Mutations in Domain I Interhelical Loops Affect the Rate of Pore Formation by the *Bacillus thuringiensis* Cry1Aa Toxin in Insect Midgut Brush Border Membrane Vesicles[▽]

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Pore formation in the apical membrane of the midgut epithelial cells of susceptible insects constitutes a key step in the mode of action of *Bacillus thuringiensis* insecticidal toxins. In order to study the mechanism of toxin insertion into the membrane, at least one residue in each of the pore-forming-domain (domain I) interhelical loops of Cry1Aa was replaced individually by cysteine, an amino acid which is normally absent from the activated Cry1Aa toxin, using site-directed mutagenesis. The toxicity of most mutants to *Manduca sexta* neonate larvae was comparable to that of Cry1Aa. The ability of each of the activated mutant toxins to permeabilize *M. sexta* midgut brush border membrane vesicles was examined with an osmotic swelling assay. Following a 1-h preincubation, all mutants except the V150C mutant were able to form pores at pH 7.5, although the W182C mutant had a weaker activity than the other toxins. Increasing the pH to 10.5, a procedure which introduces a negative charge on the thiol group of the cysteine residues, caused a significant reduction in the pore-forming abilities of most mutants without affecting those of Cry1Aa or the I88C, T122C, Y153C, or S252C mutant. The rate of pore formation was significantly lower for the F50C, Q151C, Y153C, W182C, and S252C mutants than for Cry1Aa at pH 7.5. At the higher pH, all mutants formed pores significantly more slowly than Cry1Aa, except the I88C mutant, which formed pores significantly faster, and the T122C mutant. These results indicate that domain I interhelical loop residues play an important role in the conformational changes leading to toxin insertion and pore formation.

Once ingested by susceptible insect larvae, the insecticidal crystal proteins of *Bacillus thuringiensis* are solubilized and converted to their toxic form by midgut proteases. The activated toxins bind to specific receptors on the surface of the luminal membrane of midgut columnar cells, insert into the membrane, and form pores that abolish transmembrane ionic gradients and osmotic balance, leading to the disruption of the epithelium and death of the insect (47, 51). Members of the *B. thuringiensis* Cry toxin family for which the atomic structure has been reported share a similar three-domain organization in which domain I is composed of a bundle of six amphipathic α -helices surrounding a hydrophobic helix ($\alpha 5$), and domains II and III are formed mostly of β -sheets (7, 8, 18, 26, 37, 38, 43). While domains II and III are thought to be involved in receptor binding and toxin specificity (47), domain I is believed to play a major role in membrane insertion and pore formation (51). Toxin fragments corresponding to domain I of Cry1Ac (62), Cry3Aa (53), and Cry3Ba (61) or to the first five α -helices of Cry4B (48) have been shown to form pores in model membranes. Pore formation in artificial membranes has also been demonstrated with synthetic peptides corresponding to $\alpha 5$ of

Cry1Ac (13) and Cry3Aa (19, 21) and to the $\alpha 4$ -loop- $\alpha 5$ segment of Cry3Aa (23). Spectroscopic studies have also revealed that while synthetic peptides corresponding to $\alpha 4$ and $\alpha 5$ can coassemble within a lipid bilayer, those corresponding to $\alpha 2$, $\alpha 3$, $\alpha 6$, and $\alpha 7$ adopt a membrane surface orientation (20, 22). In agreement with these findings, $\alpha 4$ was shown to line the lumen of the pores (42). On the other hand, convincing evidence supporting previous suggestions that most of the toxin molecule may become imbedded in the membrane (3, 39, 60) has recently been reported (44, 45).

Thus, several models have been proposed for the mechanism of toxin insertion and pore formation (4, 9, 28, 32, 39, 44, 52, 56). Although these models differ in the identities of the toxin segments that are suggested to insert into the membrane, they all imply that the toxin undergoes conformational changes following binding to the membrane surface. Even though such changes imply rotations about the polypeptide backbone in domain I interhelical loops, little attention has been devoted so far to the role of domain I loop residues in pore formation.

In the present study, amino acid residues strategically located within each of these loops in Cry1Aa were replaced by a cysteine using site-directed mutagenesis. The resulting mutant toxins were assayed with *Manduca sexta* midgut brush border membrane vesicles using a light-scattering technique. Mutations mapping within several of these loops altered the functional properties of Cry1Aa, suggesting the involvement of most domain I α -helices in the pore-forming process.

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TABLE 1. Toxicity of Cry1Aa interhelical loop cysteine mutants to *M. sexta* larvae

Toxin	Mutation site (loop)	Mean % mortality $\pm$ SEM (no. of independent expts) ^a
Cry1Aa	None	95 $\pm$ 2 (54)
F50C	α 1- α 2	97 $\pm$ 2 (6)
I88C	α 2- α 3	— ^b
P121C	α 3- α 4	100 $\pm$ 0 (6)
T122C	α 3- α 4	91 $\pm$ 3 (6)
N123C	α 3- α 4	94 $\pm$ 4 (6)
V150C	α 4- α 5	1.4 $\pm$ 1.3 (6)**
Q151C	α 4- α 5	100 $\pm$ 0 (6)
Y153C	α 4- α 5	42 $\pm$ 4 (6)**
W182C	α 5- α 6	64 $\pm$ 6 (6)**
W219C	α 6- α 7	97 $\pm$ 2 (6)
S252C	α 7- β 1a	99 $\pm$ 1 (6)

^a The toxins were tested at 2 μ g/ml. Values are means $\pm$ standard errors of the means, with the numbers of independent experiments indicated in parentheses. Student's *t* test differences were calculated relative to the values obtained for Cry1Aa (*, *P* < 0.05; **, *P* < 0.01).

^b —, not tested.

MATERIALS AND METHODS

Mutagenesis. At least one amino acid residue within each interhelical loop of domain I was replaced individually by a cysteine in Cry1Aa. Because α 4 is considered to play a crucial role in the mechanism of pore formation (24, 42, 59), three mutations were introduced in the α 3- α 4 and α 4- α 5 loops. All mutants tested are listed in Table 1, and the position of the mutated residues, relative to that of domain I helices, is illustrated in Fig. 1. The mutants were created by oligonucleotide-directed in vitro mutagenesis using the double-oligonucleotide method (14) (Transformer kit; Clontech Laboratories, Palo Alto, CA). All mutations were made using the double-stranded DNA expression plasmid pMP39 (41), except for the W219C mutation, which was made using pBA1 (6). Mutated genes were analyzed (50) using an Applied Biosystems (Foster City, CA) model 370A nucleotide sequencer.

Expression and purification. All protoxins were produced in *Escherichia coli*, except W219C, which was produced in *B. thuringiensis*. Insoluble inclusions were solubilized and trypsin activated as described elsewhere previously (40, 41). Activated toxins were then purified by fast-protein liquid chromatography using a Mono Q ion-exchange column (Pharmacia Biotech, Montreal, Quebec, Canada) and eluted with a 50 to 500 mM NaCl gradient in 40 mM carbonate buffer (pH 10.5) (40, 41). Fractions containing the eluted toxins were dialyzed against distilled water until they precipitated. Purified toxin precipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (35) and stored at 4°C until use. In preparation for the experiments, a stock solution of each protein was prepared by resuspending the pellet in a buffer composed 150 mM KCl and 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Caps)-KOH (pH 10.5).

Bioassays. Fertilized eggs of *Manduca sexta* were purchased from the North Carolina State University Entomology Department insectary (Raleigh, NC). Larvae were raised and bioassayed using an artificial diet supplied with the insects. Toxicity assays were performed on neonate larvae with trypsin-activated toxins as described previously (12, 58, 59) but with minor modifications. A final volume of 100 μ l containing toxin diluted in phosphate-buffered saline (8 mM Na₂HPO₄, 2 mM KH₂PO₄, and 150 mM NaCl [pH 7.4]) was layered onto the artificial diet in 1.8-cm² wells and allowed to soak into the medium. One larva was placed into each well and reared at 27°C and 70% relative humidity with a 12-h light and 12-h darkness photoperiod. Mortality was recorded after 7 days. All mutant toxins were tested at 2 μ g/ml (111 ng/cm²). Because the V150C mutant was poorly active at this concentration, the tests were repeated at 25 μ g/ml (1.4 μ g/cm²). Twenty-five larvae were used for each toxin concentration tested, and the bioassays were replicated six times. Larvae were weighed at the onset of each experiment and, for the survivors, after 7 days.

Preparation of brush border membrane vesicles. Whole midguts from fifth-instar larvae were isolated, cleared of their attached Malpighian tubules and gut contents, and stored at -80°C until use. Brush border membrane vesicles were prepared from thawed midguts with an Mg²⁺ precipitation and differential centrifugation method (63).

Light-scattering assay. Brush border membrane vesicle permeability was evaluated with an osmotic swelling assay (10). Scattered light intensity was monitored at a wavelength of 450 nm, with a photomultiplier tube at a 90° angle from the incident light beam, at 23°C in a PTI spectrofluorometer (Photon Technology International, South Brunswick, NJ). Experiments were carried out at pH 7.5 and pH 10.5 to study the effect of the additional negative charge, which is present on the thiol group of cysteine residues at the higher pH. The ability of the toxins to increase vesicle permeability to various solutes, including KCl, *N*-methyl-D-glucamine hydrochloride, potassium gluconate, sucrose, and raffinose, was assessed by performing the light-scattering assays after preincubating the vesicles with the appropriate concentration of toxin for 60 min, as described previously (12, 57–59). Membrane permeabilization kinetics were analyzed by exposing the vesicles to the toxin at the onset of the light-scattering experiments, without preincubation, as described previously (12, 57–59).

Data analysis. Percent volume recovery was calculated as follows: $100 \times (1 - I_t)$, where I_t is the relative scattered-light intensity measured at time *t*. For kinetic experiments, percent volume recovery was calculated for each experimental point, and values obtained for control vesicles, assayed without added toxin, were subtracted from the experimental values measured in the presence of toxin. The ascending portion of each resulting trace was fitted with a Boltzmann sigmoid using the Origin, version 6.1, program (OriginLab, Northampton, MA). The delay preceding vesicle swelling was defined as the time required for volume recovery to reach 5%. Maximal osmotic swelling rates were obtained from the peak value of the first derivative of the sigmoidal fits. Unless indicated otherwise, data are reported as means $\pm$ standard errors of the means for at least three experiments, each carried out with a different vesicle preparation. Data for each of these replicates consist of the average of data for five traces obtained using the same vesicle preparation. Statistical analyses were done with the two-tailed unpaired Student *t* test.

FIG. 1. Positions of the mutated residues within domain I of Cry1Aa. The three-dimensional rendering of the activated toxin crystal structure (26) was obtained using SPDV software (27). Each of the indicated residues was replaced individually by a cysteine as described in Materials and Methods. The α 2- α 3, α 4- α 5, and α 6- α 7 loops on the lower side of the illustration are most likely to contact the membrane first since they are located on the same side of the toxin molecule as the domain II loops that have been shown to bind to the receptors (47, 51).

RESULTS

Toxin production. All mutants were produced and activated with yields comparable to that observed for wild-type Cry1Aa, with the exception of the I88C mutant. For reasons that remain unclear, this mutant was expressed at very low levels and could therefore be produced in only very limited quantities. Consequently, it was tested only in selected light-scattering experiments since these experiments required much less toxin than the toxicity assays.

Toxicity. Most mutants retained strong toxicity, with mortalities exceeding 90% when assayed at 2 $\mu\text{g/ml}$ (Table 1). However, the Y153C, the W182C, and especially the V150C mutants were substantially less toxic. Nevertheless, although the Y153C and W182C mutants killed only 42% and 64% of the larvae, respectively, at this toxin concentration, the surviving insects gained only 3.7 ± 0.7 mg ($n = 6$) and 2.7 ± 0.5 mg ($n = 6$), respectively, after 7 days in the presence of these toxins, in comparison with 111 ± 6 mg ($n = 78$) for those that were incubated in the absence of toxin. In contrast, almost all larvae survived in the presence of 2 μg V150C mutant/ml (Table 1), and their weight increased by 80 ± 20 mg ($n = 6$). Even at 25 μg of this mutant per ml, only $21\% \pm 8\%$ ($n = 6$) of the larvae were killed, but the weight of the survivors increased by only 18 ± 7 mg during the same period, indicating a modest but detectable toxicity.

Pore-forming ability. Each mutant was first characterized by evaluating its ability to permeabilize *M. sexta* brush border membrane vesicles to KCl using an osmotic swelling assay (10). In this assay, vesicles are rapidly mixed with a hypertonic solution of KCl, which causes them to shrink rapidly, as evidenced by a sharp increase in scattered-light intensity during the first half-second of the experiment (Fig. 2). Vesicles preincubated for 60 min with an active toxin, such as the T122C toxin (Fig. 2A), subsequently swell in a dose-dependent manner as the solute diffuses across the membrane, and vesicle swelling causes a gradual decrease in scattered-light intensity. In the presence of an inactive mutant such as the V150C mutant (Fig. 2B), however, vesicles swell at a rate which is similar to that measured in the absence of toxin.

Figure 3 shows the percent volume recovery measured for each of the mutants after 3 s as a function of toxin concentration. With the exception of the V150C mutant (Fig. 3G), which was inactive at both pH values tested, all mutants formed KCl-permeable pores approximately as efficiently as Cry1Aa at pH 7.5 (Fig. 3). At this pH, the percent volume recovery was nevertheless significantly lower at 100 and 150 pmol toxin/mg membrane protein for the W182C mutant (Fig. 3J) and significantly higher at 5 and 15 pmol toxin/mg membrane protein for the W219C mutant (Fig. 3K). However, increasing the pH to 10.5, a procedure which causes the thiol group of cysteine to become ionized, brought about a significant reduction ($P < 0.05$) in the KCl permeability observed in the presence of at least two concentrations of all mutants except the I88C, V150C, Y153C, and S252C mutants. As pH had little effect on the activity of Cry1Aa (Fig. 3A), all mutants except the T122C mutant (Fig. 3E) displayed significantly smaller percent volume recovery values than Cry1Aa at pH 10.5 for at least one of the toxin concentrations tested (Fig. 3).

FIG. 2. Effect of interhelical loop cysteine mutants of Cry1Aa on the osmotic swelling of *M. sexta* brush border membrane vesicles. Midgut membrane vesicles isolated from fifth-instar *M. sexta* larvae and equilibrated overnight in 10 mM HEPES-KOH (pH 7.5) were preincubated for 60 min with the indicated concentrations (in pmol toxin/mg membrane protein) of the T122C (A) or V150C (B) mutant. Vesicles were rapidly mixed with an equal volume of 150 mM KCl–10 mM HEPES-KOH (pH 7.5), directly in a cuvette, using a stopped-flow apparatus. Scattered-light intensity was monitored at 90° in a PTI spectrofluorometer. Each trace corresponds to the average data for five experiments performed with the same representative vesicle preparation.

Pore properties. To test whether the functional characteristics of the pores were altered by the mutations, we next examined the ability of most of the mutants to permeabilize the membrane to the chloride salt of the relatively large cation *N*-methyl-D-glucamine (Fig. 4A), the potassium salt of the relatively large anion gluconate (Fig. 4B), the disaccharide sucrose (Fig. 4C), and the trisaccharide raffinose (Fig. 4D). In these experiments, each toxin was tested at 150 pmol/mg membrane protein. Because the amine group of *N*-methyl-D-glucamine is not ionized at a high pH, experiments with this solute were carried out only at pH 7.5. Osmotic swelling was faster with *N*-methyl-D-glucamine hydrochloride (Fig. 4A) than with potassium gluconate (Fig. 4B), in agreement with the fact that *B. thuringiensis* toxins are generally cation selective (30). Except for the Y153C and W182C mutants, which were less active, and the T122C mutant, which was very slightly but significantly more active ($P < 0.05$), all mutants permeabilized the membrane to *N*-methyl-D-glucamine approximately as well as Cry1Aa (Fig. 4A). In the presence of potassium gluconate, the Q151C, Y153C, and W182C mutants were significantly less active than Cry1Aa at pH 7.5 (Fig. 4B). At pH 10.5, however, membrane permeability to potassium gluconate was significantly lower than that observed with Cry1Aa for all mutants except the P121C and T122C mutants (Fig. 4B). Nevertheless,

FIG. 3. Pore-forming ability of domain I interhelical loop mutants. Brush border membrane vesicles equilibrated overnight in 10 mM HEPES-KOH (pH 7.5) (■) or Caps-KOH (pH 10.5) (●) were preincubated for 60 min with the indicated concentrations of Cry1Aa (A) or the F50C (B), I88C (C), P121C (D), T122C (E), N123C (F), V150C (G), Q151C (H), Y153C (I), W182C (J), W219C (K), or S252C (L) mutant. Their permeability to KCl was monitored following rapid mixing with 150 mM KCl and 10 mM HEPES-KOH (pH 7.5) (■) or Caps-KOH (pH 10.5) (●). Percent volume recovery was calculated for the 3-s time point from traces like those shown in Fig. 2. Asterisks indicate a statistically significant difference, with the corresponding value measured at the same pH for wild-type Cry1Aa (*, $P < 0.05$; **, $P < 0.01$).

only for the F50C and Q151C mutants were the percent volume recovery values (Fig. 4B) significantly different ($P < 0.01$) at pH 7.5 and 10.5. Sucrose (Fig. 4C) diffused more rapidly into the vesicles than raffinose (Fig. 4D), as expected from its smaller hydrodynamic volume. At pH 7.5, the F50C, Q151C, Y153C, W182C, W219C, and S252C mutants allowed the diffusion of sucrose at a rate which was significantly lower than that measured in the presence of Cry1Aa (Fig. 4C). At pH 10.5, however, the membrane was significantly less permeable to sucrose with all mutants tested than with Cry1Aa. This difference is due mainly to the fact that the pores formed by Cry1Aa are more permeable to sucrose at pH 10.5 than at pH 7.5, as was noted previously (12, 58). Such a difference was not observed for any of the mutants (Fig. 4C). In fact, all mutants had similar permeabilities to sucrose at pH 7.5 and 10.5 except

the F50C mutant, which was significantly less permeable ($P < 0.05$) at the higher pH. All mutants except the Q151C mutant increased membrane permeability to raffinose approximately as well as Cry1Aa at pH 7.5, but when the pH was raised to pH 10.5, the sugar flux measured in the presence of all mutants was significantly lower than that observed with wild-type Cry1Aa (Fig. 4D). However, permeability to raffinose differed significantly ($P < 0.05$) between pH 7.5 and 10.5 only in the presence of the F50C and W219C mutants.

Kinetics of pore formation. The rate of pore formation was monitored by rapidly mixing vesicles with a hypertonic 150 mM KCl solution containing 150 pmol/mg membrane protein of either one of the loop mutants without prior incubation. As shown in Fig. 5, for the I88C mutant, one of the most active mutants, the percent volume recovery increased in a sigmoidal

FIG. 4. Effect of Cry1Aa domain I interhelical loop mutants on vesicle permeability to *N*-methyl-D-glucamine hydrochloride, potassium gluconate, sucrose, and raffinose. Vesicles equilibrated in 10 mM HEPES-KOH (pH 7.5) (open bars) or Caps-KOH (pH 10.5) (hatched bars) were preincubated for 60 min with 150 pmol toxin/mg membrane protein. Their permeability was monitored following rapid mixing with either 150 mM *N*-methyl-D-glucamine hydrochloride (A) or potassium gluconate (B) or 300 mM sucrose (C) or raffinose (D) and with 10 mM HEPES-KOH (pH 7.5) (open bars) or Caps-KOH (pH 10.5) (hatched bars), as described in the legend of Fig. 2. Asterisks indicate statistically significant differences, with the corresponding values measured at the same pH for Cry1Aa (*, $P < 0.05$; **, $P < 0.01$).

manner following a lag period of about 20 s. The length of this delay, as well as the subsequent vesicle-swelling rate, varied considerably depending on the mutation and pH (Table 2). At pH 7.5, the delays and swelling rates measured in the presence

of the I88C, P121C, T122C, N123C, and W219C mutants were comparable to those observed with Cry1Aa. On the other hand, the F50C, Q151C, Y153C, W182C, and S252C mutants showed significantly longer delays or lower swelling rates. In contrast, at pH 10.5, all mutants had a significantly longer delay or lower swelling rate than Cry1Aa, except the I88C mutant, which was significantly more active, and the T122C mutant (Table 2).

DISCUSSION

The results of the present study demonstrate that the pore-forming properties of Cry1Aa can be affected by single-site mutations altering one of several of its domain I interhelical loop residues. With the sole exception of the V150C mutant, however, all mutants were able to form pores in *M. sexta* midgut brush border membrane vesicles. Although all of the active mutants, except the W182C mutant, permeabilized the membrane to KCl approximately as well as did Cry1Aa at pH 7.5, all mutants but the T122C mutant were significantly less active than the wild-type toxin at pH 10.5. In fact, all mutants were significantly less efficient than Cry1Aa in permeabilizing

FIG. 5. Kinetics of pore formation. Brush border membrane vesicles equilibrated overnight in 10 mM HEPES-KOH (pH 7.5) or Caps-KOH (pH 10.5) were mixed with an equal volume of 150 mM KCl, 10 mM HEPES-KOH (pH 7.5) or Caps-KOH (pH 10.5), and 150 pmol of I88C/mg membrane protein. Percent volume recovery was calculated for each experimental point, and control values were subtracted from those obtained in the presence of toxin. For clarity, error bars are shown for only every 50th data point.

TABLE 2. Kinetics of pore formation by Cry1Aa interhelical loop mutants in *M. sexta* midgut brush border membrane vesicles

Toxin	Mutation site	Mean delay ^{a,b} ± SEM (min)		Mean swelling rate ^{b,c} ± SEM (% vol recovery/min)	
		pH 7.5	pH 10.5	pH 7.5	pH 10.5
Cry1Aa	None	0.48 ± 0.06	0.65 ± 0.02	30 ± 7	21 ± 1
F50C	α1-α2	1.03 ± 0.07**	1.3 ± 0.1**	8.7 ± 0.8*	6.2 ± 0.3**
I88C	α2-α3	0.33 ± 0.02	0.35 ± 0.03**	37 ± 2	32 ± 2**
P121C	α3-α4	0.40 ± 0.04	0.65 ± 0.08	26.4 ± 0.5	17.6 ± 0.5*
T122C	α3-α4	0.41 ± 0.02	0.55 ± 0.07	25.2 ± 0.5	20.6 ± 0.6
N123C	α3-α4	0.56 ± 0.03	1.3 ± 0.1**	19 ± 1	4.8 ± 0.4**
Q151C	α4-α5	0.9 ± 0.4	3.4 ± 0.4**	5.1 ± 0.5*	2.2 ± 0.3**
Y153C	α4-α5	1.18 ± 0.09**	1.8 ± 0.7	6.8 ± 0.5*	5.6 ± 0.2**
W182C	α5-α6	1.8 ± 0.3*	4 ± 2	5 ± 1*	2.4 ± 0.8**
W219C	α6-α7	0.8 ± 0.2	1.1 ± 0.2	15 ± 3	7 ± 2**
S252C	α7-β1a	1.1 ± 0.2*	2.2 ± 0.5*	8 ± 1*	4.7 ± 0.6**

^a Time required for volume recovery to reach 5%.

^b A significant difference from the corresponding value for Cry1Aa is indicated by an asterisk (*, $P < 0.05$; **, $P < 0.01$).

^c Maximum slope of the Boltzmann sigmoidal curves fitted to the data points.

the membrane to all other solutes tested at the higher pH, except for the P121C and T122C mutants when assayed in the presence of potassium gluconate. In the case of the W182C mutant, this reduced pore-forming ability was apparent at both pH values even though the difference in permeability with Cry1Aa was not statistically significant for raffinose at pH 7.5. As discussed previously for a number of Cry1Aa helix α4 mutants (24, 59), because the patterns of activity were similar with all tested solutes, including large neutral and charged molecules, these observations indicate that the mutations altered mostly the number of pores formed rather than their diameter or ionic selectivity. Accordingly, these changes in pore-forming ability were accompanied by modifications in the rates of pore formation of most mutants. Even at pH 7.5, the F50C, Q151C, Y153C, W182C, and S252C mutants were significantly slower pore formers than Cry1Aa. At pH 10.5, all mutants displayed a lower rate of pore formation than Cry1Aa except the I88C mutant, which increased membrane permeability to KCl significantly faster than Cry1Aa, and the T122C mutant.

These results contrast with the fact that most mutations in interhelical loop residues that have been described so far, including, with few exceptions, those analyzed in the present study, have had relatively minor effects of toxicity. These mutations also include D120C, D120K, and D120Q from the α3-α4 loop of Cry1Aa (58); Y153C from the α4-α5 loop and W182I and G183C from the α5-α6 loop of Cry1Ab (46); and several mutations in residues N85, Q86, R87, I88, and E89 of the α2-α3 loop (64) as well as V150D from the α4-α5 loop (34) of Cry1Ac. The fact that the latter mutant was toxic is surprising in view of the very poor activity of our V150C Cry1Aa mutant. The importance of V150 in the function of both toxins should therefore be explored further in future studies. Toxicity was also not greatly affected by mutations in the α3-α4 loop of the more distantly related toxins Cry2Aa (5), Cry4Ba (33), and Cry9Ca (36). The N123Y (α3-α4 loop) and F184I (α5-α6 loop) mutants from Cry1Ab (29) and the R203A (α5-α6 loop) mutant from Cry4Ba (2) have even been reported to have higher toxicities than their respective parental toxins. On the other hand, the D222A (α6-α7 loop) mutant from Cry1Aa, a mutation that prevents the formation of an interdomain salt bridge, was considerably less toxic than the wild-type toxin even

though its in vitro pore-forming ability was not significantly altered (12). In addition, the ability of the Y153A, Y153D, and Y153R mutants from the α4-α5 loop of Cry1Ab to inhibit short-circuit current across the isolated midgut epithelium of *M. sexta* was reduced to an extent which correlated well, at least qualitatively, with their reduced toxicity (11).

The alterations in toxin activity described in the present study were most pronounced at pH 10.5. Although such highly alkaline conditions are characteristic of those found in the midgut of actively feeding lepidopteran larvae (15, 16), high pH introduces a negative charge on the side chain of cysteine and tyrosine residues and reduces the proportion of charged lysine residues. The surface of the membrane also becomes more negatively charged at a high pH. Titration of tyrosine and lysine residues does not appear to have a direct influence on the activity of the mutants since changes in pH had only minor effects on the pore-forming ability of Cry1Aa and the properties of its pores. Introducing a negative charge on interhelical loop residues, which are well exposed at the surface of the toxin, could be expected to interfere with the initial interaction of the toxin with the membrane due to electrical repulsion, as was suggested previously for the Y153D mutant of Cry1Ab (11). This explanation, however, fails to account for the substantially reduced rates of pore formation observed for the F50C, Q151C, Y153C, W182C, and S252C mutants at pH 7.5, since under these conditions, only about 13% of the cysteine residues are expected to be ionized according to the Henderson-Hasselbalch equation (49). In addition, at pH 10.5, 71% of the tyrosine residues are expected to carry a negative charge. A much higher proportion of negative charges on residue Y153 is therefore present in wild-type Cry1Aa molecules at pH 10.5 than in the Y153C mutant at pH 7.5. Furthermore, such an electrostatic repulsion between the toxin and the membrane would be expected to have stronger effects on pore formation when the added negative charge is located on the side of the toxin which comes closest to the membrane as the toxin binds to its receptor. Reduced rates of pore formation were observed with mutants bearing alterations in the loops located on either side of the toxin molecules and not only in the α2-α3, α4-α5, and α6-α7 loops, located on the same side of the toxin molecule as the apex of domain II, a well-exposed region of the

toxin which is known to play a crucial and direct role in toxin binding to its receptor (47, 51).

Our results do not directly support one of the proposed models of pore formation more than the others inasmuch as each model could suggest that the effects of mutations altering interhelical loop residues would be especially pronounced when these mutations are located within segments of the toxin molecule that are predicted to cross the lipid bilayer during membrane insertion. Reduced rates of pore formation were indeed not limited to mutants with alterations in the $\alpha 4$ - $\alpha 5$ or $\alpha 5$ - $\alpha 6$ hairpins, the insertion of which constitutes a crucial initial step in the umbrella (4, 20, 23, 42, 52) and penknife (28, 32) models, respectively. Reduced pore formation rates in mutants altered in their $\alpha 1$ - $\alpha 2$, $\alpha 3$ - $\alpha 4$, $\alpha 5$ - $\alpha 6$, and $\alpha 7$ - $\beta 1a$ loops are also not readily accounted for by the model proposed previously by Loseva et al. (39), in which all but the three first helices of domain I are inserted into the membrane along with domains II and III. The fact that the F50C mutant was both toxic to the larvae and able to form pores in midgut brush border membranes, albeit less efficiently than wild-type Cry1Aa, also contradicts the model recently proposed by Bravo et al. (9), in which the proteolytic removal of helix $\alpha 1$ from the toxin following binding to its cadherin-like receptor is considered to constitute an important step in pore formation. Because this cleavage occurs specifically at residue F50 (25) and apparently involves a chymotrypsin-like protease, it is unlikely that the F50C mutant could have been hydrolyzed at this position by the same enzyme. In agreement with the conclusion that such a cleavage is not necessarily required for pore formation, the *in vitro* pore-forming ability of the trypsin-activated Cry1Aa toxin was previously shown to be unaltered by the presence of a variety of protease inhibitors (31) or by the addition of larval midgut juice extracts (17). However, our results do not exclude the possibility that a toxin lacking helix $\alpha 1$ may be functional, as was recently demonstrated for Cry1Ab (55).

On the other hand, our results suggest that pore formation involves the extensive movement of domain I helices relative to each other. These results are therefore consistent with those of previous studies in which different domain I helices were cross-linked pairwise by engineered disulfide bonds (52, 54). The linking of either $\alpha 3$ to $\alpha 4$, $\alpha 5$ to $\alpha 6$, $\alpha 5$ to $\alpha 7$, or $\alpha 7$ to domain II prevented pore formation in artificial membranes, but full pore-forming ability was restored in the presence of β -mercaptoethanol. In contrast, the mutant in which $\alpha 2$ was cross-linked to $\alpha 3$ was equally active in the presence or absence of the reducing agent. These results indicate that most helices, with the notable exception of the $\alpha 2$ - $\alpha 3$ pair, move away from each other during pore formation. This conclusion has recently been questioned, however, as the mutant in which $\alpha 3$ is linked to $\alpha 4$ was reported to be inactive and those in which $\alpha 5$ is linked to $\alpha 6$, $\alpha 5$ is linked to $\alpha 7$, or $\alpha 7$ is linked to domain II had similar activities in the presence and absence of a reducing agent (1). The reason for this discrepancy remains to be elucidated. A stoichiometric analysis of the proportion of cysteine residues actually forming disulfide bonds in the toxin preparations used for the functional studies will undoubtedly be necessary to settle this question. In any case, the movement of domain I α -helices relative to each other during pore formation is compatible with recently proposed models (44, 56) in which most

of the toxin molecule is thought to become inserted into the membrane. In particular, the buried-dragon model (56) explicitly suggests extensive conformational changes in all three toxin domains.

Alterations in the amino acid sequence of interhelical loops could possibly affect the rate of pore formation by hindering the movement of adjacent helices relative to each other. Remarkably, mutations in each interhelical loop resulted in a reduced rate of pore formation except that which altered the $\alpha 2$ - $\alpha 3$ loop (I88C). The fact that the T122C mutant, as well as the D120C, D120K, and D120Q mutants (58), did not cause a significant reduction in toxin activity suggests that the pore-forming ability of the I88C mutant could have resulted simply from a fortuitous choice of residue to be mutated. This appears unlikely, however, in view of the fact that among the several mutations introduced into each of the $\alpha 2$ - $\alpha 3$ loop residues of Cry1Ac, the I88K mutation was the only one that resulted in reduced toxicity (64). Although further work is clearly required to fully elucidate the mechanism by which *B. thuringiensis* toxins insert into the membrane and form pores, our results underscore the involvement of domain I interhelical loops in this process.

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