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N-Acetylgalactosamine on the Putative Insect Receptor Aminopeptidase N is Recognised by a Site on the Domain III Lectin-like Fold of a *Bacillus thuringiensis* Insecticidal Toxin

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Binding of the insecticidal *Bacillus thuringiensis* Cry1Ac toxin to the putative receptor aminopeptidase N is specifically inhibited by *N*-acetylgalactosamine (GalNAc), suggesting that this toxin recognises GalNAc on the receptor. A possible structural basis for involvement of domain III of the toxin in carbohydrate-mediated receptor recognition was noted in the similarity between the domain III fold of the related toxin Cry3A and a carbohydrate-binding domain in the 1,4-β-glucanase from *Cellulomonas fimi*. This possibility was investigated by making selected mutations in domain III of the Cry1Ac δ-endotoxin. Mutagenesis of residues Asn506, Gln509 or Tyr513 resulted in toxins with reduced binding and a slower rate of pore formation in *Manduca sexta* midgut membrane vesicles compared to the wild-type Cry1Ac. These mutants also showed reduced binding to the 120 kDa Cry1Ac putative receptor aminopeptidase N. Unlike the wild-type toxin, binding of the triple mutant N506D,Q509E,Y513A (Tmut) to *M. sexta* midgut membrane vesicles could not be inhibited by GalNAc. These data indicate that GalNAc binding is located on domain III of Cry1Ac and therefore support a lectin-like role for this domain. A preliminary analysis of the Cry1Ac crystal structure locates Asn506, Gln509 and Tyr513 in a region on and adjacent to β-16 in domain III, which has a unique conformation compared to the other known Cry structures. These residues are in a favourable position to interact with either soluble or protein-bound carbohydrate.

Keywords: Bacillus thuringiensis toxin; Cry1Ac domain III; site-directed mutagenesis; carbohydrate-binding; aminopeptidase N

Introduction

Bacillus thuringiensis (Bt) Cry δ-endotoxins are highly specific protein insecticides synthesised during sporulation as protoxin crystalline inclusions. (For a recent review, see Schnepf *et al.*, 1998.) Cry toxins are referred to here according to the revised nomenclature (Crickmore *et al.*, 1998). After ingestion by a susceptible insect, the crystals are solubilised in the gut where midgut proteases process the protoxin into a stable 65 kDa active toxin (Huber & Lüthy, 1981). The toxin is capable of binding reversibly to high-affinity receptors on the midgut epithelium (Hofmann *et al.*, 1988; Van Rie *et al.*, 1989). One such putative receptor for Cry1Ac in *Manduca sexta* has been cloned and identified as a 120 kDa aminopeptidase N (Knight *et al.*, 1994, 1995). Interestingly, Cry1Ac binding to aminopeptidase N is specifically inhibited by *N*-acetylgalactosamine (GalNAc), but not by *N*-acetylglucosamine (GlcNAc) with identical charge and size (Knight *et al.*, 1994), suggesting that Cry1Ac recognises GalNAc on the aminopeptidase N. The toxin-receptor interaction very rapidly becomes irreversible as the toxin inserts into the epithelial membrane to form a 1-2 nm diameter pore (Liang *et al.*, 1995). This leads to cell

Abbreviations used: Bt, *Bacillus thuringiensis*; BBMV, brush border membrane vesicles; BSA, bovine serum albumin; Tmut, N506D,Q509E,Y513A; CBD_{N1}, N-terminal cellulose-binding domain from *Cellulomonas fimi* 1,4-β-glucanase CenC.

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death by colloid osmotic lysis, eventually followed by insect death (Knowles, 1994).

The X-ray structures of two Cry toxins, Cry3A (PDB code 1DLC) and Cry1Aa (PDB code 1CIY), show them to consist of three distinct domains (Li *et al.*, 1991; Grochulski *et al.*, 1995). Of these, the seven α-helix structure of domain I is a candidate for the irreversible binding and membrane insertion step (Li *et al.*, 1991). Mutagenesis studies on the domain II hypervariable loop regions have shown their involvement in receptor binding (Dean *et al.*, 1996; Smedley & Ellar, 1996). This is further supported by reports that domain II shares a similar structural fold to four carbohydrate-binding proteins: the vitelline membrane outer layer protein I from hen's eggs (Shimizu *et al.*, 1994; PDB code 1VMO), the plant lectin jacalin (Sankaranarayanan *et al.*, 1996; PDB code 1JAC), the *Maclura pomifera* agglutinin (Lee *et al.*, 1998; PDB code 1JOT) and the KM+ lectin from *Artocarpus integrifolia* (Rosa *et al.*, 1999). However, until now, the function of the β-jelly roll structure of domain III has remained speculative. Suggested roles have included proteolytic protection (Li *et al.*, 1991) ion channel conductance (Chen *et al.*, 1993), and as a determinant of insect specificity (de Maagd *et al.*, 1996a).

The role of domain III in receptor binding has been explored by several groups and with different toxins. In studies using recombinant proteins produced by domain swapping (Lee *et al.*, 1995; de Maagd *et al.*, 1996a,b) data from ligand blots indicated that binding and toxicity follow the movement of the corresponding domain III (de Maagd *et al.*, 1996a,b). Two investigations have shown that the binding of Cry1Ac to two 120 kDa putative receptors in the midgut epithelium of *Lymantria dispar* and *M. sexta* is mediated by domain III (Lee *et al.*, 1995; de Maagd *et al.*, 1996b). A possible structural correlation with this involvement of domain III in receptor binding may be found in the similarity of the Cry3A domain III fold and the N-terminal cellulose-binding domain from the bacterium *Cellulomonas fimi* 1,4-β-glucanase CenC (CBD_{N1}; Johnson *et al.*, 1996), as shown in Figure 1, taken from Murzin & Bateman (1997). The carbohydrate-binding region of CBD_{N1} is thought to lie in a five-stranded cleft (Johnson *et al.*, 1996), which corresponds to the outer sheet of domain III, consisting of β-strands 13b, 16, 18, 19 and 22 (Murzin & Bateman, 1997). However, in the legume lectins, the amino acid residues responsible for binding carbohydrate are situated on loops connecting the β-strands (Lis & Sharon, 1998).

The precise orientation of carbohydrate hydroxyl groups is important in the lectin-ligand interaction (Weis & Drickamer, 1996). Hence, the presence of an axial 4-hydroxyl group in GalNAc could be responsible for its dramatic ability to inhibit Cry1Ac binding to aminopeptidase N, whereas GlcNAc, which has no effect, contains an equatorial 4-hydroxyl group. In other systems, carbohydrate binding selectivity can be altered by changing a limited number of residues in the protein lectin (Drickamer, 1997). For example, three single amino acid substitutions plus a five residue insertion were sufficient to change a highly specific mannose-binding protein into one that bound galactose with significant affinity (Iobst & Drickamer, 1994). By analogy, it should be possible to locate which, if any, amino acid residues in the Cry1Ac domain III bind to GalNAc, and hence to aminopeptidase N. Here, we demonstrate that by altering specific amino acid residues in the outer sheet of Cry1Ac domain III, the ability of GalNAc to inhibit binding to aminopeptidase N is lost. Because results from a preliminary analysis of the Cry1Ac crystal structure indicate that a region on and adjacent to β-16 has a unique conformation compared with the known Cry toxin structures (D.J.D., J.L. & D.J.E., unpublished results), mutations were made in this region of the domain III outer sheet.

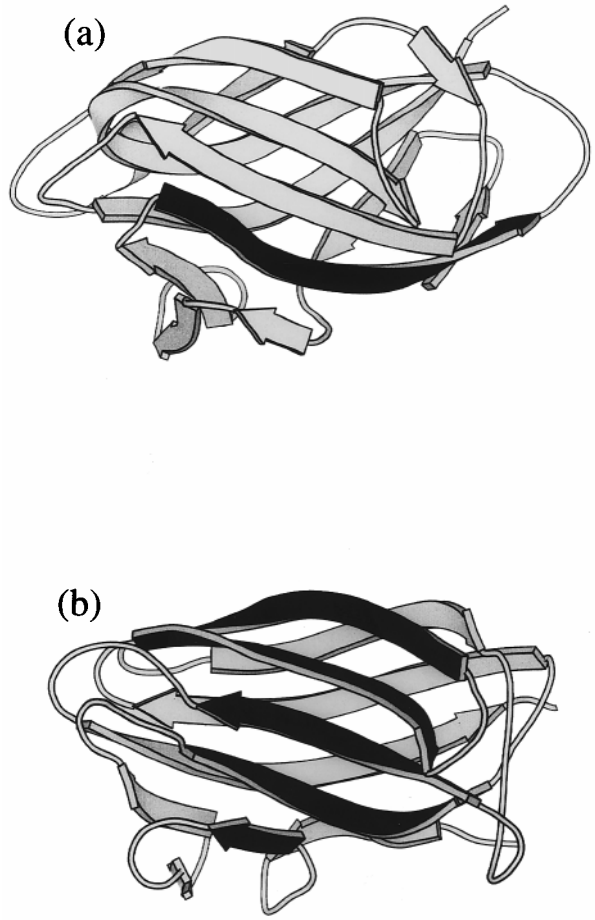

Figure 1. MOLSCRIPT (Kraulis, 1991) representations of (a) Cry3A domain III and (b) the N-terminal cellulose-binding domain of *C. fimi* 1,4-β-glucanase CenC (CBD_{N1}), PDB codes 1DLC and 1ULO, respectively. Cry3A β-16 is shaded black, as are the β-strands in sheet A of CBD_{N1}.

Results

Alignment and construction of mutant toxins

The primary sequences of the outer sheet of domain III from Cry1Ac, Cry1Aa and Cry3A were aligned (Hodgman & Ellar, 1990, with modifications) and matched in turn with sheet A of the N-terminal cellulose-binding domain (CBD_{N1}) from the bacterium *C. fimi* 1,4-β-glucanase CenC (Figure 2). The amino acid residues in the outer sheet of Cry1Ac domain III selected for mutagenesis are shown in Figure 2. One double mutant and 18 single mutants were made initially (Table 1).

Expression and stability of mutant proteins

Crystal protoxins from wild-type and mutants were analysed by SDS-PAGE. All the mutant proteins were expressed at levels similar to the wild-type, and were the standard 133 kDa size. Upon activation by either *Pieris brassicae* gut extract or trypsin, all the mutant protoxins yielded stable 65 kDa toxins. *P. brassicae* gut extract was routinely used to activate the toxin because of its consistent levels of proteolytic activity from preparation to preparation. The mutant toxins were, however, activated at least once with *M. sexta* gut extract to confirm that the products did not differ in polypeptide profile from those produced by *P. brassicae* gut extract. Mutants R511A and N544L did not produce the usual parasporal bipyramidal crystals and so were not investigated further. Representative samples of the trypsin digest analysis are shown in Figure 3.

Binding of toxin to *M. sexta* midgut BBMV

The ability of the single and double mutant toxins to bind to *M. sexta* BBMV is shown in Figure 4. In this qualitative assay, most of the toxins bound to the BBMV to a similar extent to Cry1Ac, but the binding of mutants Q509E, Q509S, Y513A and N506D,Q509E was considerably less than that of Cry1Ac. These data prompted the construction of the triple mutant N506D,Q509E,Y513A (Tmut). From this point on, only the data for Cry1Ac and mutants N506D, Q509E, Q509S, Y513A, N506D,Q509E and Tmut will be presented.

Heterologous competition binding assays were next used to measure the relative binding affinities of these mutants (Figure 5). These values are shown in Table 2. The term K_d for the dissociation binding constant is inappropriate for this two-step reversible plus irreversible binding process (Liang *et al.*, 1995) and so the term K_{com} was used to represent the binding affinity constant (Wu & Dean, 1996).

Toxin-overlay ligand blots revealed that whilst solubilised and activated wild-type Cry1Ac binds to a 120 kDa protein in *M. sexta* BBMV, no binding of Tmut to the BBMV was observed (Figure 6). Binding of mutants N506D, Q509E, Q509S, Y513A, N506D,Q509E to the BBMV was reduced (data not shown). One possible explanation of these results is that Cry1Ac antibody binds poorly to Tmut compared to Cry1Ac. However, this possibility has been ruled out by a control experiment (data not shown).

Toxicity

Bioassay of wild-type Cry1Ac against *M. sexta* neonates gave an LC_{50} value of 11.2 ng/cm^2 (95 %

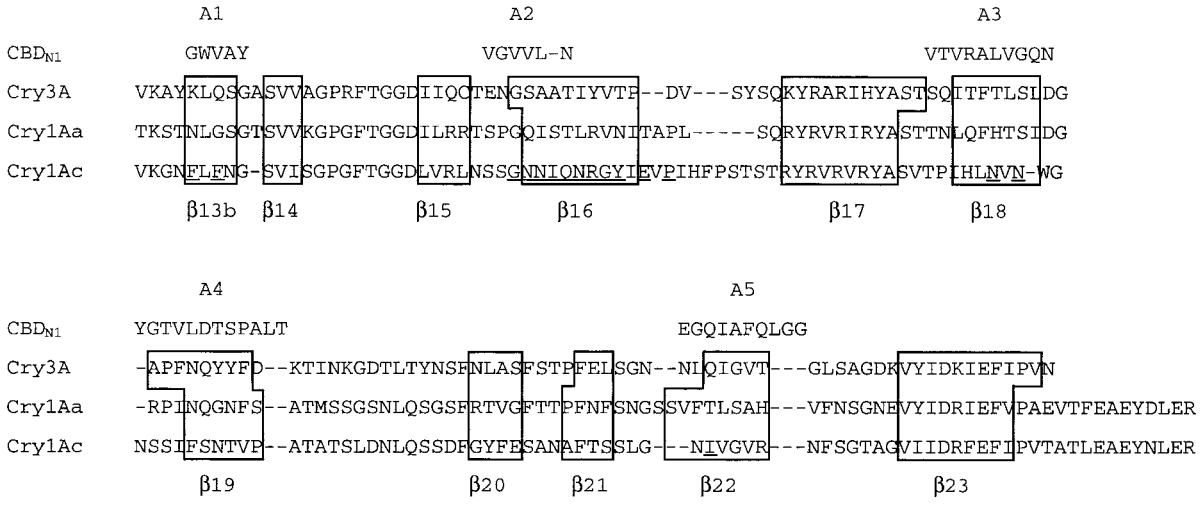

Figure 2. Alignment of the domain III outer sheet primary sequence from Cry1Ac, Cry1Aa and Cry3A, with the sequence of the β-strands of sheet A of the N-terminal cellulose-binding domain (CBD_{N1}) of *C. fimi* 1,4-β-glucanase CenC. Those residues in Cry1Ac selected for mutagenesis are underlined. Boxing indicates the regions of secondary structure in the Cry toxins, as determined from the crystal structures of Cry1Aa and Cry3A, and predicted for Cry1Ac. Numbering of the β-strands of the Cry toxins is indicated below the sequences, and that of the sheet A strands in CBD_{N1} is above the sequence.

Table 1. Sequences of mutagenic oligonucleotides for the Cry1Ac domain III mutagenesis

Mutation introduced	DNA sequence of mutagenic oligonucleotide (5′-3′)
F481R	CAGTGAAGGG**T**AAC**CG**TCTTTTTAATGGT
F483W	AGGGAAACTTTCTTT**GG**AATGG**A**TC**C**GTAATTTCAGGACC
F483G	AAGGGAAACTTTCTT**GGA**AATGG**A**TC**C**GTAATTTCAGGACC
G505A	GATTAAATAGTAG**CG**C**T**AATAACATTCAGA
N506D	AATAGTAGTGGA**G**ATAA**T**ATTCAGAATAGAG
N507A	AATAGTAGTGG**C**AAT**GC**CATTCAGAATAG
I508A	TAGTGGAAATAA**TGCG**CAGAATAGAGG
Q509E	GTAGTGGAAATAA**T**ATT**G**AGAATAGAGGG
Q509S	GTAGTGGAAATAA**T**ATT**TC**GAATAGAGGGT
N510A	AATAACATTCAG**GCTC**GAGGGTATATT
R511A	AACATTCAGAAT**GC**AGGGTATATTG
G512A	CATTCAGAATAG**GGCC**TATATTGAAGTTC
Y513A	GAATAGAGGG**GCG**ATCGAAGTTCCAAT
E515Q	GAGGGTATATT**CAGGTC**CCAATTCACTT
P517R	ATATTGAAGTTC**GGA**T**C**CACTTCCCAT
N542V	CGATTCACCTC**GTG**GTTAATTGGGG
N544L	CACCTCAACGT**GTTA**TGGGGTAATTC
I586S	ATCTTCATTAGG**G**AAT**TC**AGTAGGTGTTAG
N506D, Q509E	GATTAAATAGTAGTGGA**G**ATAA**T**ATT**G**AA**A**AATAGAGGGTATATTG

The altered bases are indicated in bold.

confidence interval 7.5-14.5 ng/cm^2). The toxicity of mutant toxins was assayed at a concentration of 40 ng solubilised toxin per cm^2 of artificial diet. This concentration causes approximately 99 % neonate death by Cry1Ac (Smedley & Ellar, 1996). All mutant toxins were of an activity similar to that of the wild-type toxin at this concentration, producing 100 % mortality. The LC_{50} value for Tmut was determined to be 16.1 ng/cm^2 (95 % confidence interval 12.2-19.8 ng/cm^2). An additional bioassay with fifth instar *M. sexta* larvae was performed with solubilised Cry1Ac and Tmut. No significant difference in the effects of the two toxins was observed (data not shown).

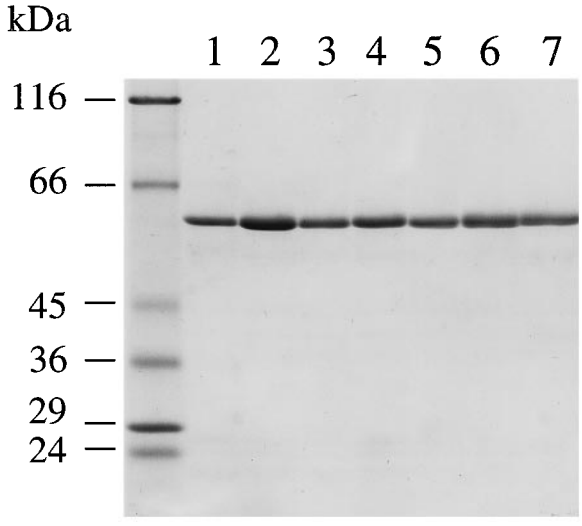

Figure 3. Comparison of the stability of trypsin-activated wild-type Cry1Ac and mutant toxins by Coomassie-blue stained SDS-13 % PAGE. Lanes 1, Cry1Ac; 2, N506D; 3, Q509E; 4, Q509S; 5, Y513A; 6, N506D,Q509E; 7, N506D,Q509E,Y513A (Tmut). Molecular mass marker positions are indicated to the left.

Toxin-induced BBMV permeability changes

Changes in the membrane permeability of BBMV were monitored using a light-scattering assay (Carroll & Ellar, 1993). A recovery in the light-scattering signal represents vesicle reswelling caused by toxin-induced lesions, allowing the entry of KCl into the BBMV, rapidly followed by water. The mixing assay monitors changes in BBMV permeability for 200 seconds immediately after the addition of activated toxin, and therefore represents the rate of pore formation, involving both the initial receptor binding and membrane penetration. Figure 7(a) shows that when introduced to *M. sexta* BBMV at the same concentration, all of mutants Q509E, Q509S, Y513A, N506D,Q509E and Tmut exert a permeabilising effect at a slower rate than wild-type Cry1Ac, with Tmut being the least efficient. This assay was repeated with different toxin concentrations. The relationship between toxin concentration and the time taken to reach 10 % volume recovery (T 10 % (seconds)) was plotted for Cry1Ac and Tmut (Figure 7(b)). The regression lines for Cry1Ac and Tmut are clearly significantly different ($P < 0.05$ by analysis of covariance), with the rate of vesicle pore formation by Tmut being significantly slower than by Cry1Ac.

Effect of carbohydrate on binding and permeability

Visual estimates of toxin binding using a modification of the spin-down binding assay described by Knowles *et al.* (1991) indicated that only 5 % of Tmut bound to the vesicles, whereas approximately 75 % of the wild-type toxin bound, under the conditions employed (Figure 8). The presence of GlcNAc does not affect the binding of the wild-type toxin, but the inclusion of GalNAc abolishes binding almost completely (Figure 8(a)). In con-

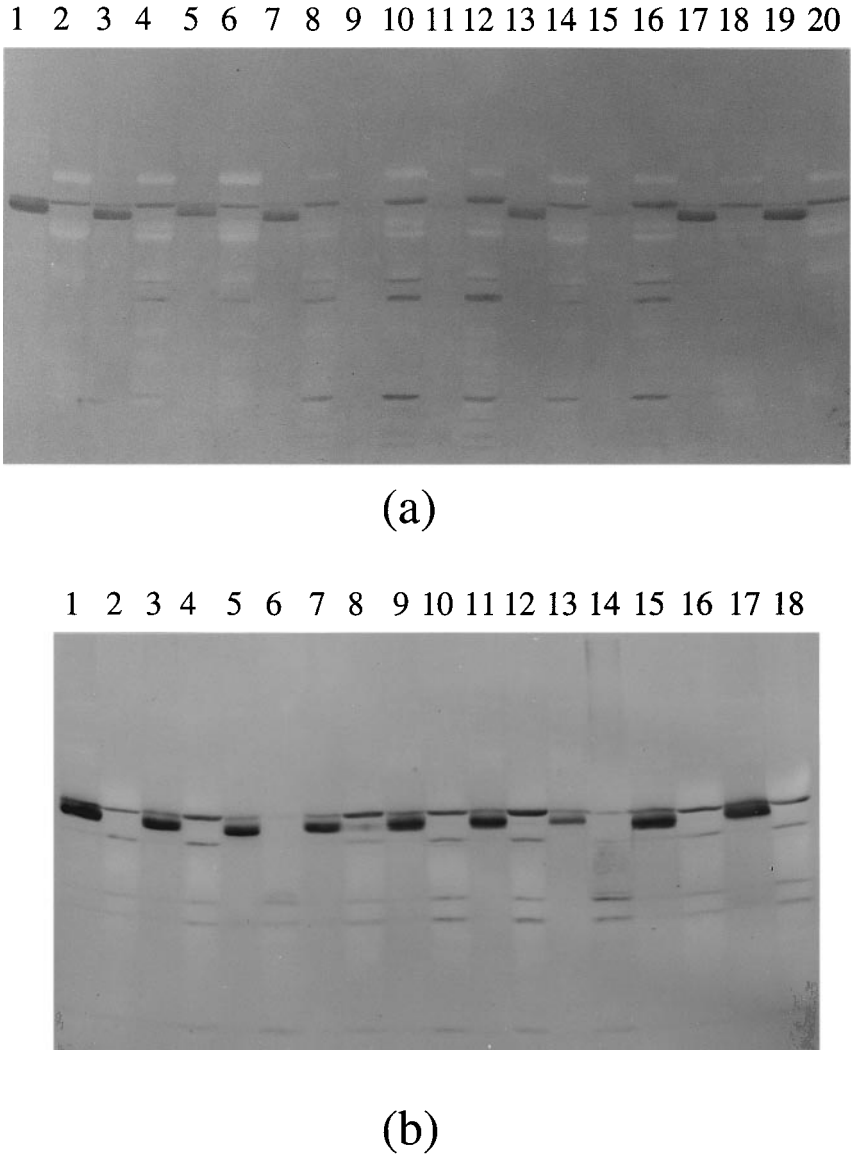

Figure 4. Qualitative binding of Cry1Ac and domain III mutants to *M. sexta* BBMV. Solubilised and activated toxin (5 μg) was incubated with 50 μg of BBMV in a final volume of 50 μl of PBS (pH 7.4), containing 0.1% (w/v) BSA. After 60 minutes incubation at room temperature (20 °C) bound toxin was separated from free toxin by centrifugation. Samples of the pellets (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19) and supernatants (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) were subjected to SDS-13% PAGE, transferred to nitrocellulose paper and the toxin detected by immunoblotting. (a) Lanes 1 and 2, Cry1Ac; 3 and 4, F481R; 5 and 6, N542V; 7 and 8, I586S; 9 and 10, Q509E; 11 and 12, Q509S; 13 and 14, N506D; 15 and 16, N506D,Q509E; 17 and 18, F483G; 19 and 20, F483W; (b) Lanes 1 and 2, Cry1Ac; 3 and 4, G505A; 5 and 6, N507A; 7 and 8, I 508A; 9 and 10, N510A; 11 and 12, G512A; 13 and 14, Y513A, 15 and 16, E515Q, 17 and 18, P517R.

trast, the association of Tmut with BBMV is not inhibited by either sugar, and is of an extent similar to that seen with wild-type Cry1Ac in the presence of GalNAc (Figure 8(b)).

The toxin-induced BBMV permeability assay was repeated with the inclusion of carbohydrate. In the presence of GalNac, the permeabilisation of *M. sexta* BBMV by Cry1Ac is greatly inhibited relative to GlcNAc (Figure 9(a)). In contrast, there was no observable difference between the Tmut-induced swelling traces for *M. sexta* BBMV in the presence of either GalNAc or GlcNAc (Figure 9(a)). The regression lines for Cry1Ac in the presence of GalNAc and GlcNAc are significantly different ($P < 0.05$). The regression lines for Tmut both plus GalNAc and plus GlcNac are significantly different from that of Cry1Ac plus GalNAc ($P < 0.05$). This indicates that the rate of pore formation by Tmut in the presence of either sugar is slower than that for Cry1Ac, even in the presence of GalNAc.

Discussion

The interaction of the Cry1Ac toxin with its binding protein aminopeptidase N is specifically inhibited by the carbohydrate *N*-acetylgalactosamine (Knight *et al.*, 1994). The third domain of Cry3A was found to have a structural fold similar to that of the N-terminal cellulose-binding domain of *C. fimi* 1,4-β-glucanase CenC (Murzin & Bateman, 1997). The individual residues in this lectin predicted to bind to its ligand were in a cleft formed by one sheet of the β-jelly roll structure (Johnson *et al.*, 1996). However, in these experiments, mutations in several residues that were assigned to β-16 by sequence alignment, but which the crystal structure shows to fall in β-16 and the adjacent β-15-β-16 loop, affected binding. Therefore, the experimentally detected site of contact between Cry1Ac and its carbohydrate ligand differs from this prediction.

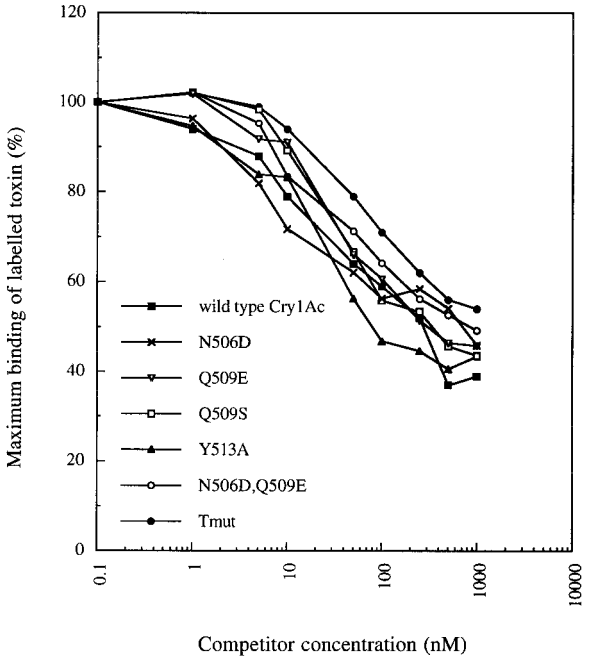

Figure 5. *M. sexta* BBMV (0.1 mg/ml) equilibrated with binding buffer were mixed with 1 nM trypsin-activated ^{125}I-labelled Cry1Ac in the presence of increasing concentrations of trypsin-activated non-labelled competitor in a final volume of 0.2 ml at 25 °C for 60 minutes. Bound toxin was separated from free toxin by centrifugation. Binding is expressed as a percentage of the amount of ^{125}I-labelled toxin bound in the absence of competitor. Each point represents the average of three separate determinations.

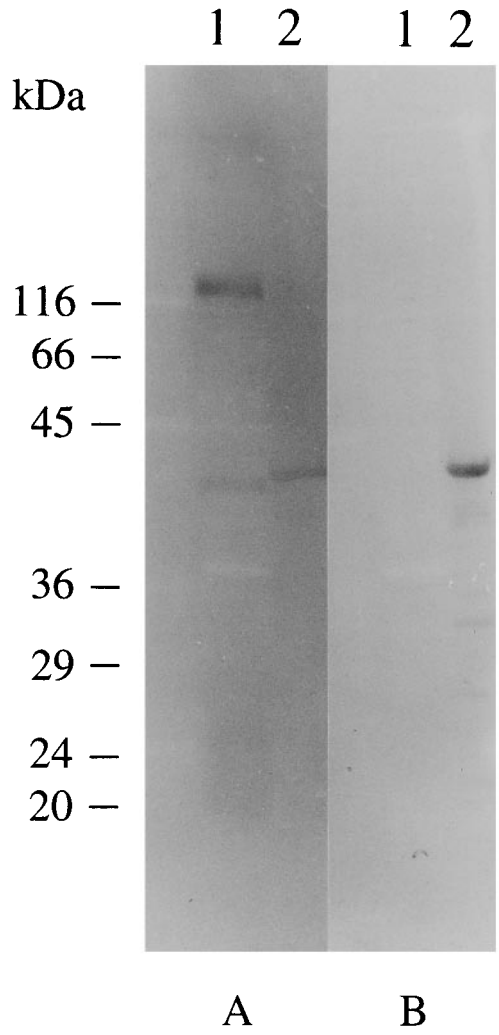

Figure 6. Detection of binding proteins in *M. sexta* midgut. BBMV proteins (30 µg, lane 1) and activated toxin (5 µg, lane 2) separated by SDS-PAGE were transferred to nitrocellulose paper and incubated sequentially with: (a) Cry1Ac or (b) Tmut, anti Cry1Ac antiserum and a secondary antibody conjugate, followed by development. Molecular mass marker positions are indicated to the left.

All of the mutants, except R511A and N544L, expressed at levels comparable to that of wild-type Cry1Ac, and were processed into stable 65 kDa toxins by both trypsin (Figure 3) and gut proteases (data not shown). This indicates that the mutant toxins were folded correctly and any change in activity would not be due to protein instability. In a qualitative assay, mutations made in residues Q509 and Y513 reduced the ability of Cry1Ac to associate with *M. sexta* BBMV (Figure 4). An assay monitoring the permeability of *M. sexta* BBMV was employed to monitor the rate of pore formation by the mutants. The mixing experiment follows changes in BBMV permeability with time, immediately after the introduction of toxin. For a toxin to cause an increase in BBMV permeability in this assay, it has to undergo both the receptor binding and membrane penetration steps. All of the single mutants N506D, Q509E, Q509S and Y513A showed a slower permeabilising rate than wild-type Cry1Ac, to varying extents (Figure 7). The double mutant N506D, Q509E was even less efficient, with the triple mutant N506D,Q509E,Y513A being the slowest. Therefore, considering the data from these two assays, it appears that residues N506, Q509 and Y513 are all involved in the binding of Cry1Ac to *M. sexta* BBMV and/or insertion into the epithelial membrane. Although mutant N506D does

Table 2. Relative binding affinities of Cry1Ac and mutants toward *M. sexta* midgut BBMV

Toxin	K_{com} (nM) ± SEM[a]
Cry1Ac	55 ± 1.5
N506D	112 ± 0.8
Q509E	111 ± 0.2
Q509S	79 ± 2.4
Y513A	81 ± 1.2
N506D, Q509E	139 ± 0.3
N506D, Q509E, Y513A	174 ± 0.4

[a] K_{com}, dissociation constant determined from heterologous competition binding experiments (except for Cry1Ac, which is from a homologous competition assay). Each value is the mean of three experiments.

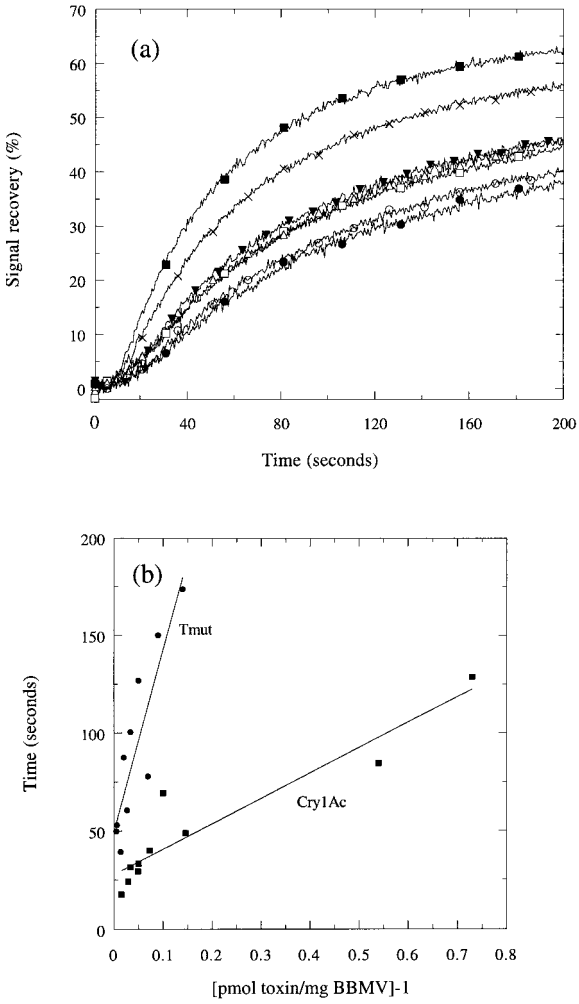

Figure 7. (a) Effect of toxin on *M. sexta* BBMV permeability to hyperosmotic KCl using a mixing assay. BBMV (0.2 mg/ml) equilibrated with 10 mM Ches/KOH (pH 9.0), 0.1% (w/v) BSA were mixed with 10 mM Ches/KOH (pH 9.0), 0.1% (w/v) BSA plus 150 mM KCl containing toxin (66 pmol toxin/mg BBMV) using a stopped-flow spectrophotometer at 20-21 °C. Reswelling was followed as the change in 90° light-scattering at 450 nm over 200 seconds. Each trace represents the average of two separate determinations corrected for control changes, showing light-scattering signal recoveries relative to the signal change observed for the control. Filled squares, Cry1Ac; crosses, N506D; filled triangles, Y513A; open triangles Q509E; open squares, Q509S; open circles, N506D,Q509E; filled circles, Tmut. (b) Linear regression analysis of toxin concentration against time taken for a toxin-induced 10% signal recovery in *M. sexta* BBMV. Conditions are as described for (a). Squares, wild-type Cry1Ac; circles, Tmut. The regression lines for wild-type Cry1Ac and Tmut are significantly different ($P < 0.05$ by analysis of covariance).

not seem to show reduced binding to BBMV in a ligand blot analysis (Figure 4), this assay is qualitative, and so very small changes in binding may not be seen.

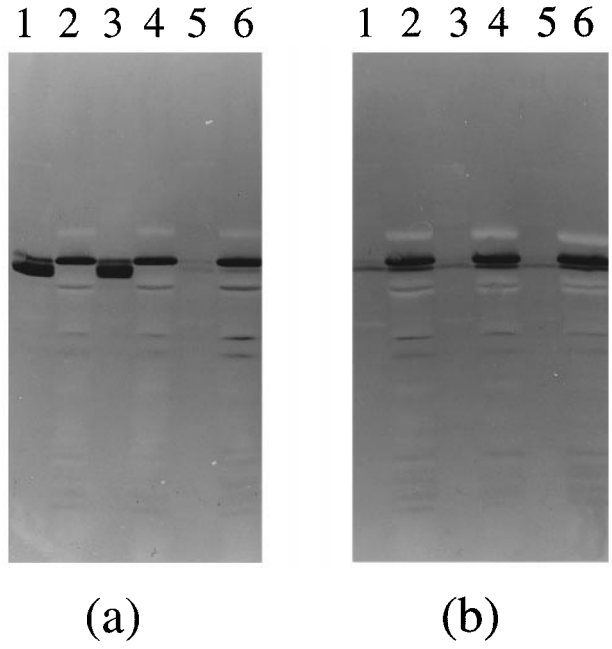

Figure 8. Effect of sugars on toxin binding to *M. sexta* BBMV. Solubilised and activated toxin was incubated with BBMV as described in the legend to Figure 4, with the inclusion of sugars, where appropriate, at 100 mM. After 60 minutes incubation at room temperature (20 °C) bound toxin was separated from free toxin by centrifugation. Samples of the pellets (lanes 1, 3 and 5) and supernatants (lanes 2, 4 and 6) were subjected to SDS-PAGE, transferred to nitrocellulose paper and the toxin detected by immunoblotting. (a) Cry1Ac; (b) Tmut. Lanes 1 and 2, BBMV and toxin with no sugars; lanes 3 and 4, BBMV and toxin with 100 mM GlcNAc; lanes 5 and 6, BBMV and toxin with 100 mM GalNAc.

Heterologous competition binding assays were carried out in order to quantify these results. All of the mutants were able to compete with the labelled Cry1Ac for binding to *M. sexta* midgut BBMV, but less efficiently than unlabelled Cry1Ac, with the K_{com} of the triple mutant being more than three times higher than that of Cry1Ac. Although these K_{com} values are from heterologous and not homologous assays, and so are not true values, they provide a means of comparing the binding affinity of Cry1Ac with the mutants. Therefore, these assays suggest that it is in fact the binding to the *M. sexta* BBMV that these domain III mutations are affecting.

Cry1Ac has previously been reported to bind to proteins of 120 kDa (Knight *et al.*, 1994) and 210 kDa (de Maagd *et al.*, 1996b; Martínez-Ramírez *et al.*, 1994). Mutants N506D, Q509E, Q509S and N506D,Q509E bound less effectively to the 120 kDa protein (not shown), with no binding by the triple mutant (Figure 6). Under the conditions used, binding to a 210 kDa protein by either Cry1Ac or mutants was not observed. It is therefore probable that residues N506, Q509 and Y513

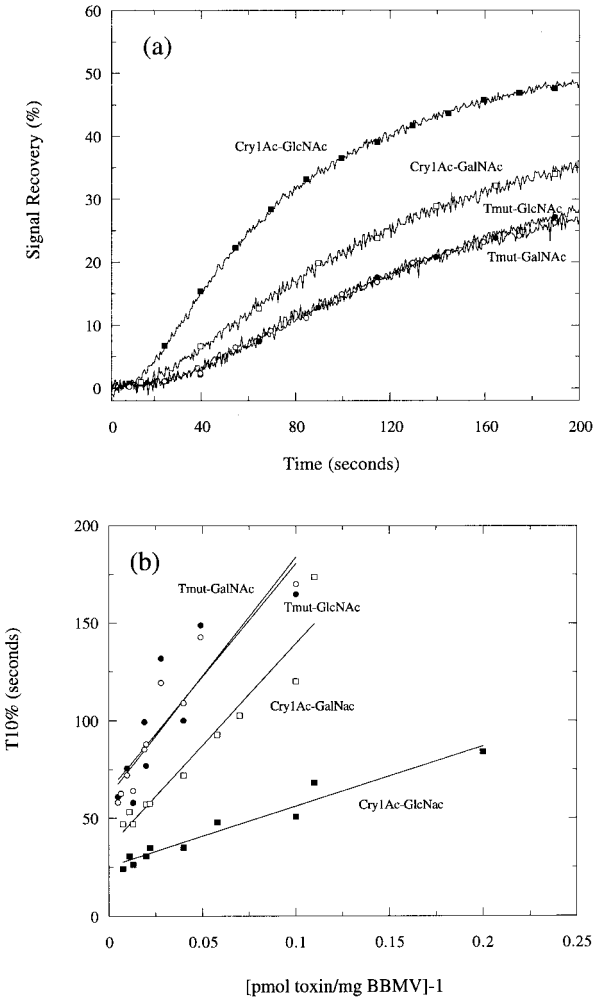

Figure 9. (a) Effect of GalNAc and GlcNAc on toxin-induced signal recoveries in *M. sexta* BBMV reswelling after mixing in hyperosmotic KCl. BBMV (0.2 mg/ml) equilibrated with 10 mM Ches/KOH (pH 9.0), 0.1 % (w/v) BSA were mixed with 10 mM Ches/KOH (pH 9.0), 0.1 % (w/v) BSA plus 150 mM KCl and 200 mM GalNAc or GlcNAc containing toxin (100 pmol toxin/mg BBMV), using a stopped-flow spectrophotometer at 20-21 °C. Reswelling was followed as the change in 90° light-scattering at 450 nm over 200 seconds. Each trace represents the average of two separate determinations corrected for control changes, showing light signal recoveries relative to the signal change observed for the control. Squares, Cry1Ac; circles Tmut. Open symbols represent GalNAc data, and filled symbols GlcNAc data. (b) Linear regression analysis of toxin concentration against time taken for a toxin-induced 10 % signal recovery in *M. sexta* BBMV. Conditions are as described in the legend to Figure 7. The regression lines for Cry1Ac-GlcNAc and Cry1Ac-GalNAc are significantly different ($P < 0.05$ by analysis of covariance). The lines for Tmut-GlcNAc and Tmut-GalNAc are both significantly different from that for Cry1Ac-GalNAc.

in Cry1Ac are involved in the binding to the 120 kDa aminopeptidase N in *M. sexta*.

GalNAc selectively inhibits the binding of Cry1Ac to *M. sexta* BBMV (Figure 8; Knowles *et al.*, 1991) and, more specifically, it inhibits the binding of Cry1Ac to aminopeptidase N (Knight *et al.*, 1994). However, even in the absence of carbohydrate, very little triple mutant binds to *M. sexta* BBMV. This can be estimated to be approximately 7 % of the amount of wild-type Cry1Ac seen to bind in the absence of carbohydrate, and is a similar quantity to the amount of wild-type Cry1Ac binding in the presence of GalNAc. Moreover, no difference in the binding of the triple mutant to BBMV in the presence or absence of sugars could be observed. This indicates that the binding of the triple mutant to *M. sexta* BBMV is not inhibited by GalNAc, and that this apparent binding is low. The inhibitory action of GalNAc on the permeabilising ability of Cry1Ac is illustrated by Figure 9(a). In contrast, no significant difference was seen between the reswelling traces induced by the triple mutant in the presence of GlcNAc or GalNAc. The regression lines for the triple mutant in either GlcNAc or GalNAc are both significantly different from that of Cry1Ac plus GalNAc, indicating that the pore formation by the triple mutant in the presence of either sugar is slower than that for Cry1Ac in the presence of GalNAc.

The data presented here allow us to conclude that residues N506, Q509 and Y513 are together involved in the binding of Cry1Ac both to the carbohydrate GalNAc and to aminopeptidase N in *M. sexta*.

Carroll *et al.* (1997) proposed that there are at least two mechanisms by which Cry1Ac causes pore formation in *M. sexta* BBMV: one a GalNAc-inhibitable aminopeptidase N-dependent mechanism located in the posterior region of the *M. sexta* midgut, and the other being GalNAc-insensitive and uniformly distributed along the length of the midgut. The lack of a significant decrease in toxicity of the triple mutant to *M. sexta* larvae appears to indicate that the aminopeptidase N-dependent mechanism of membrane pore formation is irrelevant to toxicity. However, in the absence of this mechanism *in vivo*, the GalNAc-insensitive mechanism may play a greater part such that overall toxicity does not significantly decrease. Furthermore, the duration of the *in vivo* feeding assay compared with the permeabilising and binding assays must be taken into account. A toxicity experiment takes place over a period of seven days, during which time the insect gut is exposed to solubilised toxin. However, in a binding assay activated toxin is exposed to BBMV for 60 minutes, and the permeabilising assay is followed for 200 seconds. The data of Cooper *et al.* (1998) suggest that once the toxin is irreversibly bound in the membrane, the binding site on the aminopeptidase N receptor is released, making it available to bind more toxin. A similar situation may be occurring with the GalNAc-insensitive mechanism such that the total number of pores formed in the midgut epithelium by Tmut over seven days is sufficient to give an LC_{50} value not significantly different from that of wild-type Cry1Ac.

The involvement of glycoproteins as Cry toxin receptors has been suggested for several toxin-membrane interactions (Knowles *et al*., 1984, 1991; Knowles & Ellar, 1986; Haider & Ellar, 1987; Hofmann *et al*., 1988; Knight *et al*., 1994, 1995). However, the specific inhibition by GalNAc of the Cry1Ac-aminopeptidase N interaction is so far unique among Cry toxins. We have located this carbohydrate-binding site in a region on and adjacent to β-16 in Cry1Ac domain III. In understanding the specificity determinants of these insecticidal proteins, it is significant that preliminary analysis of the Cry1Ac crystal structure indicates that the β-16 region has a unique conformation compared with the known Cry toxin structures (D.J.D., J.L. & D.J.E., unpublished results). The sequence of Cry1Ac domain III is highly dissimilar to Cry1Aa, a protein with which it shares 80% overall sequence identity (Höfte & Whiteley, 1989), and in fact is evolutionarily distinct from that of any other Bt Cry toxin (Bravo, 1997). It would be interesting to extend this work further by exchanging the domain containing the triple mutation with other Cry toxins, and evaluating the activity and receptor specificity of the resulting chimeras.

Materials and Methods

Site-directed mutagenesis and crystal purification

Site-directed mutagenesis was performed as described by Smedley & Ellar (1996) using the plasmid pMSV.Cry1Ac and the Altered-Sites *in vitro* Mutagenesis System (Promega). Oligonucleotides were synthesised by the Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge, UK. Wild-type and mutant plasmids were transformed into the acrystalliferous strain *B. thuringiensis* subsp. *israelensis* IPS-78/11 by electroporation (Bone & Ellar, 1989) and those mutant toxins that formed parasporal bipyramidal crystal proteins identical with wild-type toxin were purified according to the method of Thomas & Ellar (1983).

Solubilisation and activation

Purified δ-endotoxin crystal was solubilised by incubation in 50 mM Na_2CO_3 (pH 9.5), 10 mM DTT, at 37°C for 60 minutes. Any insoluble material was removed by centrifugation at 13,500 *g* for ten minutes. The soluble protoxin was activated by incubation either with 2.5% (v/v) *P. brassicae* gut extract (Knowles *et al*., 1984) for five minutes at 37°C, or with 1:1 (w/w) trypsin (Sigma) to protoxin at 37°C for 60 minutes, in both cases followed by centrifugation at 13,500 *g* for ten minutes. The supernatant was used as the active toxin preparation and quantified by the method of Bradford (1976) using the Bio-Rad protein assay with bovine serum albumin (BSA) as a standard.

Toxicity

M. sexta eggs were obtained from Dr S. Reynolds, School of Biological Sciences, University of Bath, Avon, UK and the insects reared on an artificial diet (Bell & Joachim, 1976). The protein concentration of solubilised protoxin was determined by the method of Bradford (1976) as described above, and diluted in phosphate-buffered saline (PBS) to the appropriate concentration; 20 µl of each concentration was applied to 1 cm^2 discs of artificial diet, allowed to dry, and a single larva placed on each disc. Bioassays were repeated a minimum of three times with eight larvae per concentration, with doses ranging between 1 and 50 ng/cm^2. Mortality was assessed after seven days at 26°C, and the LC_{50} values were determined by the program of Lieberman (1983) for probit analysis (Finney, 1952) using the results of three separate experiments.

Brush border membrane vesicle preparation

BBMV were isolated from fifth instar *M. sexta* larvae by a modification of the method of Wolfersberger *et al*. (1987), as described by Carroll & Ellar (1993). The final pellet was resuspended in 250 mM sucrose, 5 mM EGTA, 17 mM Tris (pH 7.5) and stored at −80°C. Protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay, with BSA as a standard.

SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE using a modification of the method of Laemmli & Favre (1973) as described by Thomas & Ellar (1983). Transfer of proteins from the gel to nitrocellulose paper was done with a semi-dry blot apparatus (Department of Biochemistry, University of Cambridge, UK), using a blot buffer of 39 mM glycine, 48 mM Tris (pH 9.2), 0.0375% (w/v) SDS, 10% (v/v) methanol. The location of toxin was detected with antibodies using the method of Knowles *et al*. (1991). Cry1Ac binding proteins were identified by incubating the nitrocellulose paper with gut extract-activated toxin (2 µg/ml) for 60 minutes. The toxin was then detected by immunoblotting as above.

Qualitative binding assay

Qualitative toxin binding was assayed by a modification of the method of Knowles *et al*. (1991). BBMV (50 µg) were incubated with 5 µg of activated toxin in a final volume of 50 µl of PBS (pH 7.4) containing 0.1% (w/v) BSA, and sugars, where appropriate, at 100 mM. After 60 minutes incubation at room temperature, the mixture was centrifuged at 13,500 *g* for ten minutes, and the resultant BBMV pellet containing bound toxin was washed twice with PBS containing 0.1% (w/v) BSA. Samples of the pellet and supernatant were analysed by SDS-PAGE and immunoblotting as described in the previous section.

BBMV permeability assay

BBMV solute permeability was measured using a light-scattering assay (Carroll & Ellar, 1993, with minor modifications). Freshly prepared *M. sexta* BBMV were diluted in 10 mM Ches/KOH (pH 9.0), 0.1% (w/v) BSA to a final concentration of 0.2 mg/ml, and mixed with hyperosmotic 150 mM KCl in 10 mM Ches/KOH (pH 9.0), 0.1% (w/v) BSA. Changes were monitored by 90° light-scattering at 450 nm, and the toxin mixing assay was used (Carroll & Ellar, 1993). Where appropriate, sugars were included in the mixing assay at a final experimental concentration of 100 mM. Linear regression analysis was performed using the computer program

COMPREG (Wiggans *et al.*, 1983). Quantification of the activated toxin preparation was performed by gel-scanning densitometry of SDS-PAGE-separated and Coomassie blue-stained protein with BSA as a standard using an Epson scanner and the computer programs Photoshop 3.0 and Phoretex 1D Advanced.

Iodination of toxin

Crystal protoxin was solubilised and activated with trypsin as described above. The activated toxin was precipitated out of solution with 70% (w/v) ammonium sulphate and the precipitate centrifuged at 13,500 *g* for 15 minutes at 4°C. The pellet was resolubilised in 50 mM Na_2CO_3 (pH 10.5), 200 mM NaCl, 5% (v/v) glycerol for 60 minutes at 4°C. This was then dialysed overnight at 4°C against 20 mM ethanolamine (pH 9.5) and passed through a 2 µm filter before being purified through a Pharmacia MonoQ anion-exchange column, using a Waters 650E FPLC system. The 5 mm × 50 mm column was pre-equilibrated with 20 mM ethanolamine (pH 9.5) and protein was eluted by increasing the salt concentration linearly from 0 M to 1 M NaCl, with a flow-rate of 1 ml/minute. Cry1Ac toxin was eluted between 320 and 380 mM NaCl.

Purified toxin (100 µg) was iodinated using one IODO-BEAD (Pierce) and 1 mCi of $Na^{125}I$ (Amersham), as specified by the manufacturer. The labelled toxin was separated from the free iodine by passing through a PD-10 column (Pharmacia) pre-equilibrated with binding buffer (8 mM $NaHPO_4$, 2 mM KH_2PO_4 (pH 7.4), 150 mM NaCl, 0.1% (w/v) BSA). The specific activity of the iodinated wild-type toxin was 9.5 mCi/mg.

Competition binding assays

BBMV (0.1 mg/ml) were incubated with 1 nM ^{125}I-labelled toxin in a final volume of 200 µl of binding buffer with increasing concentrations (0-1000 nM) of the same (homologous) or different (heterologous) trypsin-activated non-labelled toxin as a competitor. The reaction was left for 60 minutes at 25°C and the toxin bound to BBMV was separated from unbound toxin by centrifugation at 13,500 *g* for ten minutes at room temperature. The pellet containing the bound toxin was washed twice with binding buffer, and the radioactivity in the final pellet measured in a gamma counter (IsoSystems). Each experiment was repeated at least three times, and the mean values plotted using the CA-Cricket Graph III computer program. Affinity constants were calculated using the EBDA program (McPherson, 1983).

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