Helix 4 of the Bacillus thuringiensis Cry1Aa Toxin Lines the Lumen of the Ion Channel*

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The mode of action of Bacillus thuringiensis insecticidal proteins is not well understood. Based on analogies with other bacterial toxins and ion channels, we hypothesized that charged amino acids in helix 4 of the Cry1Aa toxin are critical for toxicity and ion channel function. Using Plutella xylostella as a model target, we analyzed responses to Cry1Aa and eight proteins with altered helix 4 residues. Toxicity was abolished in five charged residue mutants (E129K, R131Q, R131D, D136N, D136C), however, two charged (R127E and R127N) and one polar (N138C) residue mutant retained wild-type toxicity. Compared with Cry1Aa and toxic mutants, non-toxic mutants did not show greatly reduced binding to brush border membrane vesicles, but their ion channel conductance was greatly reduced in planar lipid bilayers. Substituted cysteine accessibility tests showed that in situ restoration of the negative charge of D136C restored conductance to wild-type levels. The results imply that charged amino acids on the Asp-136 side of helix 4 are essential for toxicity and passage of ions through the channel. These results also support a refined version of the umbrella model of membrane integration in which the side of helix 4 containing Asp-136 faces the aqueous lumen of the ion channel.

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polar amino acid in α4 were altered. Using diamond moth (Plutella xylostella) as a target, we tested wild-type Cry1Aa and the mutant proteins for toxicity, binding to brush border membrane vesicles (BBMVs), and ion channel formation in PLBs. We also used the substituted cysteine accessibility method (SCAM) (21) with a charged thiol-specific reagent (22) to localize cysteine-engineered amino acids relative to the channel lumen in situ. The results provide direct evidence of the role of α4 in ion channel formation and toxicity.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Protoxin Production, Toxin Activation, and Purification**—Helix 4 of Cry1Aa is composed of 26 amino acids of which five are charged (see Fig. 1A). We created nine Cry1Aa mutants at six different amino acid positions (Fig. 1B) by oligonucleotide-directed in vitro mutagenesis using the double oligonucleotide method (23) (CLONTECH TransformerTM kit, CLONTECH Laboratories, Palo Alto, CA) in the expression plasmid pMP39 (24). One mutant protein, E128K, was not expressed in sufficient quantities in either *Escherichia coli* or in *B. thuringiensis* for bioassays and thus was not included in subsequent analyses.

All protoxins were produced as insoluble inclusions in *Escherichia coli* and purified as described elsewhere (24). Trypsin activation was performed by incubating 15 μg of protoxin solubilized in 40 mM carbonate buffer, pH 10.5, with 1% (w/v) trypsin for 3 h at room temperature. Activated toxin was centrifuged for 1 h at 200,000 × g to remove lipids. Toxins were purified by fast protein liquid chromatography ion-exchange chromatography using a Mono-Q column and eluting bound toxin with a 50–500 mM NaCl gradient in carbonate buffer, pH 10.5 (24). Activated toxins were dialyzed against distilled water until precipitated. The precipitate was collected and stored in water at 4 °C. The similar biochemical properties of protoxin alkali solubility and production of a protease resistant 68-kDa toxin was taken as evidence that all mutant protein conformations resembled that of wild-type Cry1Aa toxin.

**Bioassays**—Diamondback moth larvae were reared on cabbage and bioassayed using previously described methods (25). Cry1Aa and eight mutant proteins were tested against third instar larvae of the susceptible *LAB-PS* strain (26). The protoxin form of each of the aforementioned proteins as well as the purified, trypsin-activated toxin form of E129K and D136C were tested.

Groups of 9 to 11 (usually 10) larvae were allowed to eat cabbage leaf discs dipped in distilled water dilutions of each protein (25). At least three independent groups of larvae were tested against each protein (minimum sample size = 30). Mortality was recorded after five days and adjusted for mortality of larvae that ate control disks dipped in distilled water only. In all treatments, including distilled water control and adjusted for mortality of larvae that ate control disks dipped in distilled water dilutions of each protein (25). At least three independent groups of larvae were tested against each protein (minimum sample size = 30). Mortality was recorded after five days and adjusted for mortality of larvae that ate control disks dipped in distilled water only. In all treatments, including distilled water controls, we added a surfactant (0.2% Triton A989 from Rohm & Haas).

**Binding Assays**—To determine whether loss of toxicity of some α4 mutants was caused by altered binding, we tested binding to BBMVs of Cry1Aas (positive control), four non-toxic mutant proteins, and Cry1E (a non-toxic negative control). Fourth instar P. xylostella larvae were frozen at −70 °C. Whole guts were removed from thawed larvae, placed in ice-cold buffer (300 mM mannitol, 5 mM EGTA, and 17 mM Tris-base, pH 7.5), and immediately frozen on dry ice. BBMVs were purified from approximately 1000 frozen guts by homogenization and subsequent magnesium precipitation (27). Final membrane pellets were resuspended by sonication in detergent-free HBSS (10 mM HEPES, pH 7.4, 150 mM NaCl) to a 1 mg/ml total protein concentration and stored at −80 °C. All protein concentrations were determined by the method of Bradford (28) using bovine serum albumin as a standard.

Binding was analyzed with surface plasma resonance using an optical biosensor (BIAcore 1000, Pharmacia Biosensor). Toxins were immobilized to activated carboxymethylated dextran on CM5 sensor chips by standard amine coupling (29). Toxins were dissolved as a 0.1 mg/ml stock solution in 20 mM ammonium acetate, pH 5. With one exception, we used 2300 to 2800 resonance unit (RU) surfaces of immobilized toxins. We used roughly double the amount of D136C to determine whether this increase would alter binding.

Because Bt toxins produce a biphasic event resulting in protoxin binding and membrane integration, we examined both the association and dissociation phases. To examine the association phase, we injected BBMVs (diluted 1:10 with HBS) over these surfaces for a 300-s period at 5 μl/min and recorded the increase in vesicle binding (in RU) over time. These conditions were chosen as they provided essentially surface saturating conditions on the sensor chip. After the initial 300 s, we examined the dissociation phase by injecting buffer (HBS) for 120 s. Information on toxin integration can also be observed during the dissociation phase. Toxins that do not recognize *P. xylostella* receptors (and are consequently non-toxic) will not integrate into the membrane. On a toxin-immobilized CM5 chip surface, this would cause poor cumulative association (binding) and rapid dissociation as the irreversible component (i.e., membrane integration) of the total BBMV binding would be eliminated. If a toxin binds to a receptor on the BBMV surface and subsequently integrates into the vesicular membrane, an extremely slow rate of dissociation is expected.

Binding curves were corrected for mass action by subtracting a control curve composed of injecting BBMVs over 2000 RU of immobilized bovine serum albumin. All injections of *P. xylostella* BBMVs contained bovine serum albumin (0.01% w/v) as a blocker of nonspecific binding. Surface regeneration conditions were as described previously (30).

**Ion Channel Formation and Charge Modification of Exposed Cysteine Residues in Planar Lipid Bilayers**—To determine whether loss of toxicity was caused by loss of ability to form ion channels and to determine the side of helix 4 that faces the lumen of the ion channel, we conducted experiments using the painted bilayer technique (9). PLBs are artificial membranes mimicking the natural phospholipid environment found in the natural membranes of the cells. The membranes were formed from a 7:2:1 lipid mixture of phosphatidylethanolamine, phosphatidylycholine, and cholesterol painted on a 250-μm circular aperture in a Delrin wall separating two low volume chambers (1.8 ml trans:1 ml cis). All experiments were performed at room temperature in solutions containing 150 mM KCl, 1 mM CaCl2, and buffered with 10 mM Tris, pH 9.5. Single channel currents were recorded with a BC-525C bilayer/patch-clamp amplifier (Warner Instruments, Hamden, CT). Toxin conductions were derived from the slopes of linear regression curves of single channel current data recorded for several voltages applied across the lipid bilayer (9). The probability of channels being open (NP), was obtained by dividing the sum of the total time open of N identical channels by the total recording time interval (31). Conductance data are given as means ± S.E.

To determine which side of α4 faces the lumen of the ion channel, we conducted SCAM experiments with D136C and N138C, mutants that have introduced cysteines on opposite sides of α4 (6). This powerful method has been used to map the channel-lining residues of several types of cellular ion channels (32), including the diphtheria bacterial toxin channel (33). We exploited the lack of cysteines in wild-type activated Cry1Aa by creating mutants that have introduced cysteines on opposite sides of α4 (6) (Fig. 1B). We probed accessibility of these two
individually introduced cysteines to MTSES (S67200, Toronto Research Chemicals, North York, Ontario, Canada), a reagent highly selective for cysteines. MTSES is a small molecule capable of passive diffusion into the channel lumen. If the sulfhydryl group of the cysteine is directed toward and exposed within the channel lumen, addition of MTSES should alter the channel conductance in situ by the covalent introduction of a negative charge. A 13 mM stock solution of MTSES was prepared by predissolving the lyophilized reagent in 50 μl of the bath buffer (10 mM, Tris pH 9.5, 150 mM KCl, 1 mM CaCl2) immediately before use. After formation of a lipid bilayer, electrical recording was performed for 20 min before protein addition. After injection of 0.1 mM activated protein near the membrane in the cis chamber, we monitored channel activity for another 20 min by recording step changes in the current required to hold test voltages across the PLB. With the holding potential set at −40 mV, 50 μl of MTSES stock solution were directly added to the cis chamber.

### RESULTS

**Toxicity**—Compared with wild-type Cry1Aa, mutant proteins with alterations at the charged amino acid Arg-127 (R127E, R127N) or the polar amino acid Asn-138 (N138C) showed little or no loss of toxicity to *P. xylostella* larvae (Table I). In contrast, mutant proteins with alterations of charged amino acids at Asp-136 (D136N, D136C), Arg-131, (R131D, R131Q), and Glu-129 (E129K) were not toxic.

| Mutant | Concentration | Mortality | R-group change |
|--------|---------------|-----------|----------------|
| Cry1Aa | 100 μg/ml | 95% | none |
| R127E | 100 | 98% | + to P |
| R127N | 100 | 8% | + to P |
| R131D | 100 | 0% | + to P |
| R131Q | 100 | 0% | + to P |
| E129K | 100 | 3% | + to P |
| E129K | 100 | 0% | + to P |
| D136N | 100 | 2% | + to P |
| D136C | 100 | 0% | + to P |
| N138C | 100 | 0% | + to P |

^a^ All mutants tested were protoxins unless otherwise indicated.  
^b^ Mortality after five days was adjusted for control mortality.  
^c^ + = positive; − = negative; P = polar.  
^d^ Purified trypsin-activated toxin.

**Binding**—As expected, Cry1Aa bound readily to BBMVs, and the non-toxic protein Cry1E did not (Fig. 2A). Non-toxic mutant proteins E129K and R131D showed patterns of association and dissociation that were virtually identical to those of Cry1Aa (Fig. 2A). These results imply that loss of binding was not responsible for the loss of toxicity of these two mutants.

Two other non-toxic mutants, D136C and R131Q, showed minor differences in association compared with the patterns observed for Cry1Aa (Fig. 2A). Both D136C and R131Q bound BBMVs at the same initial rate as Cry1Aa, yet their rate of binding decreased sooner than that of Cry1Aa. Although D136C and R131Q bound slightly lower levels of BBMVs and thus had a lower starting point in the dissociation phase, their rates of dissociation were similar to that of Cry1Aa. If the sulfhydryl group of the cysteine is directed toward and exposed within the channel lumen, addition of MTSES should alter the channel conductance in situ by the covalent introduction of a negative charge. A 13 mM stock solution of MTSES was prepared by predissolving the lyophilized reagent in 50 μl of the bath buffer (10 mM, Tris pH 9.5, 150 mM KCl, 1 mM CaCl2) immediately before use. After formation of a lipid bilayer, electrical recording was performed for 20 min before protein addition. After injection of 0.1 mM activated protein near the membrane in the cis chamber, we monitored channel activity for another 20 min by recording step changes in the current required to hold test voltages across the PLB. With the holding potential set at −40 mV, 50 μl of MTSES stock solution were directly added to the cis chamber.

| Mutant | Concentration | Mortality | R-group change |
|--------|---------------|-----------|----------------|
| Cry1Aa | 100 μg/ml | 95% | none |
| R127E | 100 | 98% | + to P |
| R127N | 100 | 8% | + to P |
| R131D | 100 | 0% | + to P |
| R131Q | 100 | 0% | + to P |
| E129K | 100 | 3% | + to P |
| E129K | 100 | 0% | + to P |
| D136N | 100 | 2% | + to P |
| D136C | 100 | 0% | + to P |
| N138C | 100 | 0% | + to P |

^a^ All mutants tested were protoxins unless otherwise indicated.  
^b^ Mortality after five days was adjusted for control mortality.  
^c^ + = positive; − = negative; P = polar.  
^d^ Purified trypsin-activated toxin.

**Ion Channel Formation and Charge Modification of Exposed Cysteine Residues in Planar Lipid Bilayers**—The ability to form functional ion channels was reduced or absent in four nontoxic mutants tested, but not in the toxic mutant N138C. Cry1Aa, the toxic mutant N138C, and the nontoxic mutant D136C formed ion channels at all voltages tested between −100 and +100 mV (Fig. 3). The conductance of toxic mutant N138C (481 ± 7.4 picosiemens, n = 5) did not differ from that of Cry1Aa (519 ± 19.9 picosiemens, n = 10), but the nontoxic mutant D136C had a considerably lower conductance (74 ± 14.9 pS, n = 4). With E129K, (Fig. 3, bottom trace), channel activity was only observed at high holding potentials (i.e. above 100 mV). Its conductance (16.3 ± 3.3 pS, n = 2) was 3% of that determined for Cry1Aa, and activity lasted only a few minutes. There was no channel activity observed with non-toxic mutant D136N (n = 3). Although toxic mutant N138C partitioned into the membrane as easily as Cry1Aa and had the same conductance, its probability of channels being open (NP_o) was 12% compared with 65% for Cry1Aa at −40 mV. NP_o for D136C was only 2.0%.

In SCAM experiments with D136C and N138C, addition of MTSES caused changes within minutes (Fig. 4). With D136C (Fig. 4, left, bottom), MTSES substantially increased the conductance of the channel (498.7 ± 36.4 pS, n = 4) and its NP_o,
Experiments were conducted under symmetrical conditions (150:150 mM Cry1Aa and helix 4 mutants D136N, D136C, N138C, and E129K. Exon channels formed by Cry1Aa.

Cry1Aa
-40 mV C
D136N
-40 mV C
D136C
-40 mV C
N138C
-140 mV C
E129K
20 pA 100ms

FIG. 3. Representative current recordings. Recordings are from Cry1Aa and helix 4 mutants D136N, D136C, N138C, and E129K. Experiments were conducted under symmetrical conditions (150:150 mM KCl cis:trans) with 0.1 μM protein. The applied voltage is indicated on top of each trace. The letter c on the right side of each trace indicates the closed state level of the channel.

DISCUSSION

Mutant proteins with alteration of three charged residues (Glu-129, Arg-131, and Asp-136) were not toxic, which shows that these three residues of helix 4 directly affect toxicity. Two of these charged residues (Glu-129 and Asp-136) occur on the same side of helix 4 (Fig. 1B). In contrast, mutants with alteration of a charged residue (Arg-127) or a polar residue (Asn-138) on the opposite side of helix 4 relative to Asp-136 retained full toxicity. The third nontoxic mutant, Arg-131, has an intermediate location between these two groups. Taken together, these results imply that within helix 4, some but not all charged residues are important for toxicity. Further, they suggest that the side of helix 4 containing Glu-129 and Asp-136 is important in toxicity.

Our results also suggest that loss of toxicity was caused by severely reduced ability to conduct ions through ion channels rather than disruption of binding or membrane integration. Reduced binding was not seen in two of the four non-toxic mutants examined (E129K and R131D); the other two (R131Q and D136C) showed minor changes in the associative phase only. Further, none of the non-toxic mutants showed major alterations in dissociation rate patterns, which suggests that they readily integrated into the BBMVs. These results differ from those of Chen et al. (34) who concluded that two domain I mutants, which replaced a polar or a hydrophobic residue with a negatively charged residue, lacked toxicity because of their inability to integrate into BBMVs. The mutations studied by Chen et al. (34) are not in α4 but rather in the interhelical loop region between α4 and α5 (Y153D) and the N terminus of α3 (A92E).

Results using three non-toxic mutants (E129K, D136C, D136N) in PLBs show that alteration of these charged residues either prevented ion channel formation or greatly reduced conductance. Consistent with these results, the non-toxic positively charged mutant R131Q also had a reduced conductance (approximately 250 pS, data not shown). These results suggest that Glu-129 and Asp-136 face the channel lumen and are directly involved in controlling the movement of ions through the channel. Addition in situ of the negatively charged MTSES functional group to the single cysteine introduced in D136C restored conductance to levels approaching that of Cry1Aa, which supports the hypothesis that Asp-136 faces the lumen of the ion channel and is directly involved in ion flow. Moreover, the size of the negatively charged side chain differs between Asp-136 and D136C treated with MTSES, which suggests that it is the negative charge and not the side chain per se that regulates ion flow.

Unlike the non-toxic mutants described above, mutant N138C retained both wild-type toxicity and conductance. Thus, we infer that this residue is not directly involved in ion flow. Further, structural models show that Asn-138 is on the side of helix 4 opposite from D136C (Fig. 1B). MTSES did not affect conductance of N138C, which is not surprising, because conductance of this mutant toxin without MTSES was similar to conductance of Cry1Aa. Although NPo was not correlated with toxicity, MTSES unexpectedly increased NPo for N138C as well as for D136C, suggesting both residues were accessible to MTSES.

From a different perspective, if α4 is the only helix lining the channel with the more hydrophobic α5 being anchored into the lipid membrane, Asn-138 would naturally face toward α5 (Fig. 1B), as opposed to the channel lumen. This configuration would
account for its insensitivity to both conductance and toxicity changes after mutation but yet remain accessible to the alkali-lating reagent. Further experimentation will show if α5 serves solely as a lipid anchor or plays a more direct role in ion flow. A similar topology to that described above has recently been proposed for the members of one of the two classes of tetrameric K⁺ channels in which each subunit contributes two membrane-spanning helices. These helices are arranged in the membrane such that an inner helix faces the channel lumen while an outer helix faces the lipid environment (35). Despite accessibility of N138C to MTSES and considering that conductance (i.e., the rate of ion flow) depends on the residues lining the lumen of the ion channel, the conclusion that Asn-138, unlike Asp-136, does not face the lumen of the ion channel is supported by the toxicity of N138C, the normal conductance of N138C, and the position of this residue in α4.

Comparisons with other Cry1 toxins suggest that negatively charged residues are essential in helix 4. A sequence alignment (36) shows that the 12 Cry1 toxins examined have a negatively charged residue in the middle of α4 (Fig. 1) either at position 136 (Cry1Aa, Cry1Ab, Cry1Ac, Cry1D, and Cry1E) or two residues downstream (Cry1B, Cry1C, Cry1F, Cry1G, Cry1H, Cry1J, and Cry1K). It is important to note that the crystal structures for Cry1 toxins other than Cry1Aa are unknown, therefore it remains to be determined whether the charged residues of the latter group are oriented on the side of α4 furthest from α5 (like Asp-136). Structural models suggest that in all cases except Cry1H, at least two negatively charged residues occur on the same helical face, as seen in α4 of Cry1Aa. Further experimentation using Cry1H and other toxins should reveal the roles and relative importance of negatively charged residues in different positions in helix 4.

Using the results reported here in conjunction with previous reports (1, 6, 13, 15, 18, 19), we propose an umbrella model with further refinements (Fig. 5): domain I swings away from the main body of the toxin (domains II/III). The most hydrophobic region of domain I, the C terminus of α4, and the N terminus of α5 (6) unfolds and traverses the membrane as a hairpin with the other helices spreading over the surface. The inserted hairpins of four toxin molecules aggregate so that the hydrophilic faces of four α4 helices form the lumen of the pore. The α4 helices are aligned so that the negatively charged side chains of Glu-129 and Asp-136 extend into the channel lumen forming two concentric rings of negative charges. Our proposed tetrameric structure represents the minimal configuration for a functional Bt ion channel. Channel size increases with time because of further subunit aggregation of toxin molecules with the initial tetramer.

In summary, our results provide some new insights into the architecture of Cry toxin ion channels and the ion pathway. A more detailed structure of the channel will require extensive cysteine mutagenesis and thiol-reactive reagent probing of both α4 and α5. As understanding of the mode of action of Bt toxins improves, so too will the ability to design toxins to increase toxicity and to combat evolution of resistance by pests (37, 38).

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