sexta midgut under voltage clamp conditions also followed the same order. Although the inhibition of Isc by the mutants F371C, F371V, and F371S were not measurable (Fig. 4A) at lower toxin concentrations, the differences in Isc inhibition were resolved at higher concentrations of toxin (Fig. 4B). Only a hydrophobic aromatic ring-side chain substitution such as Trp, retained the potency of native residue Phe377. Even the hydroxyl group on Tyr (sterically equivalent to Phe) results in a measurable reduction in irreversible binding. The aromatic amino acids in the loop region of CryC toxin were also reported to play a critical role in post-binding function and insect toxicity (35). However, the smaller side-chain residues (F371C, F371V, and F371S), which significantly affected the toxicity and rate of irreversible binding (insertion) to the BBMV, did not affect initial binding to M. sexta. The fact that the less toxic mutants still bound to the membrane receptor as well as the wild-type provides further evidence that these residues are not directly involved in initial recognition of the receptor molecule.

These experiments suggest that an aromatic hydrophobic residues in the surface exposed loop 2 is important for the irreversible association (insertion) of CryIAb toxin to membrane. Interestingly, F371A did not affect the toxicity to another CryIAb susceptible lepidopteran insect, H. virescens. It is likely that the toxin receptors located on the cell surface are different for the two target insects. Our ligand blot data showed that CryIAb toxin binds to a major 210-kDa BBMV protein in M. sexta and a 170-kDa toxin binding protein in H. virescens BBMV (Fig. 7). This is in agreement with previously published data (36, 37).

Thirdly, the functional role of residues 370 to 375 (370PFNIGI375) of CryIAb toxin in receptor binding and toxicity to H. virescens was analyzed. Our bioassay data showed that mutants D2, G374A, and I375A have reduced the larvicidal potency (~50, 9, and 5 times, respectively) to H. virescens. As the activation of these mutants by H. virescens gut juice is similar to the wild-type toxin, it can be assumed that the reduction in toxicity of D2, I375A, and G374A was not caused by structural instability of the mutant toxins. The reduced toxicity of these mutants were directly correlated to the reduced (4–5 times) initial binding affinity of the toxin on H. virescens BBMV as measured by competition binding studies. Furthermore, it is clear from our blot experiment that 40% of total labeled CryIAb bound to BBMV, whereas only 1% of D2 toxin bound to the BBMV prepared from H. virescens. These data are in contrast with our previous observation in M. sexta. In M. sexta, these mutations did not affect the initial binding to BBMV, but the loss of larvicidal activity of these mutants was directly attributed to the significantly reduced irreversible binding ability to BBMV (16). These results imply that although these residues are involved in toxicity and receptor binding, they perform a functionally distinct role in different insects or receptors involved in toxicity.

Determination of the role of these receptor contact residues (RRPFNIGI) in association and dissociation binding is fundamental to our understanding of the toxin-receptor molecular recognition process. Our observation that certain mutations in a surface loop of domain II affect irreversible binding (but not reversible binding) and that binding is strengthened by a hydrophobic aromatic side chain at position 371 warrants an explanation. One possibility is that toxins become tightly (irreversibly) bound through hydrophobic interaction to the receptor in a manner distinct from the insertion of the toxin to the apical membrane. The second possibility is that loop 2, and other parts of domain II, insert into the apical membrane. Further experimental evidence is necessary to distinguish between these two models.

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