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# Analysis of Mutations in the Pore-Forming Region Essential for Insecticidal Activity of a *Bacillus thuringiensis* δ-Endotoxin

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**The *Bacillus thuringiensis* insecticidal δ-endotoxins have a three-domain structure, with the seven amphipathic helices which comprise domain I being essential for toxicity. To better define the function of these helices in membrane insertion and toxicity, either site-directed or random mutagenesis of two regions was performed. Thirty-nucleotide segments in the *B. thuringiensis cry1Ac1* gene, encoding parts of helix α4 and the loop connecting helices α4 and α5, were randomly mutagenized. This hydrophobic region of the toxin probably inserts into the membrane as a hairpin. Site-directed mutations were also created in specific surface residues of helix α3 in order to increase its hydrophobicity. Among 12 random mutations in helix α4, 5 resulted in the total loss of toxicity for *Manduca sexta* and *Heliothis virescens*, another caused a significant increase in toxicity, and one resulted in decreased toxicity. None of the nontoxic mutants was altered in toxin stability, binding of toxin to a membrane protein, or the ability of the toxin to aggregate in the membrane. Mutations in the loop connecting helices α4 and α5 did not affect toxicity, nor did mutations in α3, which should have enhanced the hydrophobic properties of this helix. In contrast to mutations in helix α5, those in helix α4 which inactivated the toxin did not affect its capacity to oligomerize in the membrane. Despite the formation of oligomers, there was no ion flow as measured by light scattering. Helix α5 is important for oligomerization and perhaps has other functions, whereas helix α4 must have a more direct role in establishing the properties of the channel.**

*Bacillus thuringiensis* is unique in its capacity to produce a variety of insecticidal δ-endotoxins, which are arranged in different classes (7, 13). The structure of these toxins appears to be highly conserved (10, 13, 19), especially the seven amphipathic α helices which comprise domain I. Following binding of the toxin to specific receptors on cells lining the larval midgut (12, 15), one or more of these helices insert into the membrane and participate in the formation of an ion channel (8, 10, 16, 22). The mode of killing is believed to be colloid osmotic lysis (17), although more subtle and/or more rapid effects have not been ruled out.

Previously we had mutagenized regions of the *cry1Ac1* gene encoding residues within three of these helices, i.e., α2, α5, and α6, and found that helix α5 was the only one in which many of the mutations abolished toxicity (2, 27). As an extension of the previous studies, we have investigated the role of helix α4 and the loop connecting helices α4 and α5. Thirty-nucleotide mutagenic oligonucleotides were used to obtain random mutations in regions of the *cry1Ac1* gene encoding residues in helix α4 and the loop. Four site-specific mutations which should have altered the hydrophobic properties of helix α3 were also examined. Many mutations in helix α4 resulted in either the loss of toxicity or toxin instability, and one mutant toxin had enhanced activity. Mutations in the loop connecting helices α4 and α5 or within helix α3, however, had little effect on stability or toxicity. The nontoxic α4 mutant toxins oligomerized in the membrane as well as the wild-type toxin but did not form functional ion channels. Helices α4 and α5, which comprise a very hydrophobic loop within domain I, are both important for toxicity but have different roles in toxin aggregation and probably ion channel formation and/or function.

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## MATERIALS AND METHODS

**Bacterial strains and media.** Propagation of phage M13 clones was carried out in *Escherichia coli* JM101 at 37°C in Luria-Bertani medium (21). Other subclones were propagated in *E. coli* DH5α, using the same medium, in the presence of 50 μg of ampicillin $ml^{-1}$. The acrystalliferous derivative of *Bacillus thuringiensis* subsp. *kurstaki* HD1, strain CryB (24), was grown at 30°C on a rotary shaker in G-Tris medium (3) with or without erythromycin at 25 μg $ml^{-1}$.

**Mutagenesis and subcloning.** The *cry1Ac1* gene in M13mp19 was mutagenized as described previously (27). Thirty-nucleotide mutagenic oligonucleotides encoding residues 129 to 137 within helix α4 (5′ CATGTCATTGAATTGAATA CGCATCTC 3′ with 90% as specified plus 3% of each of the other 3 bases) and residues 145 to 155 within the carboxyl terminus of helix α4 and the loop connecting helices α4 and α5 (5′ AACTTGATAATTTTGAACTGCAAAAAG AGGAAT 3′ with 90% as specified plus 3% of each of the other 3 bases) were used to generate random mutations in each of these regions.

Four different single-amino-acid substitutions, i.e., replacement of the asparagine at position 94 by valine ($N_{94}V$) (5′ TCTAGAAATGGCTTGGACCCTA GCGAATTC 3′), $N_{94}F$ (5′ TCTAGAAATGGCTTGGAACCTAGCGATTC 3′), $N_{105}V$ (5′ GTAAATTTGATAAAGCACGCTTAGTCCTTC 3′), and $N_{105}F$ (5′ GTAAATTTGATAAAGGAAGCTTAGTCCTTC 3′), were created within helix α3 by site-specific mutagenesis (27). Double-stranded DNA was propaga ted in *E. coli* JM101, and clones were picked at random for sequencing of single-stranded DNA. Clones with one or two substitutions in helix α4 or the loop and those with specific substitutions in helix α3 were selected for further analyses.

**Immunoblotting and bioassays.** *E. coli* JM101, at a density of $1 \times 10^8$ to $2 \times 10^8$ in Luria-Bertani medium, was infected with the M13 clones, and the cultures were incubated on a rotary shaker at 37°C for 6 h. To test the stability of the mutant toxins, crude extracts of the infected cells, prepared as described previously (2), were incubated with tolylsulfonyl phenylalanylchloromethyl ketone (TPCK)-trypsin at a ratio of 1 μg of extract to 20 μg of TPCK-trypsin in 0.03 M $NaHCO_3$, pH 8.6, for 2 h. The trypsin-treated toxins were tested for stability by separation by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and immunoblotting with a polyclonal rabbit antibody against the Cry1Ac1 toxin (20).

Bioassays were done, as previously described (2), by spreading various dilutions of cells (100 μl each) expressing stable toxins onto insect diet in bioassay cups. One second- to third-instar larva of *Manduca sexta* or *Heliothis virescens* was placed on each of the diet cups, which were incubated for 7 days in an insectary. Ten replicas of each dilution (five to six per assay) were tested for toxicity with cells infected with M13 containing the wild-type *cry1Ac1* gene as a control. A portion of the cells used for the bioassays was suspended in 50 μl of 6 M urea–1% SDS–50 mM dithiothreitol–2 mM phenylmethylsulfonyl fluoride, pH 9.6, and lysed by heating in boiling water for 3 min. Ten-microliter aliquots were then electrophoresed and immunoblotted as described above to determine the amount of toxin applied to the diet. Each bioassay was repeated at least three

TABLE 1. Mutations in helix α4 and their effects on toxin stability and toxicity

| Mutation | Trypsin stability[a] | Toxicity[b] | BBMV protein binding[c] |
|---|---|---|---|
| $Q_{133}R$ | Stable | Nontoxic | + |
| $I_{132}S$ | Stable | Nontoxic | + |
| $I_{132}L$ | Stable | Nontoxic | + |
| $I_{132}V$ | Stable | Nontoxic | + |
| $I_{132}N$ | Stable | Nontoxic | + |
| $R_{131}L$ | Stable | Reduced | + |
| $R_{131}C$ | Stable | No change | NT |
| $R_{131}S$ | Stable | No change | NT |
| $M_{130}I$ | Stable | No change | NT |
| $M_{137}T$ | Stable | No change | NT |
| $F_{134}L$ | Stable | Enhanced[d] | NT |
| $Q_{133}H$ | Stable | No change | NT |
| $I_{132}V$-$D_{136}Y$ | Stable | Reduced | NT |
| $M_{130}I$-$R_{131}L$ | Unstable | | |
| $I_{132}F$-$N_{135}S$ | Unstable | | |
| $F_{134}A$-$M_{137}I$ | Unstable | | |

[a] As demonstrated in Fig. 1.

[b] $LC_{50}$ were determined with infected *E. coli* cells as described in Materials and Methods. Nontoxic means no toxicity was evident with the most concentrated suspension of either M13-infected cells or of *E. coli* clones (undiluted cells at $2 \times 10^8$ to $3 \times 10^8$ $ml^{-1}$). No change means that the $LC_{50}$ was within the same range as that of cells infected with the wild type. Reduced means that there was a >10-fold increase in the $LC_{50}$.

[c] As shown in Fig. 2. NT, not tested; +, protein binding evident.

[d] The $LC_{50}$ was $10^6$ cells ($6.6 \times 10^5$ to $1.3 \times 10^6$ for 95% confidence limits) for the $F_{134}L$ mutant versus $1.9 \times 10^6$ ($1.3 \times 10^6$ to $3.3 \times 10^6$ for 95% confidence limits) for the wild-type infected cells.

times, and the 50% lethal concentrations ($LC_{50}$s) and 95% confidence limits were calculated by employing a SAS probit program (SAS Institute, Inc.) as previously described (2). These values were corrected for any differences in the amount of toxin applied to the diet cup.

Internal fragments from all promising mutant genes were subcloned as *Xho*I-*Sph*I fragments into shuttle vector pHT3101 (1) containing the wild-type *cry1Ac1* gene (2), digested with the same enzymes. Toxin stability and alterations in toxicity ($LC_{50}$s) were confirmed by performing immunoblotting and bioassays of the *E. coli* DH5α clones expressing the mutated *cry1Ac1* genes, as described above.

**Toxin purification.** The pHT3101-*cry1Ac1* plasmids containing the various mutations were electroporated into *B. thuringiensis* CryB as described previously (2). Clones were spread on G-Tris-erythromycin agar and incubated for 72 h at 30°C. The confluent plates of spores plus inclusions were harvested in 1 M KCl–5 mM EDTA, pH 7.0. Following centrifugation at 8,000 rpm for 8 min, the pellets were each resuspended in 2 to 3 ml of deionized water and the suspensions were incubated at 65°C for 2 min to inactivate residual proteases. The cells were recentrifuged at 7,700 × *g* for 8 min, and the pellets were suspended in a minimal volume of solubilization buffer (0.03 M $NaHCO_3$–0.02% α-mercaptoethanol, pH 9.6). Suspensions in this buffer were incubated at 37°C for 20 min and then centrifuged at 7,700 × *g*, and the supernatants were saved. This extraction was repeated twice, and the pooled supernatants were dialyzed overnight at 4°C against 2 liters of 1 mM Tris, pH 8.5. After dialysis, the solubilized protoxin was incubated with TPCK-trypsin at a ratio of 1 μg of extract to 20 μg of TPCK-trypsin at 37°C for 1 h. This trypsin treatment was repeated with an additional 1-h incubation. The digested toxin was dialyzed in Spectropor dialysis tubing with a 50,000-Da-molecular-size cutoff against 1,000 volumes of 0.03 M Tris-HCl, pH 8.5, followed by dialysis against 0.03 M $NaHCO_3$–0.25 M NaCl, pH 9.6.

Further purification of the toxin was carried out with a 1-ml Mono Q cartridge (Pharmacia). Initially, the column was washed with 5 ml of 0.03 M $NaHCO_3$, pH 9.6, followed by 2 ml of 0.03 M $NaHCO_3$–0.25 M NaCl, pH 9.6, and 5 ml of 0.03 M $NaHCO_3$, pH 9.6. The toxin sample was added to the column, and 1-ml fractions were collected. The bound toxin was eluted with a linear gradient of 0.25 to 0.4 M NaCl in 0.03 M $NaHCO_3$, pH 9.6. Each fraction was assayed for protein content by the use of the bicinchoninic acid reagent (Pierce Chemical), and peak fractions were pooled. Toxin purity and concentration were determined by SDS–10% PAGE, staining the gels with Coomassie blue (18), and comparing stain intensities with those of known concentrations of bovine serum albumin (BSA).

**Ligand blotting and membrane insertion studies.** Brush border membrane vesicles (BBMV) were prepared from fifth-instar larvae of *M. sexta* according to the method of Wolfersberger et al. (26), and the protein concentration of the preparation was determined with the bicinchoninic acid reagent. Ligand blotting was performed by the method of Mohammed et al. (20). Twenty micrograms of BBMV protein (solubilized in the loading buffer) was loaded onto an SDS–8% polyacrylamide gel, and each lane was blotted separately onto a polyvinylidene difluoride membrane (Immobilon P; Millipore) strip. After nonspecific groups were blocked with 5% milk powder in Tris-buffered saline, pH 7.5, the membrane strips were incubated with either the wild-type or mutant toxin. The blots were developed after treatment with rabbit anti-Cry1Ac1 antibody followed by an anti-rabbit antibody–alkaline phosphatase conjugate.

For membrane insertion studies, the nontoxic helix α4 mutant toxins were purified from the transformed *B. thuringiensis* CryB strain as described above. A nontoxic mutant toxin with a single-amino-acid substitution in helix α5 ($A_{164}P$) (27) had been previously found to be incapable of inserting into the brush border membrane of *M. sexta* (4) and served as a negative control. BBMV (20 μg of protein) were first washed with 0.1 M $NaHCO_3$–0.25 M NaCl, pH 9.6, and then incubated with 60 ng of each of the toxins at 30°C for 1 h. The BBMV were centrifuged at 7,700 × *g* for 8 min, and the pellets were washed twice with 1 ml of 0.1 M $NaHCO_3$–0.1 M NaCl, pH 9.6, and once with 1 ml of 0.1 M $NaHCO_3$–0.25 M NaCl, pH 9.6. The washed pellets were finally each suspended in 10 μl of the latter buffer supplemented with 0.5% SDS and incubated at 65°C for 15 min. Following centrifugation, the supernatants were subjected to SDS–6% PAGE. Immunoblotting of the extracts was performed as described above.

**Light scattering assays.** The solute permeability of BBMV containing the wild-type and nontoxic-mutant toxins was analyzed by a light scattering assay as described by Carroll et al. (5) with minor modifications. BBMV (0.2 mg/ml) equilibrated with 10 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES)–KOH (pH 9.0)–1% (wt/vol) BSA were incubated with Cry1Ac1 toxin (36 pmol/mg of BBMV) for 60 min at 21°C. The treated BBMV were mixed with an equal volume of 10 mM CHES–KOH–0.1% (wt/vol) BSA containing 150 mM KCl, pH 9.0, at 21°C. Reswelling was measured by using an SpectraKinetic stopped-flow spectrophotometer (Applied Physics) with 90°C light scattering at 450 nm. All measurements were the averages of at least three replicas with errors as in Table 3.

## RESULTS

**Mutations within helix α4 affect toxicity.** Twelve different single-amino-acid substitutions and four different double-amino-acid substitutions were generated within helix α4 (Table 1). Five of the single mutants ($Q_{133}R$, $I_{132}S$, $I_{132}L$, $I_{132}V$, and $I_{132}N$) were nontoxic. The $R_{131}L$ change and the double mutation ($I_{132}V$-$D_{136}Y$) resulted in an approximately 10-fold reduction in toxicity. The $F_{134}L$ mutant, on the other hand, showed an approximately threefold increase in toxicity compared to the wild type. Extracts of the M13 clones were tested for toxin stability, and they all produced stable toxins, as shown for three of them in Fig. 1. Five other single-amino-acid substitutions in this region, i.e., $R_{131}C$, $R_{131}S$, $M_{130}I$, $M_{137}T$, and $Q_{133}H$, did not affect toxicity. Among the four mutant toxins with double-amino-acid substitutions, $M_{130}I$-$R_{131}L$, $I_{132}F$-$N_{135}S$, and $F_{134}A$-$M_{137}I$ were unstable. Those helix α4 mutant toxins which exhibited significant differences from the wild type

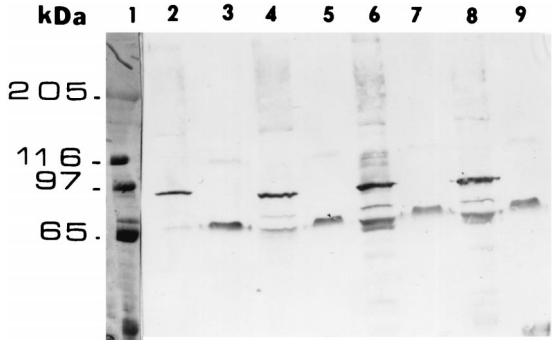


FIG. 1. Crude extracts of M13 clones digested with trypsin were electrophoresed in an 10% SDS–polyacrylamide gel and transferred to an Immobilon-P membrane for immunoblotting with the Cry1Ac1 antibody. Lane 1, molecular mass standards; lane 2, wild-type M13 crude extract; lane 3, wild-type M13 crude extract, trypsin treated; lane 4, $Q_{133}R$ M13 crude extract; lane 5, $Q_{133}R$ M13 crude extract, trypsin treated; lane 6, $I_{132}S$ M13 crude extract; lane 7, $I_{132}S$ M13 crude extract, trypsin treated; lane 8, $I_{132}L$ M13 crude extract; lane 9, $I_{132}L$ M13 crude extract, trypsin treated. All other nontoxic helix α4 mutants were also stable to trypsin digestion (data not shown).

TABLE 2. Mutations in helix α3 and the loop connecting helices α4 and α5 do not affect toxicity

| Mutation | Trypsin stability[a] | Toxicity[b] |
|---|---|---|
| $Q_{154}R$ | Unstable | |
| $I_{145}D$ | Stable | No change |
| $A_{149}V$ | Stable | No change |
| $F_{148}I$ | Stable | No change |
| $V_{150}D$ | Stable | No change |
| $I_{145}T$ | Stable | No change |
| $I_{145}V$ | Stable | No change |
| $N_{94}V$ (helix α3) | Stable | No change |
| $N_{94}F$ (helix α3) | Stable | No change |
| $N_{105}V$ (helix α3) | Stable | No change |
| $N_{105}F$ (helix α3) | Stable | No change |

[a] As demonstrated in Fig. 1.
[b] $LC_{50}$s were determined with infected *E. coli* cells as described in Materials and Methods, with values as reported for the wild type in footnote *d* of Table 1.

in terms of toxicity against *M. sexta* were further tested for toxicity against larvae of *H. virescens*. All of the mutant toxins which were nontoxic for *M. sexta* were also nontoxic for *H. virescens*.

**Mutations within the α4-α5 loop do not affect toxicity.** Seven different single-amino-acid substitutions were generated either within the carboxyl end of helix α4 or within the loop connecting helices α4 and α5 (Table 2). Only the $Q_{154}R$ substitution resulted in instability to trypsin. All of the others produced stable, fully active toxins.

**Increasing the hydrophobicity of helix α3 does not affect toxicity.** Among the seven α-helix peptides, the synthetic peptide of helix α3 exhibited the lowest level of binding to phospholipid vesicles (9). It was thought that binding could be enhanced by converting hydrophilic surface residues to hydrophobic ones, thereby increasing toxicity. Asparagines 94 and 105 were identified as being solvent exposed (14) and were mutated to either V or F (Table 2). None of any of the four single-amino-acid substitutions had any effect on toxicity or toxin stability.

**Binding to a toxin receptor and oligomerization within the membrane.** To test whether the lack of toxicity of the helix α4 mutants was due to a loss of receptor binding, ligand blot and membrane insertion studies were performed. The nontoxic helix α4 mutant toxins bound as well as the wild-type toxin to a single 120-kDa protein in *M. sexta* BBMV (Fig. 2). Nontoxic helix α4 mutant toxins were recovered from BBMV as oligomers of ca. 200 and 130 kDa to about the same extent as the wild-type toxin (Fig. 3). Results like those shown for mutants $Q_{133}R$ and $I_{132}S$ in Fig. 3 were obtained with all of the helix α4 mutant toxins. In contrast, a nontoxic helix α5 mutant toxin, $A_{164}P$, did not insert efficiently into the membrane or oligomerize as previously reported for this mutant and other nontoxic helix α5 mutants (4).

**Nontoxic helix α4 mutant toxins are affected in BBMV permeability.** Toxin-induced changes in BBMV permeability were measured by a light scattering assay (Fig. 4). The rate of decrease for the wild-type toxin was much higher than that for the $I_{132}L$ mutant, with the latter being close to the buffer control value. Similar analyses were done for all of the nontoxic helix α4 mutant toxins, and in all cases there was a seven- to eightfold difference in the initial rate compared to that of the wild type (Table 3).

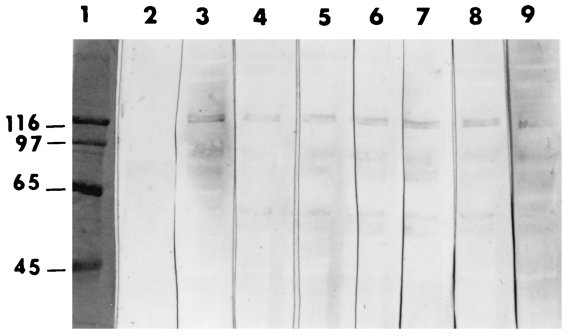


FIG. 2. Immunoblot of *M. sexta* BBMV solubilized proteins fractionated by SDS–8% PAGE and incubated with Cry1Ac1 wild-type and mutant toxins. The blots were developed after treatment with rabbit anti-Cry1Ac1 antibody followed by an anti-rabbit antibody–alkaline phosphatase conjugate. Lane 1, molecular mass standards (in kilodaltons); lane 2, BBMV not incubated with toxin; lane 3, BBMV incubated with wild-type toxin; lane 4, BBMV incubated with mutant toxin $Q_{133}R$; lane 5, BBMV incubated with mutant toxin $I_{132}S$; lane 6, BBMV incubated with mutant toxin $I_{132}L$; lane 7, BBMV incubated with mutant toxin $I_{132}V$; lane 8, BBMV incubated with mutant toxin $I_{132}N$; lane 9, BBMV incubated with mutant toxin $A_{164}P$. Mutant toxins $R_{131}L$ and $I_{132}V$-$D_{136}Y$, with reduced toxicity, also bound to the 120-kDa BBMV protein (data not shown).

## DISCUSSION

Six of 12 single-amino-acid changes within helix α4 resulted in either a total loss of or greatly reduced toxicity. This frequency is comparable to that found for random mutations in helix α5 (27). No nontoxic mutants had been isolated following random mutagenesis in the regions of the *cry1Ac1* gene encoding helices α2 and α6 (2, 27). As discussed below, mutations of surface residues within helix α3 did not affect toxicity (Table 2). Helix α1 does not bind to synthetic phospholipid vesicles (9), and it is the only part of the toxin that is susceptible to protease K after binding of the toxin to BBMV (4). It is unlikely, therefore, to play a critical role in toxin function within the membrane.

Since only mutations in helices α4 and α5, among six of the seven helices comprising domain I, affected toxicity, this portion of the toxin must contribute significantly to the formation and function of an ion channel. The α4-loop-α5 is the most

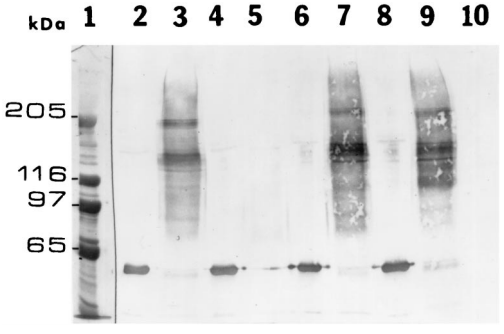


FIG. 3. Binding and oligomerization of toxins in *M. sexta* BBMV. BBMV (20 μg of protein) were incubated with 60 ng of toxin at 30°C for 1 h. The vesicles were centrifuged, washed, and extracted as described in Materials and Methods. The extracts were subjected to electrophoresis (SDS–6% polyacrylamide gels) and western blotting, and the immunoblots were treated with rabbit anti-Cry1Ac1 antibody and then an anti-rabbit antibody conjugated with alkaline phosphatase. Lane 1, molecular mass standards; lane 2, wild-type toxin; lane 3, wild-type toxin extracted from BBMV; lane 4, mutant toxin $A_{164}P$; lane 5, mutant toxin $A_{164}P$ extracted from BBMV; lane 6, mutant toxin $Q_{133}R$; lane 7, mutant toxin $Q_{133}R$ extracted from BBMV; lane 8, mutant toxin $I_{132}S$; lane 9, mutant toxin $I_{132}S$ extracted from BBMV; lane 10, BBMV. All other nontoxic helix α4 mutants also oligomerized in the membrane (data not shown).

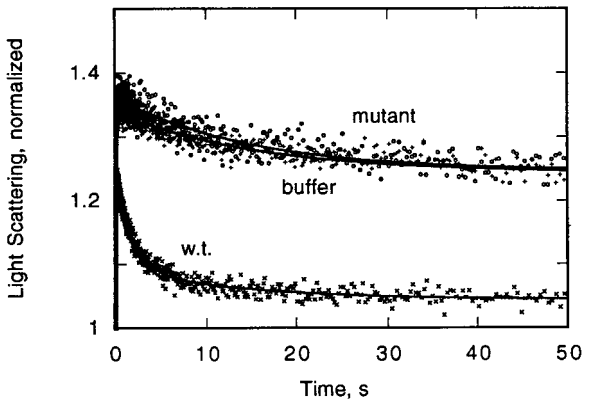


FIG. 4. Toxin-induced permeability changes in *M. sexta* BBMV as measured by a decrease in light scattering with time (5). x, wild-type toxin (w.t.); o, $I_{132}L$ mutant toxin; +, BBMV with buffer only.

hydrophobic region of the Cry1A toxins (10), and it probably inserts into the membrane, as indicated by studies with synthetic peptides of these helices (9). Helix cross-linking studies of the Cry1Aa1 toxin indicate the importance of this region of domain I in toxicity (23). Studies of mutants with single proline substitutions in helix α4 or α3 of the Cry4Ba1 toxin also suggested the importance of helix α4 in toxicity (25).

Single substitutions for 6 of the 10 residues encoded by the mutagenic oligonucleotide were identified, 4 within the hydrophobic face and 2 within the hydrophilic face of the amphipathic helix (Fig. 5). Among the former, only one in five mutations ($Q_{133}R$) resulted in the loss of toxicity. Interestingly, the $Q_{133}H$ mutant was fully toxic, as were the other endotoxins with mutations in this region, which were largely hydrophobic-to-hydrophobic changes. It was also interesting that $F_{134}L$ was about threefold more toxic than the wild type. Five mutations (affecting only two residues) in the hydrophilic face resulted in the loss of toxicity. One, $R_{131}L$, involved the loss of a charge; the other four were relatively conserved changes of $I_{132}$. None of the nontoxic mutants was affected in terms of binding to the receptor. It appears that $I_{132}$, which is a hydrophobic residue within the hydrophilic face of the helix, and $Q_{133}$, which is within the hydrophobic face, have critical functions in the properties of the ion channel. Since the $F_{134}L$ mutant showed a toxicity increase versus the wild type for two different insects, further studies are under way to characterize this mutant and to study the effect of other mutations of this residue.

None of six mutations among four residues either at the carboxyl end of helix α4 or within the loop connecting helices

TABLE 3. Light scattering measurements of the toxin-induced permeability change in *M. sexta* BBMV

| Sample tested | Rate constant of light scattering ($S^{-1}$)[a] |
|---|---|
| Wild-type toxin | 0.81 ± 0.02 |
| $Q_{133}R$ | 0.12 ± 0.01 |
| $I_{132}V$ | 0.11 ± 0.01 |
| $I_{132}N$ | 0.11 ± 0.01 |
| $I_{132}L$ | 0.11 ± 0.01 |
| $I_{132}S$ | 0.10 ± 0.01 |
| BBMV buffer only | 0.00 |

[a] Values are initial rates of decrease of light scattering as determined from the data in Fig. 4 and are averages of at least three measurements ± 1 standard deviation.

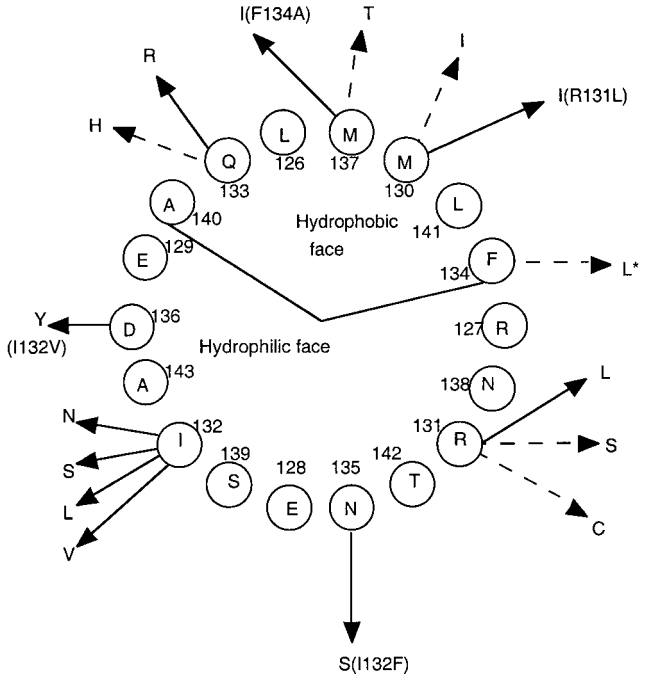


FIG. 5. Helical wheel of 18 residues in helix α4, including the 10 mutagenized residues (E129 to N138). Mutations which resulted in the loss of toxicity (including one double mutant) are indicated by solid arrows. Changes with no resultant loss of toxicity are indicated by dashed arrows. The asterisk indicates an increase in toxicity. Parentheses indicate double mutations. The hydrophobic and hydrophilic faces are demarcated by the internal lines.

α4 and α5 resulted in the loss of toxicity (Table 2). Some of these changes were relatively conserved, but others involved the substitution of a charged residue ($I_{145}D$ or $V_{150}D$) or replacement of a large residue ($F_{148}I$). Following toxin insertion into the membrane, this loop could be close to the cytoplasmic side of the membrane or might even project into the cytoplasm. It was conceivable, therefore, that there was a specific interaction of this loop with a cytoplasmic component which was important for toxicity. If there were such an interaction, it is not likely that A149 and V150 would be involved.

Site-directed mutagenesis of two N residues in helix α3 was undertaken to enhance the hydrophobic properties of this helix, since a synthetic peptide of helix α3 bound very poorly to phospholipid vesicles (9). Residues $N_{94}$ and $N_{105}$ are surface exposed (14), so the mutations should have increased the hydrophobicity of this helix and, thus, its affinity for BBMV. Since there was no change in toxicity for any of the four mutants (Table 2), binding studies were not done. It appears that the surface properties of this helix are not critical for toxicity. An unexpected result was the ability of the nontoxic helix α4 mutants to insert into BBMV and oligomerize as well as the wild-type toxin (Fig. 3). It should be noted, however, that the relative rates of insertion were not determined. An inactive helix α5 mutant toxin, $A_{164}P$, did not remain bound to the membrane, nor did other nontoxic helix α5 mutant toxins (4), although they all bound to a 120-kDa protein from BBMV in immunoblots (as in Fig. 2). All helix α5 mutant toxins which retained toxicity, except for $H_{168}R$, did oligomerize (4).

Some very large (>200-kDa) toxin oligomers have been found in purified Cry1Ac1 and other toxins, and this capacity to aggregate may be important for toxin insertion into the membrane after binding to the receptor (11). There is some

aggregation of purified Cry1Ac1, but not Cry1Ab3, toxin in solution, but in both cases the formation of ca. 200-kDa oligomers was enhanced by incubating purified toxin with BBMV (4). In addition, this oligomer is not a complex of a toxin molecule and the 120-kDa aminopeptidase N receptor (15), since antibody to the latter did not react with this oligomer (4). While interaction with other membrane components has not been ruled out, the formation of a toxin trimer is likely. The lower 130-kDa band could represent toxin dimers.

Helix α5 seems to be very important for oligomerization, perhaps among its other functions in toxicity. In contrast, mutations within helix α4 did not affect the capacity of the toxin to oligomerize in BBMV, despite the lack of permeability to ions in light scattering experiments. A different role for this helix, most likely in the function of the ion channel, is indicated. It was recently reported that a nontoxic helix α4 mutant toxin ($N_{135}Q$) of Cry1Ac1 was altered in a second phase of binding, as measured in a BIOCORE biosensor instrument with aminopeptidase N anchored in synthetic phospholipid (6). The inability to form an irreversible association implied a lack of membrane penetration by this helix α4 mutant, in contrast to the results with other helix α4 mutants reported here. The difference in results may be due to the specific mutation or, more likely, the use of BBMV, rather than synthetic phospholipids, in the present experiments.

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