Cysteine Scanning Mutagenesis of $\alpha 4$, a Putative Pore-Lining Helix of the *Bacillus thuringiensis* Insecticidal Toxin Cry1Aa[▽]

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Helix $\alpha 4$ of *Bacillus thuringiensis* Cry toxins is thought to line the lumen of the pores they form in the midgut epithelial cells of susceptible insect larvae. To define its functional role in pore formation, most of the $\alpha 4$ amino acid residues were replaced individually by a cysteine in the Cry1Aa toxin. The toxicities and pore-forming abilities of the mutated toxins were examined, respectively, by bioassays using neonate *Manduca sexta* larvae and by a light-scattering assay using midgut brush border membrane vesicles isolated from *M. sexta*. A majority of these mutants had considerably reduced toxicities and pore-forming abilities. Most mutations causing substantial or complete loss of activity map on the hydrophilic face of the helix, while most of those having little or only relatively minor effects map on its hydrophobic face. The properties of the pores formed by mutants that retain significant activity appear similar to those of the pores formed by the wild-type toxin, suggesting that mutations resulting in a loss of activity interfere mainly with pore formation.

Bacillus thuringiensis is a gram-positive spore-forming bacterium that produces a variety of insecticidal toxins which accumulate as protoxins in the form of parasporal crystals. Once ingested by susceptible insect larvae, the crystals become soluble in the midgut, and the protoxins are converted to active toxins by intestinal proteases. The activated toxins act by forming pores in the midgut luminal membrane after binding to specific receptors located at the membrane surface of the intestinal epithelium columnar cells (25). These pores are large enough to disrupt the ionic gradients established across the membrane and to cause the osmotic lysis of the cells (27).

The structures of several insecticidal Cry toxins have been elucidated by X-ray crystallography (4, 5, 13, 16, 19, 20, 24). With the exception of a recently described crystal protein of unknown toxicity (1), they all share a remarkably similar three-domain structure. Domain I is composed of a bundle of seven amphipathic α -helices, with helix $\alpha 5$ surrounded by the other helices. Domain II is composed of three β -sheets with a "Greek key" topology forming a β -prism, and domain III is composed of two antiparallel sheets forming a β -sandwich with a "jelly roll" topology. While domain I is thought to be responsible for membrane insertion and pore formation, domains II and III are thought to be involved in the binding of the toxin to its receptors (9, 16, 19, 25, 27).

Receptor binding presumably triggers a conformational change in the toxin molecule that leads to its oligomerization and insertion into the membrane. According to the umbrella model of insertion, a hairpin composed of helices $\alpha 4$ and $\alpha 5$

inserts into the membrane, while the rest of the helices are deployed over the membrane surface (2, 14, 15, 28). Results from chemical modification of preformed Cry1Aa pores in artificial membranes, using a mutant toxin possessing an aspartic acid-to-cysteine substitution at residue 136 in helix $\alpha 4$, indicated that at least part of the hydrophilic face of this helix lined the pore lumen (23). In agreement with the notion that $\alpha 4$ plays an important role in pore formation, single-site mutations in most of its charged residues cause a strong reduction in the pore-forming ability and toxicity of Cry1Aa (23, 32). Substitution of asparagine-135 by a glutamine residue in Cry1Ab and Cry1Ac disrupted the ability of these toxins to oligomerize without affecting their binding properties (7, 29). In contrast, mutants with alterations in isoleucine-132 and glutamine-133 of Cry1Ac retained the ability to oligomerize but were poorly active in bioassays and brush border membrane vesicle osmotic swelling experiments, suggesting that the mutations affected the insertion of the toxin into the membrane or the properties of the pores (18).

In order to investigate the functional role of helix $\alpha 4$ further, cysteine scanning mutagenesis was utilized in which the $\alpha 4$ residues of the Cry1Aa toxin were individually replaced by cysteine. During activation, all cysteine residues are naturally removed from wild-type Cry1Aa protoxin (16), thus leaving a single cysteine substitution in the activated mutant toxins. In this study, a detailed examination of the effects of these mutations on the properties of the pores formed, including the rate of pore formation, was determined using a light-scattering assay and brush border membrane vesicles isolated from *Manduca sexta*. Almost all of the mutants displayed a reduced toxicity and pore-forming ability. Mutations in most of the residues located on the hydrophobic face of the helix had little or only relatively minor effects on toxin activity, whereas mu-

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tations in most of the hydrophilic residues caused substantial or complete loss of activity.

MATERIALS AND METHODS

Mutagenesis and toxin preparation. Most amino acid residues from helix $\alpha 4$ of Cry1Aa have been replaced individually by cysteine by oligonucleotide-directed in vitro mutagenesis in *Escherichia coli* using the double oligonucleotide method (10) (Clontech Transformer kit; Clontech Laboratories, Palo Alto, CA) and the expression plasmid pMP39 (22). The N135C mutant toxin was prepared in the *E. coli*-*B. thuringiensis* shuttle vector pBA1 (3) using the same approach. All mutant genes were sequenced using fluorescent nucleotides and an Applied Biosystems (Foster City, CA) model 370A automated sequencer.

Wild-type Cry1Aa and mutant toxins were produced in *E. coli* except for N135C mutant toxin, which was produced in *B. thuringiensis*. *E. coli* strains were grown for 2 to 3 days at 37°C in YT broth (8 g/liter tryptone, 5 g/liter yeast extract, and 5 g/liter NaCl) containing 100 μ g/ml ampicillin, and the *B. thuringiensis* strain was grown for 2 to 3 days at 30°C in YT broth containing 10 μ g/ml tetracycline. Protoxins produced as insoluble inclusions were purified as described elsewhere (21, 22) and activated by incubating 50 mg of protoxin in 40 ml of 50 mM carbonate buffer (pH 10.5) containing 50 mM NaCl and 5 mg trypsin (Gibco BRL, Burlington, Ontario, Canada) for 90 min at 37°C. Activated toxins were centrifuged for 90 min at 200,000 $\times$ g to remove lipids and other insoluble material. Toxins were then purified by fast protein liquid chromatography using a Mono-Q anion exchange column and by eluting bound toxin with a 50 to 500 mM NaCl gradient in carbonate buffer (pH 10.5) (21). Precipitates formed by dialyzing purified toxins against distilled water were collected and stored at 4°C. The purity and integrity of each toxin preparation were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Bioassays. Fertilized eggs of *M. sexta* were purchased from the North Carolina State University Entomology Department insectary (Raleigh, NC) and raised on a standard artificial diet supplied with the eggs. Toxicity assays were performed on neonate larvae with trypsin-activated toxins. These were diluted in phosphate-buffered saline (8 mM Na₂HPO₄, 2 mM KH₂PO₄, and 150 mM NaCl [pH 7.4]). Toxin samples (100 μ l) were layered onto artificial diet in 1.8-cm² wells and allowed to stand until they were completely absorbed by the diet. One larva was placed in 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 and adjusted relative to control values with Abbott's correction. Weight gained by surviving larvae was also recorded after 7 days. Mutant toxins were systematically tested at 2 μ g/ml (111 ng/cm²), and for those toxins that were poorly active at this concentration, the tests were repeated at 25 μ g/ml (1.4 μ g/cm²). Six replicates of 25 larvae each were used for each toxin concentration tested.

Preparation of brush border membrane vesicles. Whole midguts isolated from fifth-instar *M. sexta* larvae were freed of attached Malpighian tubules, cut longitudinally to remove the peritrophic membrane and gut contents, and rinsed thoroughly with ice-cold 300 mM sucrose, 5 mM EGTA, and 17 mM Tris-HCl (pH 7.5). Brush border membrane vesicles were prepared from midgut homogenates by a procedure involving magnesium precipitation and differential centrifugation (33) and stored at -80°C until use. In preparation for light-scattering experiments, vesicles were resuspended to about 90% of the desired final volume in 10 mM HEPES-KOH (pH 7.5) or 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)-KOH (pH 10.5) and allowed to equilibrate overnight at 4°C. At least 1 h before the beginning of the experiments, they were diluted to a final concentration of 0.4 mg of membrane protein/ml with the appropriate buffer supplemented with enough bovine serum albumin to achieve a final concentration of 1 mg/ml.

Light-scattering assay. Brush border membrane permeability properties were analyzed using a light-scattering assay (6). In most experiments, vesicles were incubated for 60 min at 23°C with the specified toxin concentration, ranging from 5 to 150 pmol of toxin/mg of membrane protein. Assays were initiated by rapidly mixing the vesicles with an equal volume of a solution containing 10 mM HEPES-KOH (pH 7.5) or CAPS-KOH (pH 10.5), 1 mg/ml bovine serum albumin, and a 150 mM concentration of KCl, *N*-methyl-D-glucamine hydrochloride, or potassium gluconate, or a 300 mM concentration of sucrose or raffinose, using a stopped flow apparatus (Hi-Tech Scientific Co., Salisbury, United Kingdom). Alternatively, in experiments designed to monitor the rate of pore formation, 150 pmol of toxin/mg of membrane protein was added to the KCl solution before mixing with the vesicles, without prior incubation. Scattered light intensity was monitored at a wavelength of 450 nm, with a photomultiplier tube at an angle of 90° relative to the incident light beam, at 23°C in a Photon Technology International (South Brunswick, NJ) spectrofluorometer. Data were recorded every 0.1 s and normalized relative to the maximum value obtained for control vesicles in the absence of toxin. Percent volume recovery was defined as 100 (1 - I_t), where

TABLE 1. Toxicity of Cry1Aa $\alpha 4$ mutants toward *M. sexta* larvae

Toxin	Toxin dose of 2 μ g/ml		Toxin dose of 25 μ g/ml	
	% Mortality ^a	Wt gain (mg) ^b	% Mortality	Wt gain (mg)
Cry1Aa (wild type)	95 $\pm$ 2 (54)	— ^c	—	—
L126C	21 $\pm$ 4 (6)**	12 $\pm$ 2 (6)**	100 (6)	—
R127C	99.3 $\pm$ 0.7 (6)	—	—	—
M130C	99.3 $\pm$ 0.7 (6)	—	—	—
R131C	0 (6)**	59 $\pm$ 6 (6)*	40 $\pm$ 9 (12)**	11 $\pm$ 2 (12)**
I132C	69 $\pm$ 6 (6)**	—	—	—
Q133C	16 $\pm$ 6 (6)**	16 $\pm$ 9 (6)**	100 (6)	—
F134C	71 $\pm$ 7 (6)**	—	—	—
N135C	1.4 $\pm$ 0.9 (6)**	65 $\pm$ 9 (6)	22 $\pm$ 7 (6)**	50 $\pm$ 10 (6)**
M137C	96 $\pm$ 2 (6)	—	—	—
N138C	19 $\pm$ 2 (6)**	16 $\pm$ 3 (6)**	100 (6)	—
S139C	85 $\pm$ 5 (6)	—	—	—
A140C	11 $\pm$ 5 (6)**	32 $\pm$ 7 (6)**	84 $\pm$ 6 (6)*	—
L141C	93 $\pm$ 4 (6)	—	—	—
T142C	11 $\pm$ 4 (6)**	11 $\pm$ 2 (6)**	98.6 $\pm$ 0.9 (6)	—
A144C	0 (6)**	149 $\pm$ 6 (6)	6 $\pm$ 4 (6)**	14 $\pm$ 2 (6)**
I145C	95 $\pm$ 3 (6)	—	—	—
P146C	0 (6)**	44 $\pm$ 8 (6)**	100 (6)	—
L147C	0 (6)**	180 $\pm$ 10 (6)	0 (6)**	50 $\pm$ 10 (6)*
A149C	97 $\pm$ 2 (6)	—	—	—

^a Values, corrected by Abbott's formula, are means $\pm$ standard errors of the means with the number of independent experiments shown in parentheses. Student *t* test differences shown were calculated relative to the values obtained for Cry1Aa (*, $P < 0.05$; **, $P < 0.01$).

^b Measured when mortality was lower than 50%. Student *t* test differences shown were calculated relative to the control values measured in the absence of toxin (*, $P < 0.05$; **, $P < 0.01$).

^c —, not tested.

I_t is the relative scattered light intensity at a given time *t*. For kinetic experiments, percent volume recovery was calculated for every experimental point, and values obtained for control vesicles in the absence of toxin were subtracted from the experimental values measured in the presence of toxin. Each curve was fitted with a Boltzmann sigmoid, and kinetic parameters were extracted from the fitted curves (32). Unless indicated otherwise, data are means $\pm$ standard errors of the means (error bars) of three experiments carried out with a different vesicle preparation. Data for each of these replicates consist of the average of five traces obtained using the same vesicle preparation.

RESULTS

In Cry1Aa, helix $\alpha 4$ is composed of 26 amino acids (16) of which most were individually replaced by a cysteine residue. For reasons that remain unclear, cysteine mutations at proline-124, alanine-125, or leucine-148 were not obtained despite repeated attempts. Furthermore, only minute quantities of T143C protoxin were produced in either *E. coli* or *B. thuringiensis*, and this mutant was consequently removed from the present study. Results for mutants E128C, E129C, and D136C have been reported elsewhere (32).

Toxicity. Bioassays were performed as described in Materials and Methods with neonate *M. sexta* larvae for each of the remaining $\alpha 4$ cysteine mutants (Table 1). At 2 μ g of toxin per ml of buffer solution, at least 85% mortality was observed in the presence of Cry1Aa, R127C, M130C, M137C, S139C, L141C, I145C, and A149C. Mutant toxins I132C and F134C were significantly less toxic than Cry1Aa, but they nevertheless killed 69% and 71% of the larvae, respectively. Less than 25% mortality was observed in the presence of any of the remaining 10 mutants. For this latter group, weight gain was also measured after 7 days to monitor sublethal effects. Control larvae reared in the absence of toxin gained 111 $\pm$ 6 mg after 7 days ($n = 78$). With the exception of mutants N135C, A144C, and

FIG. 1. Effects of the Cry1Aa mutant toxins A149C (A) and S139C (B) on the osmotic swelling of brush border membrane vesicles isolated from fifth-instar *M. sexta* larvae. Vesicles (0.4 mg of membrane protein/ml) were equilibrated overnight in 10 mM HEPES-KOH, pH 7.5. Before the experiments, bovine serum albumin was added to a final concentration of 1 mg/ml, and the vesicles were incubated for 1 hour with the indicated concentrations of toxin (in picomole of toxin/milligram of membrane protein). The vesicles were rapidly mixed with an equal volume of 150 mM KCl, 1 mg/ml bovine serum albumin, and 10 mM HEPES-KOH, pH 7.5, directly in a cuvette using a stopped flow apparatus. Osmotic swelling of the vesicles was monitored by measuring scattered light intensity at an angle of 90° in a PTI spectrofluorometer. Each tracing represents the average of five experiments performed with the same representative vesicle preparation.

L147C, all mutants that caused low mortality were able to impair larval development significantly. The 10 mutants that caused low percentage mortality at 2 μ g/ml were also tested at 25 μ g/ml. At this concentration, only N135C, A144C, and L147C still showed less than 25% mortality. In all cases, the weight gained by surviving larvae was significantly lower than that measured in the absence of toxin and was lower at 25 μ g of toxin/ml than at 2 μ g/ml.

Pore-forming ability. The ability of each mutant toxin to form pores was examined by using an osmotic swelling assay (6). In this assay, brush border membrane vesicles, incubated for 60 min with different toxin concentrations, are rapidly mixed with a hypertonic solution of KCl. As a result of the osmotic gradient, vesicular shrinkage can be observed as a sharp rise in scattered light intensity (Fig. 1). The pores formed by an active toxin, such as A149C, increase membrane permeability to the solute, thus allowing the vesicles to swell back to a fraction of their original volume as observed by a reduction in scattered light intensity (Fig. 1A). Membrane permeability increases rapidly with increasing toxin concentration. In con-

trast, inactive mutants, such as S139C, have little influence on vesicle swelling rates (Fig. 1B).

The pore-forming abilities of some wild-type and mutant toxins depend on pH (30–32). Potential effects of pH on the activity of the $\alpha 4$ mutants were therefore investigated by performing the experiments at pH 7.5 and 10.5 (Fig. 2). Because the pK_a of the cysteine thiol group is 8.33 (26), the single cysteine residue of each mutant should be negatively charged at the higher pH but uncharged at the lower pH value. Only toxin mutants R127C (Fig. 2C), M130C (Fig. 2D), F134C (Fig. 2H), M137C (Fig. 2J), L141C (Fig. 2N), and A149C (Fig. 2T) had an activity which was comparable to that of the wild-type toxin (Fig. 2A). In comparison with Cry1Aa, the other mutants had various activities ranging from mildly to largely reduced. Among those with a large reduction in activity, mutants N135C (Fig. 2I), S139C (Fig. 2L), A140C (Fig. 2M), T142C (Fig. 2O), A144C (Fig. 2P), and L147C (Fig. 2S) caused volume recovery to reach levels that were not significantly different from control values measured in the absence of toxin ($P < 0.05$). The pore-forming activities of the remaining mutants, L126C (Fig. 2B), R131C (Fig. 2E), I132C (Fig. 2F), Q133C (Fig. 2G), N138C (Fig. 2K), I145C (Fig. 2Q), and P146C (Fig. 2R), were significantly lower than that of Cry1Aa, but in their presence, the vesicles were able to swell significantly more than in the absence of toxin ($P < 0.05$). For Cry1Aa and most of its mutants, toxin-induced membrane permeability was similar at both pH values tested. However, L141C (Fig. 2N), I145C (Fig. 2Q), and P146C (Fig. 2R) were significantly more active at pH 7.5 than at pH 10.5 ($P < 0.05$). Q133C (Fig. 2G) was also somewhat more active at the lower pH, but the difference was only marginally significant ($0.05 < P < 0.1$).

Pore properties. All mutants that retained a detectable pore-forming activity were further tested to investigate possible alterations in the ion selectivity and size of their pores. Ionic selectivity of the pores was tested following incubation of the vesicles with 150 pmol of toxin/mg of membrane protein in osmotic swelling experiments where potassium was replaced by the larger cation, *N*-methyl-D-glucamine (Fig. 3A), or in which chloride was replaced by the larger anion, gluconate (Fig. 3B). Experiments using *N*-methyl-D-glucamine were carried out only at pH 7.5, since this amine is not ionized at pH 10.5. As was reported earlier (8, 31, 32), the volume recovery values measured after 3 seconds in the presence of Cry1Aa are far superior in the presence of *N*-methyl-D-glucamine hydrochloride than in the presence of potassium gluconate as expected from the fact that the pores are cation selective (17). For all mutants, volume recovery was also much higher in the presence of *N*-methyl-D-glucamine hydrochloride (Fig. 3A) than in the presence of potassium gluconate (Fig. 3B).

Among the mutants that increased membrane permeability to KCl approximately as well as wild-type Cry1Aa, mutant toxins R127C, M130C, F134C, and A149C were also as effective as the wild-type toxin in permeabilizing the membrane for *N*-methyl-D-glucamine hydrochloride (Fig. 3A). However, M137C and L141C were slightly, but significantly, less active than Cry1Aa ($P < 0.05$). All mutants that were less efficient than Cry1Aa in permeabilizing the membrane to KCl also had a significantly lower ability to increase membrane permeability to *N*-methyl-D-glucamine hydrochloride ($P < 0.01$). Moreover, membrane permeability to *N*-methyl-D-glucamine hydrochlo-

FIG. 2. Dose-response curves for Cry1Aa mutant toxins. Experiments were performed as described in the legend to Fig. 1, except that 10 mM HEPES-KOH was replaced by 10 mM CAPS-KOH in the experiments carried out at pH 10.5. Values of percent volume recovery after 3 s were derived from curves similar to those shown in Fig. 1 as described in Materials and Methods. Significant differences with the corresponding values obtained for Cry1Aa are indicated (*, $P < 0.05$; **, $P < 0.01$).

ride was not significantly higher in the presence of I132C or N138C than in the absence of toxin ($P > 0.05$) (Fig. 3A).

In the presence of potassium gluconate, mutants R127C, M130C, M137C, and L141C were as effective as Cry1Aa at both pH 7.5 and 10.5 (Fig. 3B). Significantly lower volume recovery values were observed for mutants F134C and A149C

at pH 10.5 ($P < 0.05$), but these mutants demonstrated wild-type activity at pH 7.5. Significant differences in osmotic swelling measured at pH 7.5 and 10.5 ($P < 0.05$) were recorded for L126C, L141C, I145C, and P146C (Fig. 3B). The difference in osmotic swelling observed for R131C, Q133C, F134C, and A149C at pH 7.5 and 10.5 were only marginally significant

FIG. 3. Effects of mutant toxins on the permeability of brush border membrane vesicles to different salts. Membrane permeability was assayed using the experimental protocol described in the legend to Fig. 1 but replacing KCl with *N*-methyl-D-glucamine hydrochloride (A) or potassium gluconate (B) following incubation of the vesicles with 150 pmol of toxin/mg of membrane protein. Only those mutants that were able to permeabilize the membrane to KCl were tested for their effects on membrane permeability to the other solutes. Significant differences with the corresponding values obtained for Cry1Aa are indicated (*, $P < 0.05$; **, $P < 0.01$).

($0.05 < P < 0.1$). R131C, I145C, and P146C were active at pH 7.5, albeit significantly less active than Cry1Aa ($P < 0.05$), but inactive at pH 10.5.

Sucrose (Fig. 4A) and raffinose (Fig. 4B), two large uncharged solutes, were used to investigate possible alterations in the size of the pores formed by the mutant toxins. Osmotic swelling measured in the presence of sucrose was much larger than that measured in the presence of raffinose as expected from the larger size of the latter sugar. At both pH values, only toxin mutants R127C, M130C, and L141C permeabilized the membrane for sucrose as efficiently as Cry1Aa did. Among the other mutants tested, only M137C was able to increase significantly membrane permeability to sucrose at both pH values. While L126C had a significant effect only at pH 7.5, F134C and A149C significantly increased membrane permeability to sucrose only at pH 10.5. In the case of F134C, at pH 7.5, the osmotic swelling values were only marginally different ($0.05 < P < 0.1$) from those measured in the absence of toxin or in the presence of Cry1Aa. For all mutants, volume recovery values measured in the presence of raffinose were very low in comparison with those measured in the presence of the other solutes. Only Cry1Aa, R127C, and M130C displayed significant

FIG. 4. Effects of mutant toxins on the permeability of brush border membrane vesicles to oligosaccharides. Membrane permeability to the disaccharide sucrose (A) and the trisaccharide raffinose (B) was assayed using the experimental protocol described in the legend to Fig. 1 following incubation of the vesicles with 150 pmol of toxin/mg of membrane protein. Only those mutants that were able to permeabilize the membrane to KCl were tested for their effects on membrane permeability to these solutes. Significant differences with the corresponding values obtained for Cry1Aa are indicated (*, $P < 0.05$; **, $P < 0.01$).

activity at pH 7.5, and only Cry1Aa and R131C were significantly active at pH 10.5.

Kinetics of pore formation. Determination of pore formation rates was carried out by exposing the vesicles to toxin at the moment they were diluted with the hypertonic KCl solution. Such assays were performed only for mutants that retained significant activity in incubation experiments using KCl. As illustrated in Fig. 5, volume recovery, calculated for every experimental point, increased in a sigmoidal fashion after a delay period in the presence of active toxins, such as Cry1Aa and A149C, and somewhat more slowly in the presence of a less active toxin, such as I145C. Kinetic parameters, including the delay time and the maximal osmotic swelling rate, were estimated from Boltzmann sigmoidal curves fitted to the experimental values (Table 2). Mutants R127C, M130C, F134C, and M137C displayed parameters similar to those of Cry1Aa at both pH levels with the exception of F134C for which the delay at pH 10.5 was slightly, but significantly, longer than that observed with Cry1Aa. The kinetic parameters estimated for L141C and A149C were comparable to those of Cry1Aa at pH 7.5, but these mutants were significantly less active than

FIG. 5. Kinetics of pore formation by Cry1Aa and the mutant toxins I145C and A149C at pH 7.5. Permeability of the vesicles to KCl was assayed as described in the legend to Fig. 1 except that the vesicles were not preincubated with the toxin. Instead, 150 pmol of toxin/mg of membrane of the indicated toxin was added to the KCl solution before mixing with the vesicles. Percent volume recovery was calculated for every experimental point, and the values obtained for control vesicles, assayed without added toxin, were subtracted from those obtained in the presence of toxin. For clarity, error bars are shown only every 50th experimental point.

Cry1Aa at pH 10.5. The remaining mutants, L126C, R131C, I132C, Q133C, N138C, I145C, and P146C, were all much less active than Cry1Aa was at both pH 7.5 and 10.5. The delay periods measured for Q133C, M137C, L141C, I145C, and A149C were significantly longer at pH 10.5 than at pH 7.5 ($P < 0.05$) and the maximal swelling rates measured for R127C, Q133C, and L141C were significantly lower ($P < 0.05$) at the higher pH.

DISCUSSION

Most mutations analyzed in the present study caused a strong reduction in both the toxicity of Cry1Aa toward *M. sexta* larvae and its ability to increase the permeability of midgut

TABLE 2. Kinetic parameters of pore formation by Cry1Aa mutant toxins

Toxin	Delay (min) ^{a,b}		Rate (% vol recovery/min) ^{b,c}	
	pH 7.5	pH 10.5	pH 7.5	pH 10.5
Cry1Aa (wild type)	0.48 ± 0.06	0.65 ± 0.02	30 ± 7	21 ± 1
L126C	>6	>6	1.11 ± 0.07*	1.8 ± 0.6**
R127C	0.38 ± 0.08	0.6 ± 0.1	26 ± 1	19 ± 2
M130C	0.35 ± 0.08	0.59 ± 0.07	25 ± 3	23 ± 1
R131C	>6	NA ^d	4.2 ± 0.9*	NA
I132C	1.0 ± 0.2	NA	5 ± 2*	NA
Q133C	2.2 ± 0.6*	>6	2.5 ± 0.2*	0.2 ± 0.3**
F134C	0.5 ± 0.1	0.79 ± 0.04*	16 ± 1	9 ± 5
M137C	0.49 ± 0.06	0.78 ± 0.08	20 ± 3	16 ± 4
N138C	>6	>6	0.9 ± 0.2*	0.8 ± 0.8**
L141C	0.42 ± 0.06	1.0 ± 0.1*	20 ± 3	6 ± 3**
I145C	1.1 ± 0.1**	3.8 ± 0.5**	4 ± 2*	1.3 ± 0.6**
P146C	>6	NA	0.9 ± 0.4*	NA
A149C	0.59 ± 0.06	1.17 ± 0.07**	15.2 ± 0.6	11 ± 2*

^a Delay is defined as the time required to reach 5% volume recovery. >6, mutants for which 5% volume recovery was not reached within the time course of the experiments.

^b Significantly different from the corresponding value for Cry1Aa (*, $P < 0.05$; **, $P < 0.01$).

^c Osmotic swelling rates correspond to the maximal slopes of the curves.

^d NA, not active.

124PALREEMRIQFNDMNSALTTAIPLLA149

FIG. 6. Summary of mutant pore-forming properties. Polar amino acid residues are circled, and nonpolar residues are boxed. The effect of their replacement by a cysteine residue on toxin pore-forming ability, deduced from osmotic swelling experiments, is indicated by the color of the circles and boxes: residues shown in black forms correspond to mutants that retained wild-type or near wild-type activity, those shown in white forms, correspond to mutants that retained negligible or very low activity, and those shown in gray forms correspond to mutants that retained an intermediate activity. Data for E128C, E129C, and D136C toxin mutants are derived from a previous study (32), and mutants corresponding to residues identified by crossed-out forms were not available for the present study.

brush border membrane vesicles isolated from *M. sexta* to a variety of small solutes. In general, the toxicity of each mutant correlated well with its pore-forming ability compared to that of wild-type Cry1Aa, as was observed previously for a variety of Cry1Aa $\alpha 4$ mutants with alterations in one or the other of its charged residues (32). Notable exceptions were identified, however, including I132C, S139C, and I145C mutant toxins, for which levels of toxicity only slightly lower than that of Cry1Aa were observed (Table 1) despite pronounced reductions in their ability to increase the permeability of the vesicles (Fig. 2). Differences between the in vivo and in vitro activities of Cry1Aa mutants have been described previously (8, 31). In all former cases, however, the mutants had lost substantial toxicity while retaining a pore-forming ability that was comparable to that of the wild-type toxin. Although further work will be required to precisely identify the reasons for these apparent discrepancies, they probably reflect important differential effects of the complex midgut microenvironmental conditions, which are only partially reconstituted in the in vitro experiments, on the pore-forming ability of the mutants (11, 12, 30).

As summarized in Fig. 6, only 6 of the 19 mutants tested in the present study and none of the three cysteine mutants studied previously (32) retained a pore-forming ability that was comparable to that of wild-type Cry1Aa. These comprise five mutants (M130C, F134C, M137C, L141C, and A149C) in which a nonpolar residue was replaced by a cysteine. Except for the A149 mutant, these mutated residues map close to one another on a face of the helix, delimited by residues L126 and F134, in which all residues are nonpolar. Only one mutant retained wild-type activity after the substitution of a polar residue (R127C). Other mutants with alterations at residue

R127 (R127E and R127N) also retained a pore-forming ability that was comparable to that of wild-type Cry1Aa (32). Although this residue is immediately adjacent to the hydrophobic side of the helix, it is located near its N-terminal end and may therefore not be inserted in the membrane at the level of the lipid bilayer.

All other mutants tested had a substantially reduced (L126C, E128C, Q133C, D136C, I145C, and P146C) or negligible (E129C, R131C, I132C, N135C, N138C, S139C, A140C, T142C, A144C, and L147C) pore-forming ability (Fig. 6). In contrast with R131C, other mutants with alterations at residue R131 (R131D, R131E, R131H, and R131Q) retained a readily detectable level of activity, at least at pH 7.5 (32). On the other hand, the very poor activity of N135C correlates well with the inactivity of the N135Q mutants of Cry1Ab and Cry1Ac (7, 29), two toxins in which helix $\alpha 4$ is identical to that of Cry1Aa except for one amino acid at position 148. In a previous study, the inactivity of the T142D mutant was tentatively attributed to a deleterious effect of introducing a negative charge in a region of the helix that is normally devoid of charged residues (32). However, the results of the present study indicate that the sensitivity of the toxin to alterations at residue T142 is independent of the charge, since the T142C toxin was inactive at pH 7.5 and 10.5 despite the fact that its thiol group should have been uncharged at the lower pH but negatively charged at the higher pH. Introducing a negative charge on the cysteine of some mutants nevertheless had a disruptive effect on toxin activity, which was especially pronounced for I145C and P146C, two mutants in which the residue that was altered was nonpolar. It should be pointed out, however, that both of these mutants had a considerably reduced pore-forming activity when measured at pH 7.5 so that the added negative charge accentuated, rather than caused, the deleterious effect of the mutation. In addition, minor effects of pH were observed in the kinetic experiments for a few of the mutants. These were most apparent for the Q133C and L141C mutants, for which both the delay and osmotic swelling rate were affected significantly, even though this effect was much less evident following preincubation of the vesicles with the toxin for an hour before the onset of the assay.

Notwithstanding such subtle differences, the activities of the $\alpha 4$ mutants followed a remarkably similar pattern in all osmotic swelling experiments carried out with and without preincubation in the presence of toxin. These reductions in activity cannot be attributed to major changes in the ionic selectivity of the pores, since for each mutant, the ratio of its activity relative to that of Cry1Aa is similar in experiments performed with potassium chloride, with a larger cation, *N*-methyl-D-glucamine, and with a larger anion, gluconate (Fig. 3). Similarly, these reductions in activity cannot be attributed to major changes in the size of the pores formed by the mutant toxins, since a similar pattern is also observed in the presence of the disaccharide sucrose and the trisaccharide raffinose (Fig. 4). Although minor effects on the ionic selectivity and size of the pores cannot be completely excluded, these observations, as well as the results of the experiments analyzing the kinetics of pore formation, indicate that the reduction in the activity of the mutants is mainly due to an alteration in the number of pores formed, rather than to a change in their properties.

A similar conclusion was reached from the study of Cry1Aa

$\alpha 4$ mutants in which alterations in charged residues caused a sharp reduction in the pore formation of the toxin (32). It is also consistent with earlier results in which the ion channel properties of the pores formed by the Cry1Aa mutant N138C in planar lipid bilayers were examined in detail (23). Their conductance was indeed similar to that of the pores formed by the wild-type toxin, but the probability of the channels being open was considerably lower (12.2% for N138C versus 65.0% for Cry1Aa). It should be pointed out, however, that the probability of the channels being open does not necessarily reflect the pore-forming ability of the toxin, although it must contribute to the overall permeability of the membrane. In fact, even though single-channel lipid bilayer experiments allow precise measurements of the pore properties, once these have formed in the membrane, the kinetics of pore formation are much more reliably studied in osmotic swelling experiments involving a large population of vesicles, each possessing the toxin receptors of the insect host.

In summary, the results of the present study are consistent with our earlier hypothesis that part of $\alpha 4$ lines the lumen of the pores formed by Cry1Aa (23). They also stress the importance of this helix in the mechanism of pore formation by this toxin. Its pore-forming ability is indeed very sensitive to alterations, not only of charged residues of $\alpha 4$, as was documented previously (23, 32), but of most of its polar residues as well. Further work will be required to establish whether the reduced activity caused by such alterations is due to decreased receptor binding, reduced oligomer formation, and/or membrane insertion. The mutants described herein should be particularly useful in exploring the role of each of these steps in the overall mechanism of action of *B. thuringiensis* toxins.

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