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Substitution of Residues on the Proximal Side of Cry1A *Bacillus thuringiensis* δ-Endotoxins Affects Irreversible Binding to *Manduca sexta* Midgut Membrane

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Substitution of a positively charged residue (R93F) or addition of a negatively charged residue (A92D) at the N-terminal of α3 helix of domain I of the Cry1Ac δ-endotoxin resulted in a substantial reduction in toxicity against *Manduca sexta.* The N-terminal residues of helix 3 are considered to be on the same (proximal) surface of the toxin as the loops in domain II which are involved in the binding of the toxin to the receptor. The loss of toxicity was not caused by a decrease in the initial binding but rather by reduced irreversible binding. Only 65 and 75% of the A92D and R93F mutant toxin, respectively, bound to midgut vesicles irreversibly, compared to 94% of the wild type toxin. On the other hand, replacing A119 in a loop on the distal side of the helices with negatively charged residues (A119D or A119E) did not affect toxicity or irreversible binding.

The entomophathogenic crystalline inclusions produced by *Bacillus thuringiensis* have been widely used in the control of agricultural pests because of their specificity and lack of harmful side effects. These parasporal crystals are comprised of δ-endotoxins, or Cry proteins, which are expressed as protoxins. Upon ingestion by susceptible insect larvae, the 130-140 kDa protoxins are solubilized in the insect midgut and converted to active toxins by midgut proteases (1). The 65 kDa activated toxins pass through the peritrophic membrane and bind to receptors located on the surface of midgut epithelia cells of the larvae (2) and then insert into the membrane. The inserted toxins kill the insect by disrupting the ionic balance, but the mechanism is not clear, being described as either forming small non-specific pores (3) or ion channels (4).

Most of the Cry proteins contain five conserved blocks of amino acids (5). Based on the crystal structures of the Cry3A and the recently elucidated Cry1Aa toxins (6,7) it is likely that all the Cry proteins have a similar tertiary structure comprised of three domains. Domain I is a bundle of seven amphipathic α-helices with α5 in the center. Domain II is comprised of three β-sheets with loops at the apex of the β-hairpin extensions. Beta-sheet three has an axis of pseudo-symmetry nearly parallel to the α5 helical axis. Domain III is a sandwich of two anti-parallel β-sheets (7). It has been postulated that domain I (or certain α-helices) inserts into the membrane and either interacts with a pre-existing channel or forms an ion channel (8-11).

Studies with synthetic peptides corresponding to the α5 and α6 helices have supported the role of domain I in insertion and ion-channel formation (12). In addition, the effects of mutations in various regions of domain I are consistent with this model. In particular, it has been observed that in Cry1Ab and Cry1Ac introduction of a negatively charged

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residue at position Y153 in the loop between helices α4 and α5 and at the N-terminal of helix α3 at positions A92 and R93, drastically reduces toxicity for target insects (13). This reduction in toxicity is directly correlated with a reduction in irreversible binding and channel formation (14).

It is interesting that these particular domain I mutations are located on the surface of the as certain loops in domain II which have been implicated in the initial reversible binding to the receptor. In this communication we have further analyzed amino acid residues of the Cry1Ab and Cry1Ac toxins on both the proximal and distal sides of the α-helices of domain I relative to these loops in domain II, for their importance in toxicity and irreversible binding. Only mutations on the proximal side resulted in reduced irreversible binding and toxicity.

MATERIALS AND METHODS

Construction, expression and activation of mutant proteins. Site-directed mutagenesis was carried out according to the method of Kunkel (15) using a uracil-containing pSB033 vector and *E. coli* MV1190 as a host (14). This system was used both for mutations and the expression of proteins. Mutations in the *cry1Ac* gene (A92C, A92D and R93F) were prepared as previously described (13). Mutant colonies were screened by the dideoxy sequencing method using Sequenase Version 2.0 kit (US Biochemicals). Purification, solubilization and trypsin activation of the crystal protein were performed as described previously (16).

Toxicity assay. Bioassays were performed with *Manduca sexta* neonate larvae by surface contamination method (14). Artificial diet was prepared following manufactures instruction (BioServ Inc. Frenchtown New Jersey). Various dilutions of toxin solution (50μl) was poured on the diet surface and allowed to dry. Two day old *M. sexta* larvae were placed on the dried surface and the mortality was monitored after five days. The effective dose estimate (LC_{50}) was calculated using the Probit program (17).

Preparation of brush border membrane vesicles (BBMV). M. sexta brush border membrane vesicles (BBMV) were prepared from midguts of fifth instar larvae by the differential magnesium precipitation method of Wolfersberger *et al.* (18). The final BBMV pellet was resuspended in binding buffer (150mM NaCl, 8mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) and stored in liquid nitrogen until further use.

Ligand binding assays. Iodination of trypsin-activated toxins and heterologous competition assays were performed as described earlier (19). For the competition binding assay 20 μg of *M. sexta* BBMV was incubated for 1h with 0.5 nM ^{125}I-labeled toxin in 100ml of binding buffer in the presence of varying concentrations (1.0 to 800nM) of unlabeled toxin. Data was analyzed using the LIGAND computer program (20).

Dissociation binding assays. Dissociation assays were performed according to the procedure of Chen *et al.* (14). Briefly, 0.5 nM of ^{125}I-labeled wild type or mutant toxin was first incubated with 20μg of BBMV for 1h at room temperature. After association reaction, a 500 fold excess of unlabeled toxin was added and the amount of reversibly bound toxin was measured at different time intervals (0 to 60 min). The pellet was washed twice with binding buffer and counted in a gamma counter (Beckman Instruments). Non-specific binding was determined by adding labeled toxin and a 500 fold excess of unlabeled toxin together to the BBMV and was subtracted from the total binding.

RESULTS AND DISCUSSION

Effect of Mutations on the Stability of Protoxin and Toxin

The double mutant A2 ($_{147}$LF$_{148}$--AA), in which leucine and phenylalanine were replaced by alanines, did not produce stable toxin upon trypsin digestion. Another double mutant, A3 ($_{180}$QR$_{118}$--AA), failed to yield any detectable protoxin in *E. coli.* Other Cry1Ab mutants, R115A, A119D, A119E, A119G, and A1($_{117}$WE$_{118}$--AA) produced stable toxin upon trypsin digestion. Similarly, the selected Cry1Ac mutants, A92D, A92C, and R39F, all formed toxins as stable as the wild type toxin when incubated with *M. sexta* midgut juice (data not shown).

Location of Mutant Proteins

Mutations in domain I of the Cry1Ab and Cry1Ac toxins are shown in Fig. 1. The mutated residues 92-93 (A92D and R93F) and 147-148 (A2) are present at the N-terminal of α3 and C-terminal of α4 helices, respectively. Based on a structural alignment with the Cry1Aa toxin, this region is on the same side as the potential receptor binding loops in domain II (7). Mutations of amino acid residues 115-119 (R115A, A1, A119D,E or G) and 180-181 (A3),

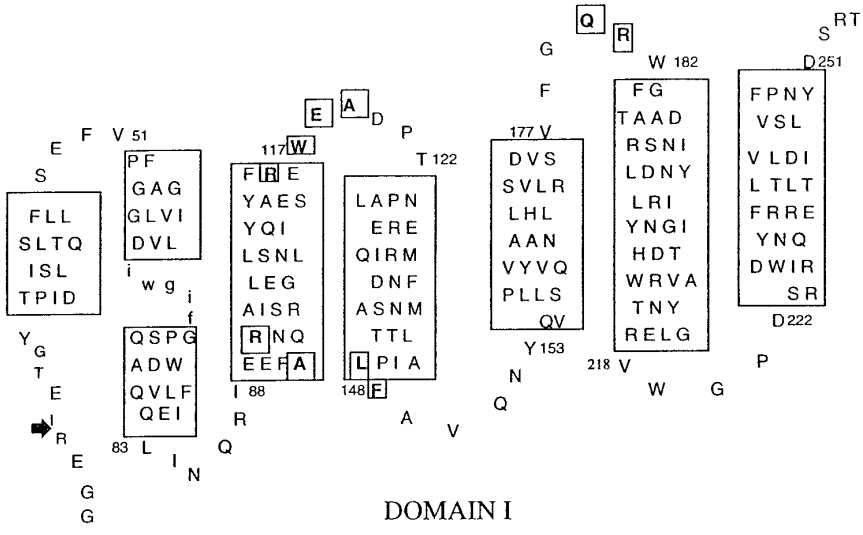

FIG. 1. Schematic of Cry 1Ab seven helical bundles of domain I based on the alignment with Cry1Aa. Box letters indicate the position of the residues mutated in Cry1Ab and Cry1Ac. R115A, A119D, A119E, A119G, A1 ($_{117}$WE$_{118}$--AA), A2 ($_{147}$LF$_{148}$--AA), and A3 ($_{180}$QR$_{181}$--AA) are Cry1Ab mutants. A92C, A92D and R93F are Cry1Ac mutants. Arrow shows the site for trypsin cleavage.

however, are located in loops between helices α3-α4 and α5-α6, respectively. This region is on the side opposite to the loops of domain II. The face of domain I aligned with the loops in domain II is designated the proximal side, whereas the opposing face is considered the distal side.

Effect on Insecticidal Activity

Bioassays results showed that there was no significant variation in the LC_{50} values for the wild type Cry1Ab and distal side mutants R115A, A119D, A119E, A119G, and A1 towards *M. sexta* (Table 1). On the other hand, mutation A92D in Cry1Ac does result in the loss of toxicity, while, A92C was as toxic for *M. sexta* as the wild type toxin (13). In the case of Arg93, any mutation that removes the positive charge, such as R93F, results in reduced toxicity (13). Similar results were obtained with A92E and Y153D in Cry1Ab (14). These bioassay results showed that mutations which removed a positive charge on the presumptive distal side of the toxin did not affect toxicity.

Effect of Mutations on Initial Binding

To determine whether the low toxicity of mutants A92D and R93F was due to reduced initial binding to the membrane receptor, heterologous competition assays were determined. ^{125}I-labeled Cry1Ab or Cry1Ac toxin was competed with unlabeled mutant toxins for binding to *M. sexta* BBMV (Fig. 2A and B). None of the mutations had any significant effect on the initial binding as reflected by their binding affinities [K_{com}] (Table1). Studies with a BIA Core surface plasma resonance biosensor (Pharmacia) have also shown that the A92D toxin does not affect the rate of initial binding (L. Masson, personal communication). Similar observations have been made with the Cry1Ab toxin when Ala92 was mutated to Glu (A92E) (14).

Effect of Mutations on Irreversible Association

To determine if the post-initial binding events had been altered in mutants with no toxicity for *M. sexta*, irreversible binding experiments were performed. In the presence of an excess

TABLE 1
Effect of Cry1Ab and Cry1Ac Mutations on the Toxicity and Initial Binding to the *M. sexta* BBMV

Toxin	LC_{50}[a] (ng/cm^2)	Kcom[b] (nM)	B_{max} (pMole/mg BBMV)
Cry1Ab*	12.5 (8.7–18.5)	8.1 ± 0.53	58.6 ± 1.3
R115A	17.2 (9.5–23.2)	4.7 ± 1.25	38.7 ± 2.5
A119D	10.7 (7.5–17.9)	5.2 ± 1.45	42.5 ± 1.7
A119E	11.6 (8.7–17.2)	6.0 ± 0.75	60.2 ± 1.2
A119G	12.2 (8.5–18.1)	6.9 ± 0.8	60.1 ± 1.2
A1 ($_{117}$WE$_{118}$- -AA)	18.2 (9.5–14.2)	6.9 ± 1.24	55.3 ± 2.1
Cry1Ac*	7.8 (6.3–11.2)	2.75 ± 0.53	55.2 ± 1.8
A92C	8.2 (6.8–13.4)	2.92 ± 0.08	42.1 ± 2.7
A92D	>700[c]	2.95 ± 0.13	47.5 ± 1.7
R93F	>500[c]	1.85 ± 0.15	60.1 ± 1.6

[a] LC_{50} value is the average of three assays.
[b] K_{com} (binding affinity) has earlier been described by Wu and Dean (23) to define the binding constant derived from the competition assay for Cry toxins.
[c] No mortality observed at these concentrations.
* Classification of δ-endotoxins is based on the revised nomenclature (25).

amount of unlabeled toxin, ^{125}I-labeled A92D and R93F showed greater dissociation than wild type Cry1A or mutant A92C toxins (Fig. 3A). More than 94% of the Cry1Ac toxin remained bound to the BBMV after 80 min of chase. On the other hand, the non-toxic mutants, A92D and R93F, showed greater dissociation, with only 65% and 76%, respectively, remaining irreversibly bound after 80 min. These observations are in agreement with earlier reports demonstrating that in the Cry1Ab domain I mutants, A92E and Y153D (with reduced toxicity), there is reduced irreversible binding although the initial binding is not affected (14).

To confirm these findings, we performed dissociation assays with the fully toxic Cry1Ab mutant toxins, A119D and A119G, and compared the results with those obtained with the non-toxic mutant, A92E. As noted previously, the percentage of active mutant toxin bound irreversibly to BBMV was similar to the wild type toxin, while the A92E toxin had a sharp decline in the percentage that remained bound (Fig. 3B). It was observed, however, that the Cry1Ab mutant toxin, A92E, was displaced more than the Cry1Ac mutant toxin, A92D. This may be due to the greater propensity of Cry1Ac to aggregate and thus greater difficulty in removing surface bound toxin from BBMV (Lee and Dean, unpublished data).

Irreversible binding is indicative of toxin insertion into the membrane (21-24). Reduced irreversible binding implies that the membrane insertion process has been disturbed resulting in a reduction in ion-conductance and toxicity (14). A defective insertion process as observed with mutants A92E, A92D and R93F (Fig. 3 A and B) can be explained on the basis of the recently determined crystal structure of the Cry1Aa toxin (greater than 90% sequence identity with the Cry1Ab and Cry1Ac toxins). Arg93 is involved in salt bridges with Glu81 and Asp74 (9), so a mutation of Arg93 to Phe (R93F) would be destabilizing to this charge interaction. Similarly, Ala92 is not solvent exposed but rather faces the apolar α4-α5 loop. Introduction of a negatively charged residue may have a repulsive effect when the toxin comes in contact with the negatively charged environment of the insect membrane.

On the other hand, when a negatively charged residue was introduced at position A119 (A119D and A119E), which lies in the loop between α3-α4 on the distal side of domain I, no effect on toxicity or irreversible binding was found (Table 1, Fig. 3B). Arg115 is reported to form a salt bridge with Asp112 (9) but mutating Arg115 to Ala (R115A) did not result in

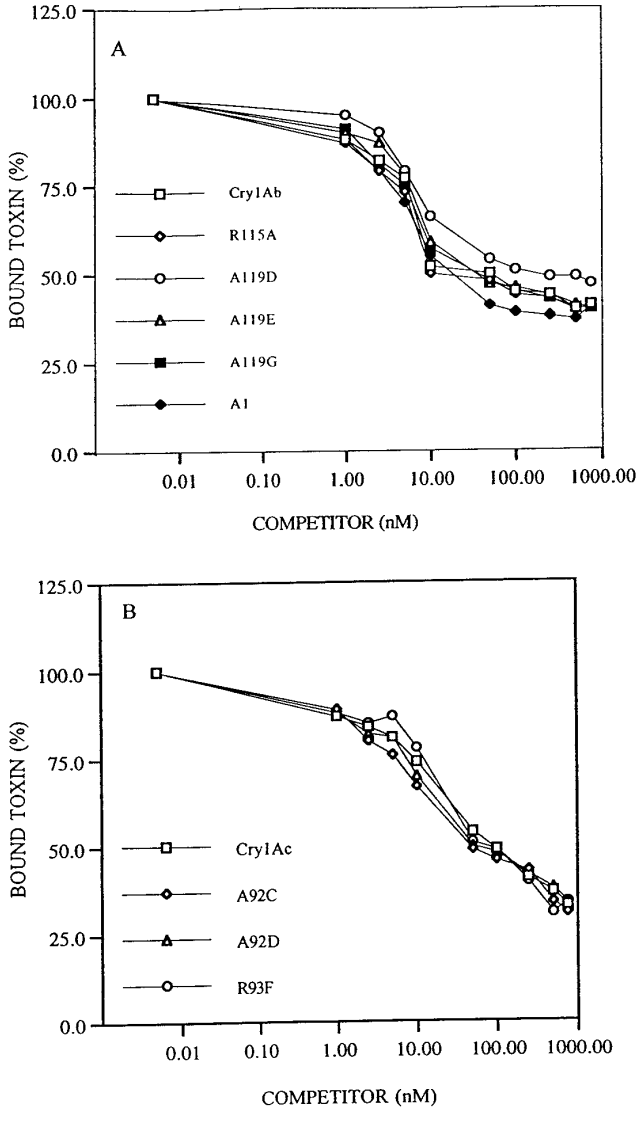

FIG. 2. Competition binding assay of ^{125}I-labeled Cry1Ab (A) and Cry1Ac (B). Binding is expressed as the percentage of labeled toxin bound to the *M. sexta* BBMV (200μg/ml) after competition with increasing amounts of mutant toxins.

a reduction of the toxicity or irreversible binding of the mutant toxin for *M. sexta* (Table 1, Fig 3A.). This indicates that, unlike the positively charged residue on the proximal site (R93), a positive charge (R115) on the distal surface does not appear to have as critical a role in toxin insertion.

We have noted that the proximal surface of domain I is the same as the loops in domain II which are involved in the initial reversible binding to the toxin to the receptor. This correlation implies that this orientation of the toxin is critical for the first two steps of binding to the receptor and toxin insertion into the membrane. This latter step may appear to involve ionic interactions with the membrane of these loops in domain I, an interaction which is essential for the subsequent insertion into the membrane of the amphipathic, pore-forming helices.

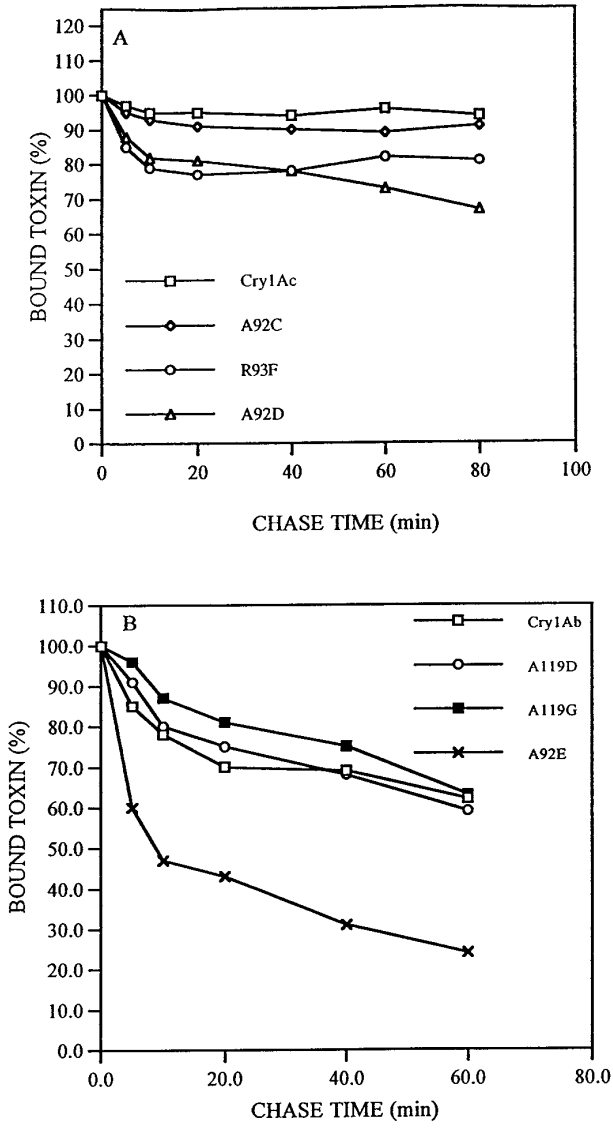

FIG. 3. Dissociation assay of Cry1Ac mutants (A) and Cry1Ab mutants (B). Binding is expressed as the percentage of toxin remaining bound to the BBMV after adding 500 fold excess of corresponding unlabeled toxin. Non-specific binding has been subtracted from the total binding.

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