Role of Helix 3 in Pore Formation by the *Bacillus thuringiensis* Insecticidal Toxin Cry1Aa†

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ABSTRACT: Helix 3 of the Cry1Aa toxin from *Bacillus thuringiensis* possesses eight charged amino acids. These residues, with the exception of those involved in intramolecular salt bridges (E90, R93, E112, and R115), were mutated individually either to a neutral or to an oppositely charged amino acid. The mutated genes were expressed, and the resultant, trypsin-activated toxins were assessed for their toxicity to *Manduca sexta* larvae and their ability to permeabilize *M. sexta* larval midgut brush border membrane vesicles to KCl, sucrose, raffinose, potassium gluconate, and *N*-methyl-D-glucamine hydrochloride with a light-scattering assay based on osmotic swelling. Most mutants were considerably less toxic than Cry1Aa. Replacing either E101, E116, E118, or D120 by cysteine, glutamine, or lysine residues had only minor effects on the properties of the pores formed by the modified toxins. However, half of these mutants (E101C, E101Q, E101K, E116K, E118C, and D120K) had a significantly slower rate of pore formation than Cry1Aa. Mutations at R99 (R99C, R99E, and R99Y) resulted in an almost complete loss of pore-forming ability. These results are consistent with a model in which α-helix 3 plays an important role in the mechanism of pore formation without being directly involved in determining the properties of the pores.

Since the publication of the atomic structures of Cry3A and Cry1Aa, two entomocidal toxins isolated from the intracellular crystal-forming bacterium *Bacillus thuringiensis* (*1, 2*), there have been intensive efforts directed at understanding the mechanisms by which these toxins bind to and permeabilize target cell membranes (*3*). These structurally similar toxins have three domains (D) of which DII and DIII are involved in toxin specificity (*4*−*6*). Various reports have shown that DI, composed of seven α-helices, is responsible for membrane insertion and permeabilization through pore formation (*2, 7, 8*). Two theoretical models for this membrane insertion have been put forward, the umbrella model and the penknife model (*9, 10*). Evidence supporting an umbrella-like model of membrane integration was provided by Schwartz et al. (*11*). The creation of strategically placed, reversible, disulfide bridges between different helices within DI or between DI and DII of Cry1Aa indicated that DI initially separates from DII and DIII whereupon a hairpin, formed by helices α4 and α5, presumably inserts into the membrane to create an active pore. It was conjectured that the remaining DI helices spread on the membrane surface (*11, 12*). Consistent with this hypothesis, mutational analyses have shown that single residue substitutions in α4 or α5, but not α2 or α6, severely affect larval toxicity, thus confirming an important role for the α4/α5 hairpin in pore formation (*13*−*16*). It has also been established that a nine-residue segment of the Cry1Ac α7 helix could be replaced by a similar hydrophobic peptide from diphteria toxin without a loss in toxicity or pore-forming activity (*17*). Several studies using synthetic peptides demonstrated that α5 could adopt a transmembrane configuration, self-aggregate, and form large conductance pores (*18*−*20*). It was subsequently shown that synthetic α4 and α5 peptides could co-assemble in a lipid membrane, thus providing further support for an umbrella-like membrane integration model, and that the other helices, with the exception of α1, could adopt a membrane surface orientation (*21*). By in situ monitoring of cysteine accessibility in active toxin channels, Masson et al. (*22*) provided evidence showing that charged residues within α4 played a direct role in regulating ion flow, thus establishing the helical face of α4 lining the pore lumen in planar lipid bilayers.

In Cry1Aa, α3 is composed of 30 residues, including 3 positively charged arginine residues and 5 negatively charged glutamic acid residues (Figure 1), making it the second largest helix after α6 (33 residues) in DI (*2*). Various reports have shown that α3 could withstand numerous single amino acid substitutions without overt deleterious effects on toxicity. Although it was reported that A92D and most mutations at

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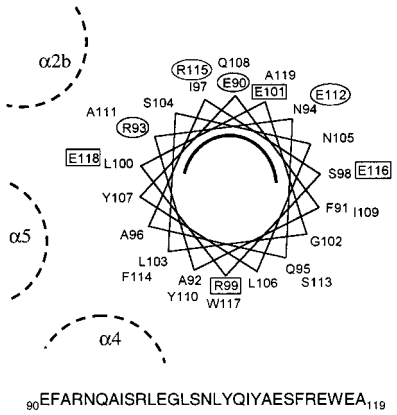

FIGURE 1: Schematic representation of the structure of α-helix 3 from Cry1Aa. Charged residues involved in intramolecular salt bridges, identified by circles, were left intact. Mutations were introduced in each of the other charged residues, identified by boxes. The thick line inside the helical wheel indicates the hydrophilic half of the helix. The approximative position of the helices facing α3, within the crystal structure, is shown by dashed lines.

R93 in Cry1Ac either abolished or greatly reduced toxicity (*23*), 10 other A92 mutants were fully toxic, and 6 out of 7 mutants, positioned within the beginning part of this helix (residues 90 and 91), were comparable to the parental toxin in terms of toxicity. Subsequent work showed that alterations at the proximal end of α3 (A92D, A92E, and R93F) correlated with a loss of toxicity (*14*) but not at residues 94 (N94F and N94V) and 105 (N105F and N105V) (*24*) nor at the distal end of the helix (R115A, A119D, A119E, and A119G) (*14*).

Inasmuch as the only α3 mutants reported to have substantially diminished toxicity were those where an electrical charge had been either added (A92D and A92E) or removed (R93F and others altered at R93), and based on the important role these charged residues have in α4 (*22*), we systematically replaced the charged residues throughout α3 that were not involved in salt bridges, to further explore the structural constraints on this helix and to obtain further data on the role of charged residues in promoting the transition from the aqueous to the membrane-associated form of the Cry1Aa toxin.

EXPERIMENTAL PROCEDURES

Mutagenesis. The negatively charged residues E101, E116, and E118, and the positively charged residue R99 were targeted for mutagenesis. Mutations at E90, R93, E112, and R115 were avoided as these charged residues form either interhelical (R93 with α2b), intrahelical (E112−R115), or other (E90 with R87, located within the α2b−α3 interhelical loop) salt bridges which could be important for protein stability (*2*). Since D120 is the first residue past the C-terminal end of α3 (in the α3/α4 loop), it was included as part of the study. Mutants were created by oligonucleotide-directed in vitro mutagenesis using the double oligonucleotide method (*25*) (Clontech Transformer kit, Clontech Laboratories, Palo Alto, CA) and the double-stranded expression plasmid pMP39 (*26*). All mutants were sequenced using an Applied Biosystems (Foster City, CA) model 370A automated fluorescent sequencer.

Expression and Purification. All recombinant *Escherichia coli* strains were grown at 30 °C in double-strength YT broth containing ampicillin at 100 μg/mL for 2−3 days. The cells were harvested, washed once in 0.1 M phosphate buffer, pH 6.0, and disrupted by two passages through a French pressure cell at 11 000 psi internal pressure. The insoluble protoxin-containing inclusions were harvested and treated as described elsewhere (*26*). Insoluble inclusions were solubilized in 0.4 M carbonate buffer, pH 10.5, and activated by the addition of 1% (w/v) trypsin for 3 h at room temperature. Contaminating colloidal lipids from *E. coli* were removed by centrifuging the activation mixture for 1 h at 200000*g*. All toxins were purified by fast protein liquid chromatography using a Mono Q ion exchange column (Pharmacia Biotech, Montreal, Quebec) as described elsewhere (*26*) and stored at −20 °C until use. The purity and integrity of all Cry toxins were verified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (*27*) and protein concentrations determined by the method of Bradford (*28*) using bovine serum albumin as a standard.

Bioassays. Fertilized eggs of *Manduca sexta* were purchased from the Carolina Biological Supply Co. (Burlington, NC). Toxicity assays were performed on neonate larvae with trypsin-activated toxins. A final volume of 100 μL containing toxin diluted in phosphate-buffered saline (8 mM Na_2HPO_4, 2 mM KH_2PO_4, and 150 mM NaCl, pH 7.4) was layered on an artificial diet (*29*) in each 2 cm^2 well of a 60 well plate. 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 rates were recorded after 7 days. All mutant toxins were tested at 2 μg/mL, and, depending on the mortality rate observed at this concentration, the tests were repeated at either 50 ng/mL or 50 μg/mL. Thirty larvae were used for each concentration tested, and the bioassays were replicated 5 times.

Preparation of Brush Border Membrane Vesicles. *M. sexta* larvae were raised to the fifth instar on the artificial diet supplied with the insects (Carolina Biological Supply Co.). Whole midguts were isolated, cleaned of their contents and attached Malpighian tubules, and stored at −80 °C until use. Brush border membrane vesicles were prepared from thawed midguts using a magnesium precipitation and differential centrifugation method (*30*). The final pellet was suspended to 0.44 mg of protein/mL in 10 mM of either 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)[1]/KOH, pH 7.5, or 3-cyclohexylamino-1-propanesulfonic acid (Caps)/KOH, pH 10.5, and allowed to equilibrate overnight at 4 °C.

Light-Scattering Assay. Brush border membrane permeability properties were analyzed with a light-scattering assay as described by Carroll and Ellar (*31*). Because the highly alkaline conditions encountered in the lepidopteran insect midgut (*32*, *33*) have been suggested to play an important role in *B. thuringiensis* toxin function (*3*, *15*, *34*), experiments were carried out at both pH 7.5 and pH 10.5. At least 1 h prior to the experiments, vesicle suspensions were further diluted to 0.40 mg of protein/mL with 10 mg/mL bovine serum albumin in either 10 mM Hepes/KOH, pH 7.5, or 10 mM Caps/KOH, pH 10.5. Vesicles were preincubated at 23 °C for 60 min with the indicated toxin concentrations, except

[1] Abbreviations: Caps, 3-cyclohexylamino-1-propanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SEM, standard error of the mean.

Table 1: Toxicity of Cry1Aa α-Helix 3 Mutants toward *M. sexta* Larvae

	% mortality[a]		
toxin	50 ng/mL	2 μg/mL	50 μg/mL
Cry1Aa	45 ± 1	−	−
R99C	−	5 ± 1	2 ± 1
R99E	−	3 ± 1	2 ± 1
R99Y	−	3 ± 1	13 ± 4
E101C	3 ± 1	93 ± 1	−
E101K	−	4 ± 1	69 ± 1
E101Q	3 ± 1	92 ± 1	−
E116K	3 ± 1	98 ± 1	−
E116Q	9 ± 1	95 ± 1	−
E118C	8 ± 1	30 ± 1	97 ± 1
E118K	25 ± 2	97 ± 1	−
E118Q	17 ± 1	65 ± 1	−
D120C	32 ± 2	92 ± 1	−
D120K	18 ± 1	98 ± 1	−
D120Q	12 ± 2	67 ± 1	−

[a] Values are means ± SEM of five independent experiments.

for kinetic experiments where the preincubation step was omitted. Assays were initiated by rapidly mixing the vesicles with an equal volume of either 150 mM KCl, *N*-methyl-D-glucamine hydrochloride, or potassium gluconate, or 300 mM sucrose or raffinose using a Hi-Tech stopped-flow kinetics apparatus (Salisbury, U.K.). Exposure of the vesicles to these hypertonic solutions results in their osmotic shrinking which is detected by a rapid rise in scattered light intensity. As the solutes diffuse into the vesicles, these swell at a rate that depends on the permeability of the membrane. Osmotic volume changes were monitored by measuring the 90° scattered light intensity at 23 °C in a Spex CMIII spectrofluorometer (Jobin Yvon Horiba, Edison, NJ) with both monochromators set at 450 nm.

Data Analysis. Percent volume recovery was calculated as $100 \times (1 - I_t)$, where I_t is the relative scattered light intensity measured at time *t*. For kinetic experiments, percent volume recovery was calculated for each data point, and values obtained for control vesicles, assayed without toxin, were subtracted from the experimental values measured in the presence of toxin. Data are reported as means ± SEM of at least three experiments, each performed in quintuplicate, with different vesicle preparations. Statistical comparisons were made with the two-tailed unpaired *t* test using the Instat version 1.13 program (Graphpad Software, San Diego, CA).

RESULTS

Toxicity. Most α3 mutants analyzed in the present study were considerably less toxic to *M. sexta* larvae than Cry1Aa (Table 1). The three mutants with alterations at amino acid position 99, R99C, R99E, and R99Y, were essentially nontoxic. With the exception of E101K and E118C, the other mutants retained the ability to kill over 50% of the larvae at 2 μg/mL. At 50 ng/mL, the mutants with alterations of E101 or E116, as well as E118C and D120Q, were poorly active, but E118K, E118Q, D120C, and D120K were able to kill more than 15% of the larvae.

Pore-Forming Ability. The ability of these mutants to permeabilize *M. sexta* brush border membrane vesicles to KCl was evaluated using a light-scattering assay (*31*). As shown in Figure 2A, using the active wild-type Cry1Aa toxin, both the rate and extent of vesicle swelling increased rapidly

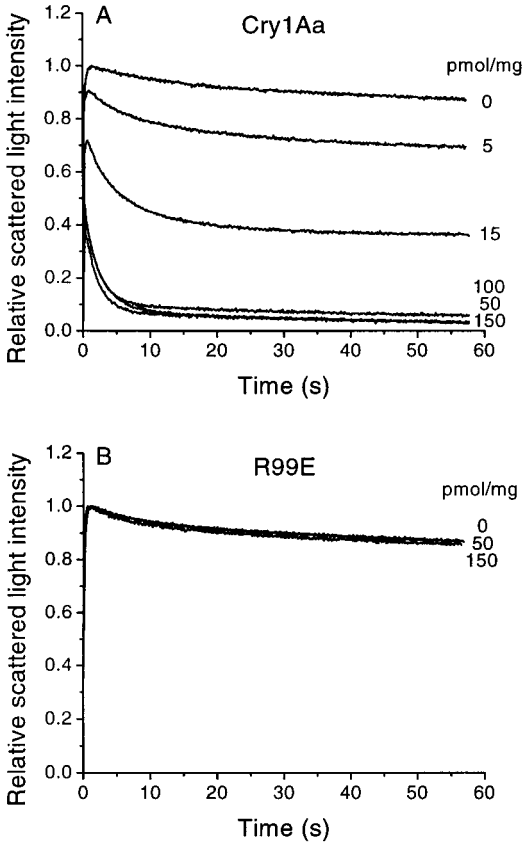

FIGURE 2: Effect of Cry1Aa and mutant R99E on the osmotic swelling of *Manduca sexta* brush border membrane vesicles. Vesicles isolated from the midguts of fifth-instar *M. sexta* larvae and equilibrated in 10 mM Hepes/KOH, pH 7.5, were preincubated for 60 min with the indicated concentrations of wild-type Cry1Aa (A) or R99E (B). Vesicles were rapidly mixed with an equal volume of 150 mM KCl and 10 mM Hepes/KOH, pH 7.5, using a stopped-flow apparatus, and scattered light intensity was monitored at an angle of 90°. Each trace corresponds to the average of 5 experiments.

with increasing toxin concentration. Vesicle volume reached a near-steady-state after approximately 30 s. In contrast, in the presence of R99E, a mutant which has completely lost toxicity to *M. sexta*, the vesicles swelled slowly, at a rate matching that observed in the absence of toxin (Figure 2B).

To compare the pore-forming ability of different mutants, percent volume recovery reached at the 3 s time-point was calculated from individual curves such as those shown in Figure 2 and plotted against toxin concentration (Figure 3). In agreement with their lack of toxicity, the three mutants with alterations at R99 were unable to form channels in brush border membrane vesicles at either neutral or alkaline pH (Figure 3A,B). At pH 7.5, all the other mutants permeabilized the vesicles to KCl approximately as well as Cry1Aa (Figure 3C,E,G,I). This was also the case at pH 10.5 for most mutants, with the exception of E101C (Figure 3D), E116K and E116Q (Figure 3F), and E118C (Figure 3) which were significantly less active at pH 10.5 than at pH 7.5.

Pore Properties. To further characterize the pores formed by the active mutant toxins, their ability to increase the permeability of the vesicles to the large uncharged solutes, sucrose and raffinose (Figure 4), and to the salts of relatively large ions, gluconate and *N*-methyl-D-glucamine (Figure 5), was assayed using the same experimental approach as for KCl. All mutants increased membrane permeability to both

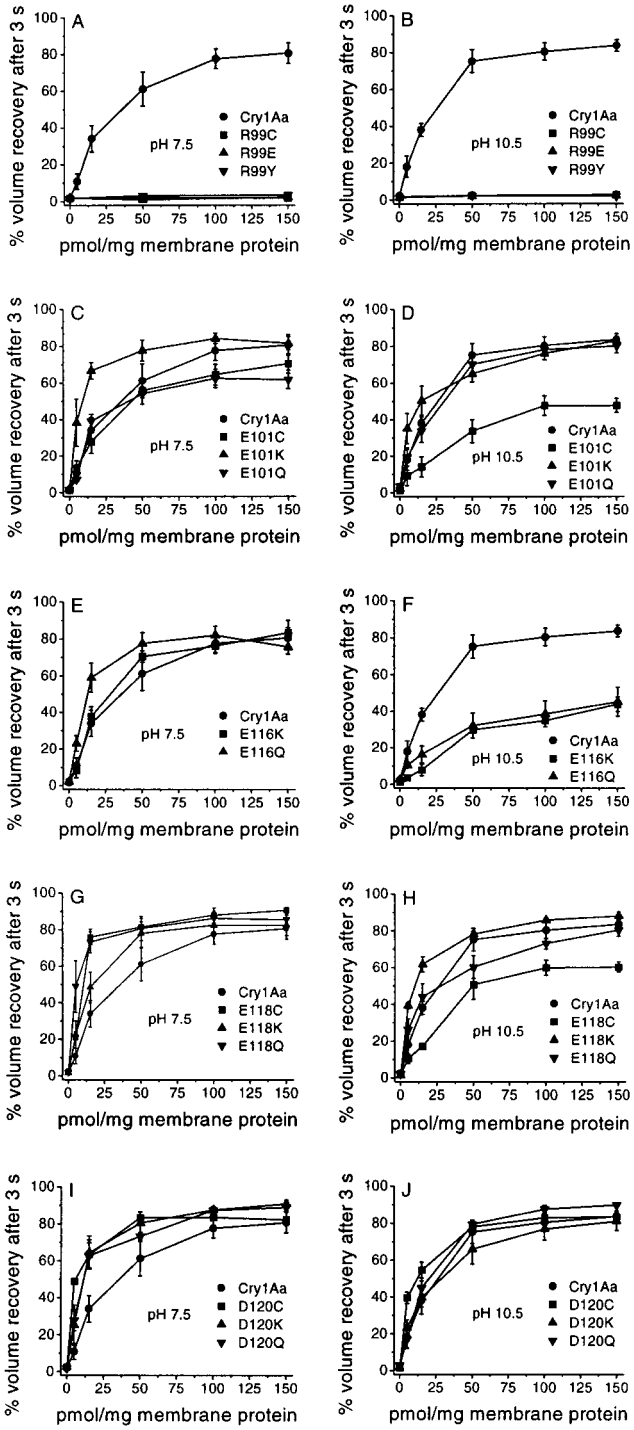

FIGURE 3: Pore-forming ability of α-helix 3 mutants. Midgut brush border membrane vesicles equilibrated in 10 mM Hepes/KOH, pH 7.5 (A, C, E, G, and I), or Caps/KOH, pH 10.5 (B, D, F, H, and J), were preincubated 60 min with the indicated concentrations of Cry1Aa or either one of its mutants with alterations at amino acid position 99 (A and B), 101 (C and D), 116 (E and F), 118 (G and H), or 120 (I and J). Their permeability to KCl was monitored following rapid mixing with 150 mM KCl and 10 mM Hepes/KOH, pH 7.5 (A, C, E, G, and I), or Caps/KOH, pH 10.5 (B, D, F, H, and J), as described in the legend of Figure 2. Percent volume recovery at the 3 s time point was calculated from traces such as those shown in Figure 2 as described under Experimental Procedures.

sugars approximately as well as Cry1Aa, at pH 7.5 (Figure 4A,C), although a slight, but significant, reduction in the permeability to sucrose was observed for E101Q (Figure 4A). All mutants also allowed diffusion of sucrose and raffinose at pH 10.5, but the permeability to sucrose of E101C, E101K, E116K, E116Q, and E118Q and the permeability to raffinose of E101C, E116K, and E116Q were significantly smaller than those of Cry1Aa (Figure 4B,D). Interestingly, the permeability to sucrose of D120Q was significantly higher than that of Cry1Aa at pH 10.5, suggesting that this mutant forms slightly larger pores.

Permeability to potassium gluconate was generally similar to that observed in the presence of Cry1Aa, but E116K and E116Q showed a minor reduction, and E118K, D120C, and D120Q a minor increase, at pH 10.5 (Figure 5A,B). Permeability to *N*-methyl-D-glucamine hydrochloride at pH 7.5 did not differ from that observed in the presence of Cry1Aa except for a very slight increase in the presence of E118C, E118K, and D120Q (Figure 5C). This salt was only tested at pH 7.5 because the *N*-methyl-D-glucamine ion has a pK_a of 9.63 (*35*) and is therefore present mainly in its uncharged form at pH 10.5.

Kinetics of Pore Formation. Monitoring of the percent volume recovery following exposure of the vesicles simultaneously to a KCl gradient and toxin, without preincubation as in the previous experiments, revealed that several of the mutants had significantly slower pore-forming kinetics than wild-type Cry1Aa (Figure 6). This was true at pH 7.5 and 10.5 for all three mutants at position 101 (Figure 6A,B) as well as for E116K (Figure 6C,D), 118C (Figure 6E,F), and D120K (Figure 6G,H). E116Q differed significantly from Cry1Aa at pH 10.5 (Figure 6D) but not at pH 7.5 (Figure 6C).

DISCUSSION

The introduction of single amino acid changes in Cry1Aa has highlighted the critical importance of charged amino acid residues within α4 for proper ion channel function (*22*). In that study, alteration of charged residues in α4 led to a severe decrease in membrane permeabilization in lipid bilayers and a complete loss of toxicity to susceptible larvae. Based on those results, we hypothesized that α3, which has membrane association rather than transmembrane properties (*21*) and thus would be more peripherally involved in channel formation, should exhibit a greater tolerance to charged amino acid mutations. In a multimeric pore assembly, it is reasonable to expect that some α3 mutations will affect toxin activity due to either steric or mechanistic reasons, but these alterations should be minor in comparison with those observed for α4, a pore-lining helix. The present light-scattering assay and toxicity data confirmed our notion that α3 can tolerate a broader range of mutations than α4 and α5. With the sole exception of those modified at R99, all mutants had a pore-forming ability similar to that of Cry1Aa when preincubated with the vesicles at pH 7.5. In addition, at this pH, only minor differences were observed in the ability of these mutants to permeabilize the brush border membrane to larger solutes, in comparison with Cry1Aa, indicating that the pores formed by these toxins had similar properties.

Although a strong case was made, in recent review articles (*3, 15*), for an important role of the highly alkaline conditions characteristic of the lepidopteran insect midgut in *B. thuringiensis* toxin activity, pH had very little effect on the pore-forming properties of Cry1Aa, and only four mutants, E101C,

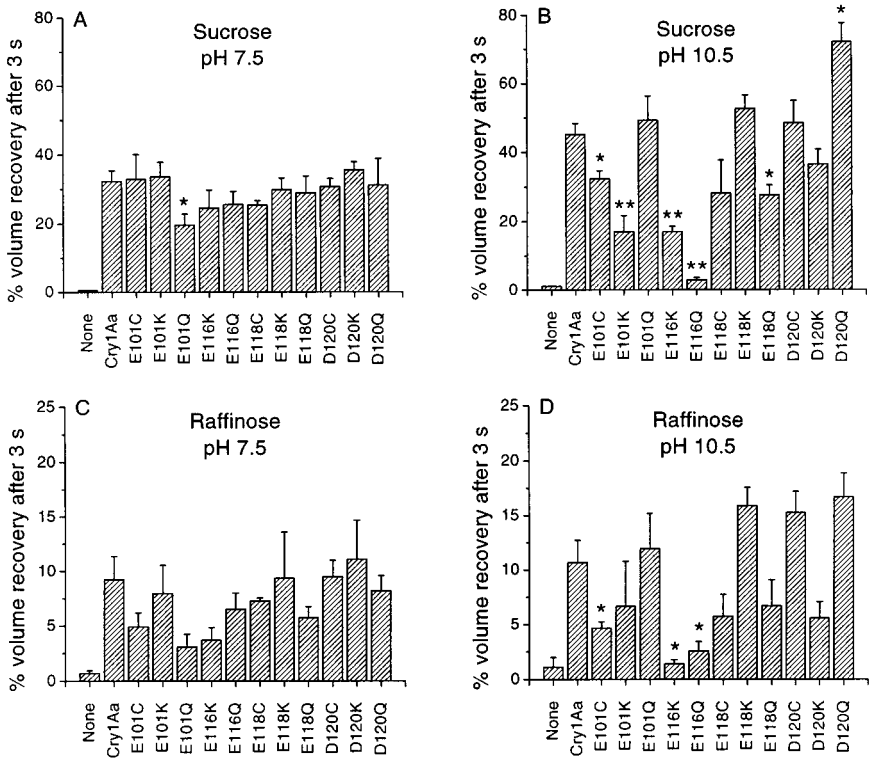

FIGURE 4: Effect of α-helix 3 mutants on vesicle permeability to oligosaccharides. Midgut brush border membrane vesicles equilibrated in 10 mM Hepes/KOH, pH 7.5 (A and C), or Caps/KOH, pH 10.5 (B and D), were preincubated 60 min with 150 pmol of toxin/mg of membrane protein. Their permeability to sucrose (A and B) and raffinose (C and D) was monitored following rapid mixing with 300 mM sucrose or raffinose and 10 mM Hepes/KOH, pH 7.5 (A and C), or Caps/KOH, pH 10.5 (B and D), as described in the legend of Figure 2. Significance level: *, $p < 0.05$; **, $p < 0.01$.

E116K, E116Q, and E118C, displayed a weaker activity at pH 10.5 than at pH 7.5. These mutants had all lost a negatively charged residue, but so had the other mutants modified at positions 101 and 118 which were not significantly affected by the alkaline pH. In addition, the negative charge should have been restored in both cysteine mutants as the pH was raised to 10.5. The increased sensitivity to high pH introduced by the mutations at these two positions is therefore not a simple consequence of removing a negative charge, but must involve more subtle and indirect alterations in the structure and function of the toxin.

The mode of action of *B. thuringiensis* insecticidal crystal proteins is a complex, multistep process involving protoxin solubilization and activation in the larval midgut, binding of the activated toxin to specific cell surface receptors, pore formation, and eventual loss of gut integrity through epithelial cell lysis (*3*, *10*, *15*, *36*). A mutation affecting any one of these steps will alter the overall toxicity of the resulting protein. Irreversible binding to the brush border membrane, indicative of toxin insertion, has been suggested to constitute a better index of toxicity than the initial, reversible binding of the toxin to its receptor (*14*, *37*, *38*). In the present study, all mutants capable of forming pores, and therefore able to associate irreversibly with the membrane after binding to a receptor, retained a reduced but measurable degree of toxicity. However, from a quantitative point of view, reduced toxicity was not paralleled by an equivalent reduction in pore-forming ability. Because in vivo bioassays were carried out with in vitro solubilized and activated toxins, α3 mutants appear to be affected at a step following toxin activation but preceding pore formation in the toxicological process.

When the focus was shifted from the overall pore-forming ability to a kinetic analysis of pore formation in real time by eliminating the preincubation step, considerable differences were observed in the rate at which these mutant toxins increased the permeability of the vesicles. In general, reductions in the rate of pore formation and toxicity were more pronounced for mutations at E101 and E116 than at E118 and D120. There was nevertheless only a partial correlation between the kinetics of pore formation and toxicity for the active mutants. For instance, among the E101 mutants, E101C showed the slowest kinetics (Figure 6A,B), yet its toxicity was equal to that of E101Q and higher than that of E101K, although much below that of Cry1Aa (Table 1). Similarly, E116K, although significantly slower than E116Q (Figure 6C,D), was equally toxic (Table 1). Only for E118 mutants (Figure 6E,F) was it found that the slowest mutant, E118C, was also the least toxic (Table 1), with the toxicity difference staying within a factor of 2−3. A reduced rate of pore formation could contribute to a decrease in toxicity by increasing the time during which toxins are exposed to proteases in the insect midgut, but this was clearly not the case for almost half of the mutants analyzed in the present study. Some of the mutated toxins were incubated with midgut juice before light-scattering experiments, but their ability to permeabilize the vesicles was not significantly altered by such treatments (data not shown). Although all mutant protoxins showed normal trypsin activation in vitro, the possibility cannot be excluded that mutations in α3 could have rendered the toxins more susceptible to proteolysis in the insect midgut after binding to the brush border membrane.

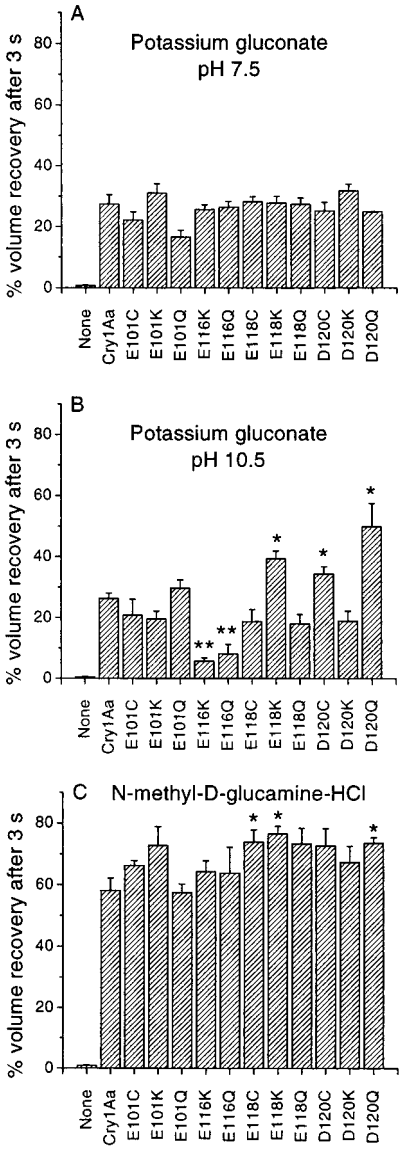

FIGURE 5: Effect of α-helix 3 mutants on vesicle permeability to potassium gluconate and *N*-methyl-D-glucamine hydrochloride. Vesicles equilibrated in 10 mM Hepes/KOH, pH 7.5 (A and C), or Caps/KOH, pH 10.5 (B), were preincubated 60 min with 150 pmol of toxin/mg of membrane protein. Their permeability to potassium gluconate (A and B) and *N*-methyl-D-glucamine hydrochloride (C) was monitored following rapid mixing with 150 mM potassium gluconate or *N*-methyl-D-glucamine hydrochloride and 10 mM Hepes/KOH, pH 7.5 (A and C), or Caps/KOH, pH 10.5 (B), as described in the legend of Figure 2. Significance level: *, $p < 0.05$; **, $p < 0.01$.

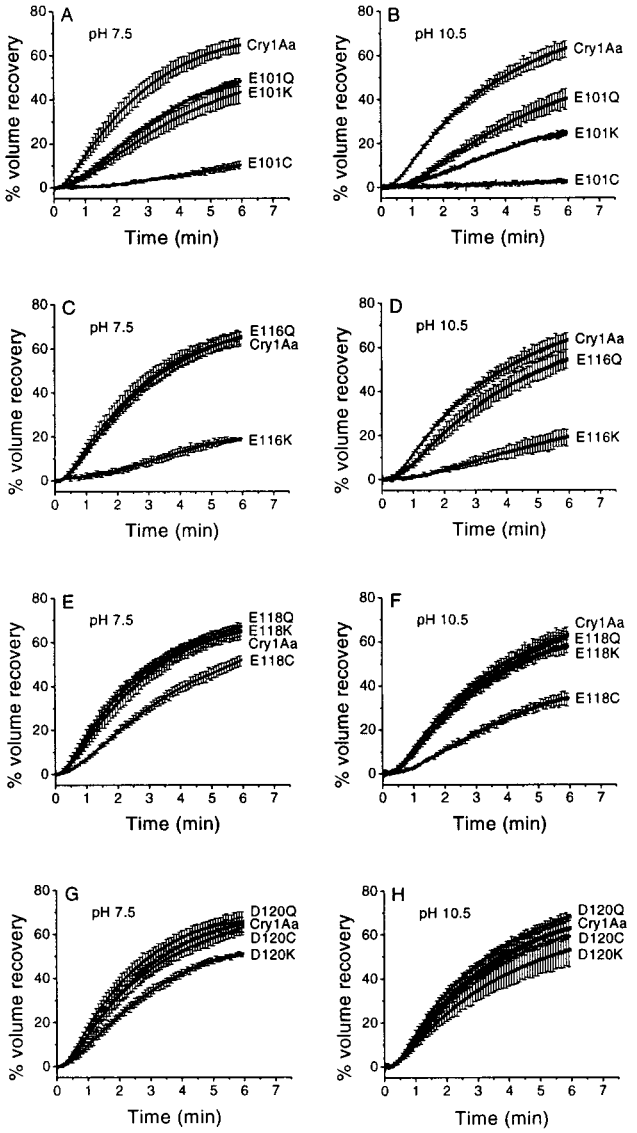

FIGURE 6: Kinetics of pore formation. Vesicles equilibrated in 10 mM Hepes/KOH, pH 7.5 (A, C, E, and G), or Caps/KOH, pH 10.5 (B, D, F, and H), were mixed with an equal volume of 150 mM KCl, 10 mM Hepes/KOH, pH 7.5 (A, C, E, and G), or Caps/KOH, pH 10.5 (B, D, F, and H), and 150 pmol/mg of membrane protein of Cry1Aa or either one of its mutants with alterations at amino acid position 101 (A and B), 116 (C and D), 118 (E and F), or 120 (G and H), without preincubation with the toxin. 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 only shown for every 50th data point.

Unfortunately, attempts to demonstrate an increased proteolysis of these mutant toxins once bound to the brush border membrane were unsuccessful due to a rapid loss of the vesicles' osmotic properties in the presence of trypsin or midgut juice extract (data not shown).

All mutants in which R99 was replaced by another amino acid (C, E, or Y) lost the capacity to form functional pores in midgut brush border membrane vesicles. This correlates with a complete loss of toxicity and suggests an important role for this residue in the mechanism of pore formation. At the structural level, R99 is located on the hydrophobic face of α3, in contrast with all other charged residues, and directly opposite to R93 (Figure 1), a position where the presence of a positively charged amino acid is also thought to be essential for pore formation and toxicity (*14*, *23*). R93 has been shown, however, to form a complex salt bridge with two other residues located within α2b (*2*). Elimination of this salt bridge could therefore lead to an inactive protein due to a disruption of the structural integrity of DI.

Previous studies have shown that certain mutations within DI can cause the inactivation of Cry toxins through the inability to irreversibly associate with midgut brush border membrane vesicles (*14*, *37*) or by altered aggregation (*24*, *39*). Interestingly, in the latter study, reduced aggregation was observed primarily for nontoxic α5 mutants and not for nontoxic α4 mutants. Because the light-scattering assay measures membrane permeability, we cannot determine whether the lack of pore formation observed with the R99

mutants was due to a defect in membrane integration or in another step of the mechanism leading to the assembly of a functional pore. In contrast, all of the other mutants analyzed in the present study were clearly able to insert into the membrane since they retained the capacity to form pores. The reduced rates of pore formation observed for several of these mutants suggest that α3 contributes to pore formation.

In summary, most mutations altering charged residues in α3 had only minor effects on the properties of the pores formed by the toxin. With the exception of those with alterations at R99, and even though several had a reduced rate of pore formation, all mutants retained a pore-forming ability that was comparable to that of wild-type Cry1Aa following a 1 h preincubation with the vesicles, at least at pH 7.5. Although these data are consistent with the results published by Gazit et al. (*21*) that this helix does not integrate into the membrane but associates with its surface, in agreement with an umbrella model of membrane integration of *B. thuringiensis* Cry toxins (*11*), they strongly suggest an active role of α3 in the mechanism of pore formation.

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