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Chemical modification of *Bacillus thuringiensis* Cry1Aa toxin single-cysteine mutants reveals the importance of domain I structural elements in the mechanism of pore formation

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ABSTRACT

Bacillus thuringiensis Cry toxins form pores in the apical membrane of insect larval midgut cells. To investigate their mechanism of membrane insertion, mutants in which cysteine replaced individual amino acids located within the pore-forming domain of Cry1Aa were chemically modified with sulfhydryl-specific reagents. The thiol group of cysteine was highly susceptible to oxidation and its reactivity was significantly increased when the toxins were purified under reducing conditions. Addition of a biotin group to the cysteine had little effect on the ability of the toxins to permeabilize *Manduca sexta* brush border membrane vesicles except for a slight reduction in activity for S252C and a large increase in activity for Y153C. The activity of Y153C was also significantly increased after modification by reagents that added an aromatic or a charged group to the cysteine. When permeability assays were performed in the presence of streptavidin, a large biotin-binding protein, the pore-forming activity of several mutants, including Y153C, where the altered residue is located within the hairpin comprising helices α4 and α5, or in adjacent loops, was significantly reduced. These results support the umbrella model of toxin insertion.

1. Introduction

Bacillus thuringiensis produces a variety of proteins with insecticidal activity [1,2]. These include the Cyt and Cry proteins, which are most often produced as crystalline inclusions during sporulation, and the Vip proteins, which are secreted during the vegetative phase of growth. Cyt proteins are specifically toxic to certain species of Diptera in vivo and cytolytic to most cultured cells in vitro. Cry toxins form a large family of proteins that are collectively active against insects of several orders and other invertebrate species. Although the mode of action of all these toxins has not been fully elucidated, members of each family appear to interact with the apical membrane of the intestinal epithelium of susceptible insects.

In the case of the three-domain Cry toxins, the protein first binds to specific receptors located at the surface of the intestinal membrane. This interaction is mediated by elements of domains II and III, mainly composed of β-sheets [3,4]. Receptor binding triggers the insertion of the toxin into the membrane and the formation of large pores that abolish vital transmembrane ionic gradients. These pores allow the diffusion of a wide variety of charged and uncharged solutes, but display a slight cationic selectivity [5]. Their formation is attributable to domain I, composed of six amphipathic α-helices surrounding a hydrophobic helix (α5) [6–8].

During the course of studies aimed at evaluating the contribution of different domain I structural elements in the mechanism of pore formation, a large number of mutant Cry1Aa toxins have been created in which a single, strategically located amino acid was replaced by cysteine. The altered residues include charged amino acids of α3 [9], numerous amino acids located in the inter-helical loops of domain I [9,10] and almost all residues of α4, the helix that is thought to line the pore lumen [11–13]. The activity of these mutants, analyzed with an osmotic swelling assay based on scattered light intensity measurements, varied considerably. Although most α3 and domain I loop mutants showed a near wild-type activity at pH 7.5, several of them were less efficient than Cry1Aa when tested at pH 10.5 [9,10]. On the other hand, most α4 mutants were considerably less active than the unaltered Cry1Aa toxin, especially when the substituted amino acid was located on the hydrophilic face of the helix [11–13].

In the present study, we have taken advantage of the unique chemical properties of the thiol group of cysteine and of the fact that this amino acid is absent from the wild-type activated Cry1Aa toxin [7]. The toxins selected for these experiments include those inter-helical loop mutants that were toxic and displayed a pore-forming activity comparable to that of Cry1Aa at pH 7.5 and that could be produced in sufficient quantities [9,10], as well as R127C, the only α4 cysteine mutant

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with wild-type activity in which the altered residue was hydrophilic and surface-exposed [11–13]. The effects of thiol-specific reagents on the pore-forming activity of these mutants was only evident when the mutated amino acid was part of the α4–α5 hairpin, or of the adjacent α3–α4 and α5–α6 loops, indicating that these structural elements play a major role in the mechanism of pore formation.

2. Materials and methods

2.1. Mutagenesis and toxin preparation

The Cry1Aa cysteine mutants used in this study were prepared in *Escherichia coli* with the double oligonucleotide site-directed mutagenesis method [14] and the pMP39 plasmid [15] except for W219C which was prepared in the *E. coli–B. thuringiensis* shuttle vector pBA1 [16]. Their pore-forming properties have been reported previously [9–13]. Toxins were produced in *E. coli*, or *B. thuringiensis* for W219C, activated with trypsin, and purified by fast protein liquid chromatography with a Mono-Q anion exchange column as described previously [15,17]. For most experiments, however, the purification was performed under reducing conditions to minimize the oxidation of the thiol group of cysteine. This was done by adding 2 mM EDTA as soon as the bacterial growth was completed and during all subsequent steps, and 5 mM dithiothreitol during all purification steps following the incubation with trypsin. Each toxin was concentrated by ultrafiltration with 30-kDa cutoff Amicon regenerated cellulose membranes (Millipore, Bedford, MA) after purification. In preparation for the experiments, EDTA and dithiothreitol were removed by dialysis against buffer containing 100 mM KCl and 10 mM HEPES–KOH, pH 7.5, from which oxygen had been removed by extensive bubbling of nitrogen gas through the solution. Toxin samples were then stored in nitrogen-flushed sealed vials until use.

2.2. Chemical modification

Chemical modification with N-biotinylaminoethyl methanethiosulfonate (MTSEA-biotin) was performed on each of the mutants in the buffer used for dialysis. Depending on the mutant, the reaction mixture contained 260 to 1570 μg of toxin per ml and the reagent in a toxin:MTSEA-biotin molar ratio of 1:10. The toxins thus modified were used for osmotic swelling experiments after having been incubated for at least an hour with or without 2 μg of streptavidin (Sigma-Aldrich, Oakville, Ont.) per μg of toxin. Other reagents, 2-aminoethyl methanethiosulfonate (MTSEA), (2-sulfonatoethyl) methanethiosulfonate (MTSES), 4-(hydroxymethyl)benzyl methanethiosulfonate (MTSHMB) were also used with mutant Y153C at the same molar ratio as was used for MTSEA-biotin. All methanethiosulfonate reagents were obtained from Toronto Research Chemicals (Toronto, Ont.) and all reactions were carried out at room temperature for at least 1 h.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% acrylamide gels under standard conditions [18]. Each toxin (3 μg per well) was diluted in sample buffer (final concentrations of 50 mM Tris–HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) sodium dodecyl sulfate, and 0.1% (w/v) bromophenol blue) in the presence or absence of 100 mM dithiothreitol and boiled for 5 min before electrophoresis. Gels were either stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL) following the manufacturer's instructions, or used for western blot analysis (section 2.4).

2.4. Western blot

The reactivity of the cysteine thiol group of each mutant protein was analyzed by western blot. Each mutant (100 ng) was first modified with MTSEA-biotin as described in section 2.2. After electrophoretic migration in a 10%-acrylamide sodium dodecyl sulfate-polyacrylamide gel in the presence or absence of dithiothreitol, the proteins were transferred onto a Immobilon-P polyvinylidene fluoride membrane (Millipore) in an EC140 mini blot module transfer cassette (E-C Apparatus, Milford, MA) under a 200 V potential difference for 2 h. The buffer (25 mM Tris–HCl, pH 8.3, 192 mM glycine and 10% (v/v) methanol) was maintained at 4 °C throughout the transfer. Non-specific binding sites were blocked with a solution containing 20 mM Tris–HCl, pH 8.0, 250 mM NaCl, 2.5% (w/v) bovine serum albumin and 0.1% (w/v) Tween-20. The membrane was then incubated with 5 μg of horseradish peroxidase-conjugated streptavidin (Pierce) in 10 ml of TGG buffer (0.5% (w/v) Tween-20, 10% (w/v) glycerol, 1 M glucose, 250 mM NaCl, and 100 mM sodium phosphate, pH 7.2) during 1 h at room temperature. The membrane was finally rinsed four times for 10 min with a buffer containing 20 mM Tris–HCl, pH 7.6, 137 mM NaCl and 0.1% (w/v) Tween-20. Labeled proteins were detected by enhanced chemiluminescence using the reagents from Amersham (Uppsala, Sweden) according to the manufacturer's instructions.

2.5. Preparation of brush border membrane vesicles

Fertilized eggs of *M. sexta* were purchased from the North Carolina State University Entomology Department insectary (Raleigh, NC) and larvae were raised on a standard artificial diet supplied with the eggs. Brush border membrane vesicles were prepared from whole midgut homogenates with a procedure involving magnesium precipitation and differential centrifugation [19].

2.6. Osmotic swelling assay

Kinetics of pore formation in *M. sexta* brush border membrane vesicles were analyzed with an osmotic swelling assay based on scattered light intensity measurements [20]. Vesicles (0.4 mg membrane protein per ml) suspended in 10 mM HEPES–KOH, pH 7.5, and 1 mg/ml bovine serum albumin were rapidly mixed with an equal volume of a solution containing 150 pmol of the desired toxin per mg of membrane protein, 150 mM KCl, 10 mM HEPES–KOH, pH 7.5, and 1 mg/ml bovine serum albumin using a stopped-flow apparatus (Hi-Tech Scientific, Salisbury, UK). Volume changes were monitored at room temperature by measuring the intensity of scattered light at an angle of 90° from the incident beam at a wavelength of 450 nm with a photomultiplier tube in a Photon Technology International (South Brunswick, NJ) spectrofluorometer.

Data were recorded every 0.1 s for 6 min and normalized relative to the maximum value obtained for control vesicles in the absence of toxin. Scattered light intensity is an inverse function of vesicle volume and percent volume recovery was defined as $100\,(1-I_t)$, where I_t is the relative scattered light intensity at a given time t. Volume recovery values were calculated for every experimental point and control values obtained in the absence of toxin were subtracted from those measured in the presence of toxin.

3. Results and discussion

The goal of the present study was to investigate the effects of sulfhydryl-specific reagents on the pore-forming properties of selected Cry1Aa cysteine mutants. In initial experiments, however, these effects appeared very modest for all mutants tested and tended to become gradually less evident as the samples were stored for long periods before they were chemically modified (data not shown). It was therefore suspected that the reactivity of our mutants could be lowered because their cysteine residues were being converted to cysteic acid derivatives. The thiol group of cysteine is indeed susceptible to oxidation to sulfenic (–SOH), sulfinic ($-SO_2H$) and

sulfonic (–SO_3H) acids [21–23]. These derivatives cannot react with thiol-specific reagents, and their formation, in addition to being very poorly reversible, is favored by several factors which are part of the conditions generally used for the purification of *B. thuringiensis* Cry toxins. These factors include exposure to molecular oxygen, due to extensive vortexing of the samples, the presence of metallic cations which catalyze the reaction, and high pH [21–23]. To minimize oxidation of the thiol group, all mutants were therefore produced in the presence of a chelating agent, EDTA, and a reducing agent, dithiothreitol, under the conditions described in section 2.2. The latter compound was added only after the activation step as it interferes with the catalytic activity of trypsin.

Mutant reactivity was analyzed by western blotting after chemical modification with MTSEA-biotin. Following migration on polyacrylamide gels in the absence of dithiothreitol and transfer to polyvinylidene fluoride membranes, biotinylated proteins were labeled with horseradish peroxidase-conjugated streptavidin and detected by chemiluminescence (Fig. 1A). All mutants prepared under reducing conditions were labeled with MTSEA-biotin although the level of their reactivity varied. P121C, N123C and R127C displayed the most intense signal, but T122C, Y153C, W219C and S252C also showed a strong reactivity. On the other hand, F50C, D120C, Q151C and W182C were less intensely labeled than the other toxins. These differences may be the result of lower cysteine accessibility to the solvent, and hence to the reagent, or to a higher level of oxidation in those mutants with lower reactivity. All reactions could be reversed by the addition of dithiothreitol in the sample buffer, indicating that the labeling resulted specifically from the formation of a disulfide bond between cysteine and the methanethiosulfonate reagent (Fig. 1B). When similar experiments were carried with mutants produced under our usual conditions [15,17], their reactivity was considerably weaker than when they were produced under reducing conditions (data not shown). Toxins prepared with the new procedure were therefore used in all subsequent experiments.

The ability of each mutant to increase the permeability of *M. sexta* midgut brush border membrane vesicles was tested at pH 7.5 using an osmotic swelling assay (Fig. 2). All mutants permeabilized the membrane at a rate which was comparable to that observed with wild-type Cry1Aa (Fig. 2A), except for Q151C (Fig. 2H) which was inactive, and for Y153C (Fig. 2I) and W182C (Fig. 2J) which were less active than the others. The activity of D120C (Fig. 2C) [9], P121C (Fig. 2D), T122C (Fig. 2E), N123C (Fig. 2F) and W219C (Fig. 2K) [10], as well as R127C (Fig. 2G) [13] was similar to that reported earlier for the same mutants produced under standard conditions. However, F50C (Fig. 2B) and S252C (Fig. 2L), produced under reducing conditions, were significantly more active than previously reported [10]. This difference was particularly evident for S252C which was as active as wild-type Cry1Aa in the present study. Because the substitution of serine by a cysteine residue is rather conservative, this mutation could be expected to have little or no effect on the activity of the toxin. This was only observed when the toxin was produced under reducing conditions however, and the lower activity reported previously [10] is likely explained by the presence of oxidized species when it is produced under standard conditions. Although the oxidation of the thiol group modifies only slightly the size of the residue, it introduces a negative charge that could reduce toxin activity. While the substitution of phenylalanine by cysteine is much less conservative, the lower activity of F50C (Fig. 2B), when produced under standard conditions, could also be due to the oxidation of its thiol group.

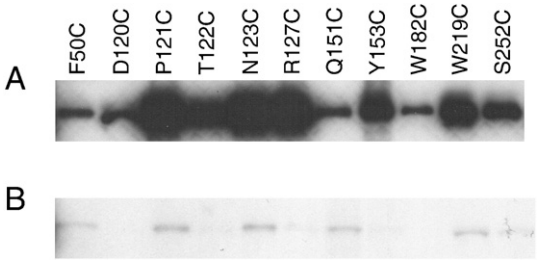

Fig. 1. Chemical reactivity of Cry1Aa domain I cysteine mutants. Toxins were first incubated with a ten-fold excess of MTSEA-biotin for at least 1 h. Following migration on 10%-acrylamide sodium dodecyl sulfate-polyacrylamide gels in the absence (A) or presence (B) of dithiothreitol, 100 ng of each mutant was transferred onto a polyvinylidene fluoride membrane. Biotinylated proteins were labeled with streptavidin-conjugated horseradish peroxidase and revealed by enhanced chemiluminescence.

In contrast, Q151C produced under reducing conditions was almost completely inactive (Fig. 2H) despite the fact that it had a readily detectable activity, albeit reduced relative to that of wild-type Cry1Aa, when produced under standard conditions [10]. Because dithiothreitol, present during the purification steps, was removed before the experiments, a possible explanation for the inability of Q151C permeabilize the membrane is the formation of a disulfide bridge between two toxin molecules as was observed previously for other Cry1Aa cysteine mutants [24]. The presence of a disulfide bond was demonstrated by re-incubating the toxin with dithiothreitol (Fig. 3). Under these conditions, the ability of mutant Q151C to permeabilize the vesicles was restored to a level which was even superior to that observed when the protein was produced under standard conditions [10]. The possibility that homo-dimers were formed between toxin molecules was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence and absence of dithiothreitol (Fig. 4). Only Q151C and W182C formed detectable amounts of dimers (Fig. 4A) which dissociated in the presence of the reducing agent (Fig. 4B). For both mutants, however, the proportion of dimers appears insufficient to fully account for their rather small activity when compared with the wild-type toxin (Fig. 2). Because these mutants were among those that were biotinylated less efficiently (Fig. 1), the possibility that they were partially oxidized before the chemical modification could explain their limited reactivity and contribute, along with the formation of homo-dimers, to their modest pore-forming activity. This explanation cannot be extended to all mutants that were poorly labeled, however, since F50C and D120C formed pores as efficiently as wild-type Cry1Aa despite the fact that their reactivity was comparable to that of Q151C and W182C. The consequences of the presence of a cysteic acid residue on pore formation could therefore vary considerably depending on its position within the structure of the toxin.

Except for Q151C, which was inactive, all mutants were incubated with a ten-fold excess of MTSEA-biotin for at least 1 h and their activity was subsequently tested with the osmotic swelling assay (Fig. 2). Except for Y153C and S252C, this chemical modification had no significant effect on the ability of the toxin to alter the permeability of the vesicles. The activity of S252C was slightly reduced (Fig. 2L), but the effect of Y153C on membrane permeability was strongly increased when the toxin was biotinylated (Fig. 2I). The presence of a strategically located biotin group on each of the mutants was further exploited by repeating the osmotic swelling experiments after incubating the biotinylated toxins with streptavidin, a 60-kDa protein which binds to biotin non-covalently, but with high affinity [25]. The presence of streptavidin had no significant effect on the pore-forming activity of F50C, D120C, T122C, W219C and S252C (Fig. 2B, C, E, K, L), but considerably reduced that of the remaining mutants, P121C, N123C, R127C, Y153C and W182C (Fig. 2D, F, G, I, J). Interestingly, in this latter group of mutants, the altered residues are located in the loop linking helices α3 and α4 (P121C and N123C), near the N-terminal extremity of helix α4 (R127C), in the loop linking helices α4 and α5 (Y153C), or in the loop linking helices α5 and α6 (W182C) (Fig. 6). These residues are thus located near or within the hairpin composed of helices α4 and α5, a region thought to be involved in toxin insertion according to the umbrella model [26–29]. In agreement with this model, binding of streptavidin to a residue located within the α4–α5 loop could prevent the hairpin from interacting with the membrane, and binding of this large protein to the loops located at the N-terminal end of α4 or at the

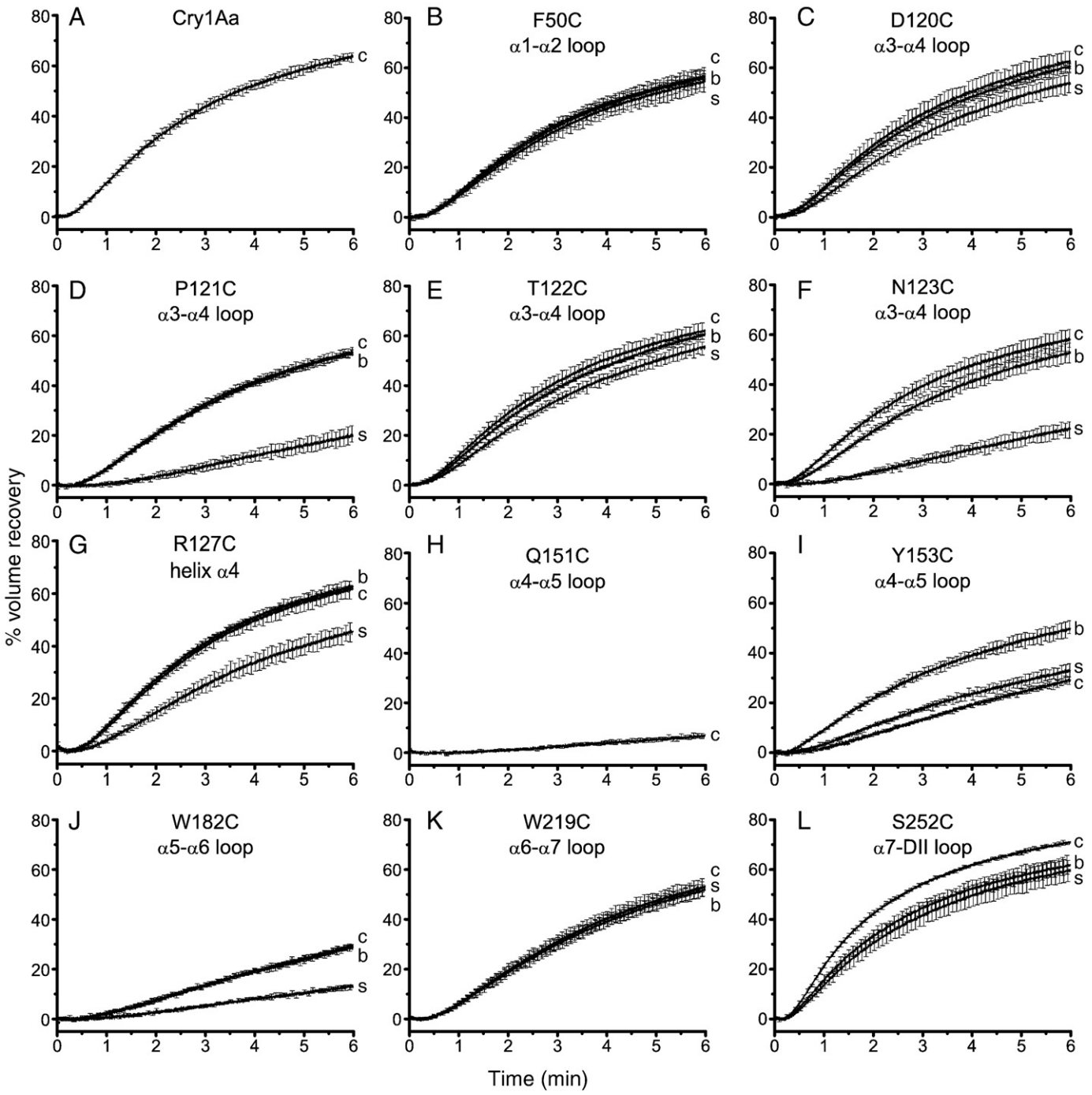

Fig. 2. Effect of chemical modification on the pore-forming activity of domain I cysteine mutants. Mutant toxins were tested after treatment with MTSEA-biotin (b) and subsequent incubation with streptavidin (s) or without chemical modification (c). Vesicles (0.4 mg membrane protein/ml) isolated from 5th-instar larvae 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. 150 pmol of mutant toxin/mg of membrane protein were added to a solution containing 150 mM KCl, 1 mg/ml bovine serum albumin and 10 mM HEPES/KOH, pH 7.5. The vesicles were then rapidly mixed with an equal volume of the KCl solution 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 Photon Technology International spectrofluorometer. Percent volume recovery was calculated for every experimental point and values obtained for control vesicles, assayed without added toxin, were subtracted from those obtained in the presence of toxin. For clarity, error bars representing the standard error about the mean of three independent experiments are shown for every 50th experimental point.

C-terminal end of α5 could hinder the conformational change that allows these two helices to move away from the other domain I helices before inserting into the membrane.

D120C and T122C did not show a similar reduction in activity even though their altered residue is located in the same loop as those of P121C and N123C (Fig. 6). In the case of D120C, this lack of effect could possibly be related to its rather modest reactivity with MTSEA-biotin noted above (Fig. 1). As for T122C, its biotin group appears to be less readily accessible to the bulky streptavidin molecule in the intact protein (Fig. 2E) than after being denatured for the western blot experiment (Fig. 1). It remains possible that the lack of any streptavidin effect on F50C activity could be due to the latter's low level of biotinylation. However, a clear reduction in activity was observed for W182C even though both mutants displayed a similar reactivity (Fig. 1). The lack of any streptavidin effect on the activity of F50C, W219C and S252C could therefore be due to poor accessibility of the biotin group or to the

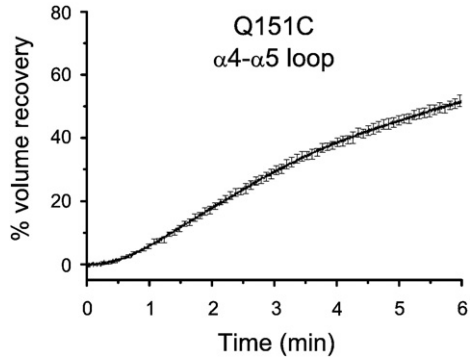

Fig. 3. Effect of dithiothreitol on the formation of pores by mutant Q151C. Experiments were conducted as described in the legend of Fig. 2 except that the toxin was incubated with 5 mM dithiothreitol and 2 mM EDTA for at least 1 h before the beginning of the osmotic swelling assay.

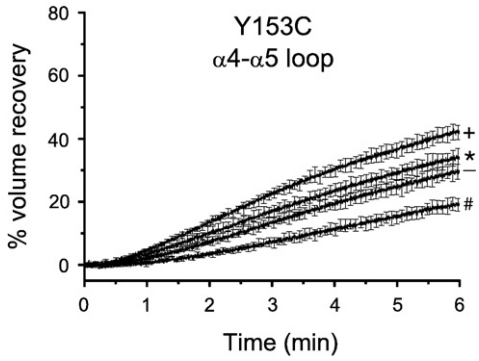

Fig. 5. Chemical modification of mutant Y153C. Experiments were performed as described in the legend of Fig. 2 except that the toxin was either modified with MTSEA (+), MTSES (−) or MTSHMB (*) for at least 1 h, or not modified (#), before the beginning of the osmotic swelling assay.

possibility that streptavidin binding to their biotinylated residue does not alter the function of the toxin. This latter hypothesis appears more likely, however, in the light of the fact that these mutated residues are surface-exposed in the crystal structure of Cry1Aa [7].

The reduced activity of Y153C relative to wild-type Cry1Aa may suggest that the presence of an aromatic residue in position 153 is important for toxin insertion since aromatic amino acids are thought to facilitate the translocation of protein segments through biological membranes and to contribute to the anchoring of membrane proteins by interacting with phospholipid head groups at the water–lipid interface [30]. In agreement with this hypothesis, the Cry1Ab mutants Y153A, Y153R and Y153D [31], as well as Y153C [32], also display reduced pore-forming activity, although the reduction is more pronounced for Y153D than for the other mutants. Furthermore, substitution of the structurally equivalent tyrosine at position 202 of the more distantly related toxin Cry4Aa by alanine or cysteine resulted in an almost complete loss of toxicity, but the Y202F mutant was only slightly less active than the wild-type toxin [33].

In this context, the two-membered heterocyclic moiety of biotin could possibly interact with the membrane in a manner similar to that of the side chain of aromatic residues and thus stimulate pore formation by Y153C. This hypothesis and the importance of tyrosine at position 153 were tested by modifying this mutant with MTSHMB, a reagent that adds a 4-(hydroxymethyl)benzyl group, similar to the side chain of tyrosine, on the cysteine residue via a disulfide bond. This treatment indeed stimulated the activity of the toxin (Fig. 5). Pore formation by Y153C was also increased, however, after chemical modification with MTSES and MTSEA which adds a negative and a positive charge, respectively, on the cysteine (Fig. 5). It should nevertheless be pointed out that charged residues are often found on membrane proteins at the water–lipid interface and may contribute to anchoring the protein in the membrane [30]. None of the chemical modifications restored toxin activity to a wild-type level, possibly because the added side chains were all considerably longer than that of the tyrosine residue normally present at this position.

In conclusion, steric constraints introduced by biotinylation of single cysteine residues followed by streptavidin binding interfered with pore formation when the introduced cysteine was located within the α4–α5 hairpin, or in the adjacent α3–α4 and α5–α6 loops, but had no significant effect when it was located elsewhere in domain I (Fig. 6).

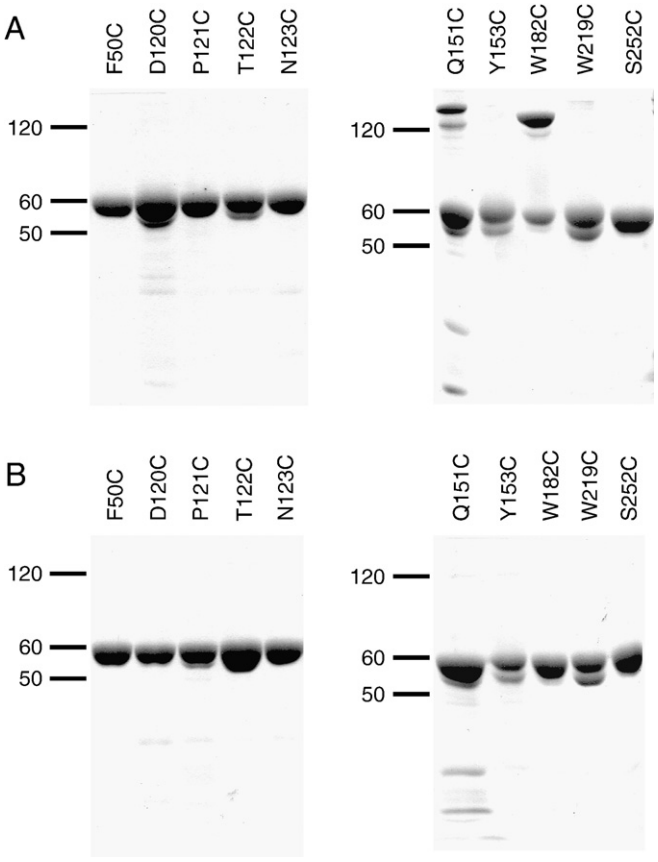

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Cry1Aa domain I cysteine mutants in the absence (A) or presence (B) of dithiothreitol. Each well contained 3 μg of the indicated toxin.

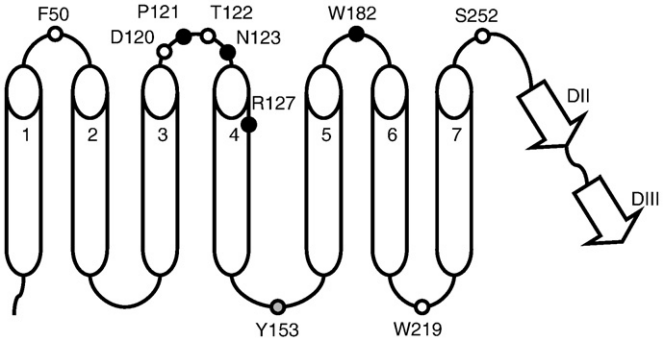

Fig. 6. Summary of results. The toxin structure is represented schematically with emphasis on Domain I. Helices and domains (D), as well as the position of each mutated residue that was chemically modified, are identified by their respective numbers. The activity of mutants for which the substituted amino acid is identified with a black circle was strongly inhibited in the presence of streptavidin, whereas those identified with a white circle were not affected. The activity of mutant Y153C, identified with a grey circle, was increased by the biotinylation, but this stimulation was abolished by the subsequent addition of streptavidin.

The activity of Y153C, in which the mutated amino acid is located at the tip of the α4–α5 hairpin, was stimulated when it was modified with reagents that added a biotin, a positive or negative charge, or an aromatic group that resembled the tyrosine side chain normally present at this location (Fig. 6). Taken together, these results stress the importance of these domain I structural elements and provide further support for the umbrella model of toxin insertion [26–29].

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