Mutations in the Bacillus thuringiensis Cry1Ca toxin demonstrate the role of domains II and III in specificity towards Spodoptera exigua larvae

Salvador HERRERO*, Joel GONZÁLEZ-CABRERA†, Juan FERRÉ†, Petra L. BAKKER* and Ruud A. de MAAGD*†

*Business Unit Bioscience, Plant Research International B.V., Wageningen University and Research Centre, 6700 AA Wageningen, The Netherlands, and †Department of Genetics, University of Valencia, Burjassot, Spain

INTRODUCTION

Strains of Bacillus thuringiensis, a Gram-positive entomopathogenic bacterium, produce different kinds of crystal inclusions during sporulation. These crystal inclusions are composed of one or various Cry proteins (also called δ-endotoxins). Some of these proteins are highly toxic to certain insects, but they are harmless to most other organisms, including wildlife and beneficial insects [1,2]. The mode of action of B. thuringiensis Cry1 and related proteins follows, after ingestion by the insect, a complex process of multiple steps. These include: (i) solubilization of the crystal to release the Cry proteins in their protoxin form, (ii) activation of the protoxins by midgut proteases to their active form, (iii) binding of the toxin to midgut receptors, and (iv) pore formation in the brush border cell membranes, eventually killing the insect. Recently, an intermediate step has been suggested to link protoxin activation and binding to specific receptors. This step would include the interaction of the not fully activated toxin with the midgut of the insect through specific binding sites involved in the activation and oligomerization of the toxin. Oligomeric forms have been suggested, in that model, as being responsible for insertion into the cell membrane, leading to pore formation. The more variable domain II has been shown to be involved in the interaction with toxin binding sites in the midgut of the insect, playing an important role in determining insect specificity. Interaction of domain II with the binding sites is thought to take place through three main loops identified in its structure. Mutagenesis of some residues localized in these loops leads to reduced binding and, as a consequence, decreased toxicity (reviewed in Schnepf et al. [1]). Domain III is a β-sandwich with a jelly roll topology. Domain III exchange in hybrid toxins has resulted in new toxins with altered specificity and increased toxicity compared with the parental toxins, revealing the importance of domain III in specific binding to putative receptors and specificity [10,11]. For instance, hybrid toxins containing domains I and II of Cry1Ea or Cry1Ab and domain III of Cry1Ca (G27 and H04 respectively) showed an increase in activity against Spodoptera exigua compared with Cry1Ab or Cry1Ea [10]. Furthermore, domain III was shown to be responsible for the N-acetylglactosamine-inhibited binding of Cry1Ac to M. sexta aminopeptidase N [13,14].

Yamamoto and Dean [15] reported that Cry1Ca toxin containing a substitution in the hypothetical loop 2 (Gln125Ala) together with a substitution in loop 3 (Thr460Ala), both in domain II, had reduced toxicity against S. exigua. They also showed

Several mutations of the Bacillus thuringiensis Cry1Ca toxin affected with regard to specific activity towards Spodoptera exigua were studied. Alanine was used to replace single residues in loops 2 and 3 of domain II (mutant pPB19) and to replace residues 541–544 in domain III (mutant pPB20). Additionally, a Cry1Ca mutant combining all mutations was constructed (mutant pPB21). Toxicity assays showed a marked decrease in toxicity against S. exigua for all mutants, while they retained their activity against S. exigua. Toxins containing domains I and II of Cry1Ea or Cry1Ab showed no binding to BBMV [6–9], revealing a similar three-domain structure. The N-terminal domain I consists of seven α-helices, and is thought to be responsible for toxin insertion into the cell membrane, leading to pore formation. The more variable domain II has been shown to be involved in the interaction with toxin binding sites in the midgut of the insect, playing an important role in determining insect specificity. Interaction of domain II with the binding sites is thought to take place through three main loops identified in its structure. Mutagenesis of some residues localized in these loops leads to reduced binding and, as a consequence, decreased toxicity (reviewed in Schnepf et al. [1]). Domain III is a β-sandwich with a jelly roll topology. Domain III exchange in hybrid toxins has resulted in new toxins with altered specificity and increased toxicity compared with the parental toxins, revealing the importance of domain III in specific binding to putative receptors and specificity [10,11]. For instance, hybrid toxins containing domains I and II of Cry1Ea or Cry1Ab and domain III of Cry1Ca (G27 and H04 respectively) showed an increase in activity against Spodoptera exigua compared with Cry1Ab or Cry1Ea [10]. Furthermore, domain III was shown to be responsible for the N-acetylglactosamine-inhibited binding of Cry1Ac to M. sexta aminopeptidase N [13,14].

Yamamoto and Dean [15] reported that Cry1Ca toxin containing a substitution in the hypothetical loop 2 (Gln125Ala) together with a substitution in loop 3 (Thr460Ala), both in domain II, had reduced toxicity against S. exigua. They also showed

These data together suggest a strong similarity (although with some differences) in the modes of action of Cry1A and Cry1C toxins.

The crystal structures of several trypsin-activated Cry proteins (Cry1Aa, Cry3Aa, Cry3Bb and Cry2Aa) have been elucidated [6–9], revealing a similar three-domain structure. The N-terminal domain I consists of seven α-helices, and is thought to be responsible for toxin insertion into the cell membrane, leading to pore formation. The more variable domain II has been shown to be involved in the interaction with toxin binding sites in the midgut of the insect, playing an important role in determining insect specificity. Interaction of domain II with the binding sites is thought to take place through three main loops identified in its structure. Mutagenesis of some residues localized in these loops leads to reduced binding and, as a consequence, decreased toxicity (reviewed in Schnepf et al. [1]). Domain III is a β-sandwich with a jelly roll topology. Domain III exchange in hybrid toxins has resulted in new toxins with altered specificity and increased toxicity compared with the parental toxins, revealing the importance of domain III in specific binding to putative receptors and specificity [10,11]. For instance, hybrid toxins containing domains I and II of Cry1Ea or Cry1Ab and domain III of Cry1Ca (G27 and H04 respectively) showed an increase in activity against Spodoptera exigua compared with Cry1Ab or Cry1Ea [10]. Furthermore, domain III was shown to be responsible for the N-acetylglactosamine-inhibited binding of Cry1Ac to M. sexta aminopeptidase N [13,14].

Yamamoto and Dean [15] reported that Cry1Ca toxin containing a substitution in the hypothetical loop 2 (Gln125Ala) together with a substitution in loop 3 (Thr460Ala), both in domain II, had reduced toxicity against S. exigua. They also showed

Abbreviations used: BBMV, brush border membrane vesicles; GI, growth inhibition; LC50, concentration causing 50 % mortality.

1 To whom correspondence should be addressed (email ruud.demaagd@wur.nl).
that such a mutant competes less effectively with biotin-labelled Cry1Ca for binding to \(S.\ exigua\) BBMV (brush border membrane vesicles). Alanine substitution of residues 541–544 of domain III of Cry1Ca in the hybrid G27 toxin abolished the activity of this hybrid against \(S.\ exigua\), but not against \(M.\ sexta\) [16]. These results suggested an important role for domain III, as well as for domain II, in the mode of action of Cry1Ca, including insect specificity.

In the present study, we took a more detailed look at the effects of mutations in domain II and/or domain III on the target insect specificity and mode of action of Cry1Ca. In particular, we studied how these different mutations can modify the interaction of the toxin with different elements involved in the complex process of binding to the midgut receptors. Additionally, for the first time, we demonstrated that mutations in domain II loops affect, in an insect-specific manner, the ability of the toxin to form oligomers.

**EXPERIMENTAL**

**Mutagenesis and protein isolation**

Cry1Ca mutants were constructed by introduction of the mutations Gln\(^{374}\)Ala and Thr\(^{440}\)Ala (domain II mutant; pPB19), Ser\(^{431}\)Ala, Thr\(^{432}\)Ala, Gly\(^{433}\)Ala and Val\(^{434}\)Ala (domain III mutant; pPB20), or all mutations combined (mutant pPB21), in the cry1Ca gene in expression plasmid pBD150 [16], using the QuickChange mutagenesis kit (Stratagene, Amsterdam, The Netherlands).

Wild-type Cry1Ca and mutant protoxins were expressed in *Escherichia coli* strain XL-1 Blue, extracted, solubilized and trypsin treated as described previously [16]. Briefly, each 1 g of cell culture expressing the different toxins was resuspended in 3 ml of lysis buffer (50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl) by vigorous shaking, and then 800 \(\mu\)g of lysozyme was added per g of pellet. After incubation at room temperature for 20 min, deoxycholic acid was added to a final concentration of 1 mg/ml and incubated at \(37^\circ C\) for 30 min. Then DNase I was added to a final concentration of 50 \(\mu\)g/ml and incubation at \(37^\circ C\) was continued for another 30 min. The mixture was clarified by centrifugation at 40,000 \(g\) for 20 min. Pellets containing the protoxin inclusion bodies were washed several times with washing buffer (20 mM Tris/HCl, pH 8.0, 5 mM EDTA, 100 mM NaCl). Protoxin was solubilized by incubation of the inclusion bodies at \(37^\circ C\) in solubilization buffer (50 mM sodium carbonate, pH 10.0) containing 10 mM dithiothreitol. After at least 2 h of solubilization, soluble protoxin was separated from the insoluble fraction by centrifugation at 40,000 \(g\) for 20 min. Then the pH of the solution was lowered to pH 9 using 1 M Tris/HCl, pH 8.0, and 0.2 \% (w/v) streptomycin was added and incubated for 1 h on ice. When necessary, activation of protoxin was performed by adding trypsin at a ratio of 1:10 (trypsin/protoxin, w/w) and incubating for 2 h at \(37^\circ C\). Protoxins and trypsin-activated toxins were dialysed overnight against 50 mM sodium hydrogen carbonate, pH 9.0, 150 mM NaCl.

Toxins for binding assays were purified by anion-exchange chromatography. Activated toxins were dialysed against Tris/NaCl buffer (20 mM Tris/HCl, 150 mM NaCl, pH 9.0) overnight at \(4^\circ C\). The dialysed solution was filtered and loaded on to a MonoQ HR 5/5 anion-exchange column (FPLC system; Pharmacia, Uppsala, Sweden) equilibrated previously with Tris/NaCl buffer. Toxic was eluted from the column by increasing the NaCl concentration. For iodine labelling of Cry1Ca, an additional purification step was performed, following the procedure described in Zhao et al. [17], to remove adsorbed protoxin fragments that block iodination.

**Insect bioassays**

Trypsin-activated toxins were assayed by diet surface contamination of a solid artificial diet. Neonate larvae of \(S.\ exigua\) and 1-day-old larvae of \(M.\ sexta\) were used for bioassays, and mortality was scored after 6 days at \(28^\circ C\). The LC\(_{50}\) (concentration causing 50 \% mortality) and its 95 \% fiducial limits were estimated by Probit analysis [18] of results from three or more independent experiments using the POLO-PC program (LeOra Software, Berkeley, CA, U.S.A.).

A GI (growth inhibition) assay was performed by diet surface contamination with 2 \(\mu\)g/cm\(^2\) toxin. Early last-instar larvae of \(S.\ exigua\) were weighed and allowed to eat contaminated diet. After 48 h, larvae were weighed again and the percentage GI was calculated as described previously [19]. Values of GI higher than 100 mean a loss of weight of the larvae during the assay.

**BBMV preparation**

BBMV to be used in binding assays were prepared according to the method described by Woltersberger [20]. The same protocol was used for the preparation of the BBMV used in oligomerization assays, except that EGTA was excluded from the buffers.

**\(^{125}\)I-labelling of Cry1Ca and binding assays**

Cry1Ca was labelled with \(^{125}\)I using Iodo-beads\(^{\text{\textregistered}}\) reagent (Pierce Biotechnology, Rockford, IL, U.S.A.) following the manufacturer’s recommendations. The specific radioactivity of labelled Cry1Ca was 0.57 mCi/mg, as estimated by sandwich ELISA. To determine specificity of binding and to select the concentration of BBMV for competition assays, BBMV from \(S.\ exigua\) (0–0.3 mg of total vesicle protein/ml) were incubated with 710 pM \(^{125}\)I-Cry1Ca in 100 \(\mu\)l of PBS containing 0.1 \% BSA at room temperature for 60 min. \(^{125}\)I-Cry1Ca bound to BBMV was separated from free toxin by centrifugation at 16,000 \(g\) at 4 \(^\circ\)C for 10 min. The pellet was washed twice with 500 \(\mu\)l of ice-cold PBS/BSA. The radioactivity in the pellet was then measured in a 1282 Compgamma CS Universal gamma counter (LKB- Wallac Pharmacia, Turku, Finland) and taken as total binding. Non-specific binding was estimated by adding a 1000-fold excess of unlabelled toxin to the reaction mixture. Specific binding was calculated as the difference between total and non-specific binding.

For competition assays, increasing amounts of unlabelled wild-type Cry1Ca, pPB19, pPB20 or pPB21 toxins were added to the reaction mixture containing 125I-Cry1Ca and 100 \(\mu\)g/ml BBMV in PBS/BSA. The estimation of dissociation constant (\(K_d\)) and binding site concentration (\(R_0\)) was performed with the LIGAND software package [21]. The parameter \(K_d\) refers to the apparent dissociation constant and not to the true equilibrium dissociation constant, since the binding of Cry proteins to BBMV is a two-step process involving reversible plus irreversible binding [22]. Statistical tests (Student’s *t* test) were performed and charts made using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, U.S.A.). Binding experiments were independently performed at least twice.

**Biotin labelling of Cry1Ca and binding assays**

Cry1Ca was labelled with biotin using biotin N-hydroxysuccinimide ester (Boehringer) as described previously [23]. For binding experiments, BBMV (10 \(\mu\)g of protein) were mixed with 1 ng of biotin-labelled toxin and increasing amounts of unlabelled Cry1Ca (1, 10, 100 and 1000 ng) in 50 \(\mu\)l of BBS (100 mM sodium borate, 150 mM NaCl, pH 8.0) containing 0.1 \% Tween-20.
After incubation for 1 h at room temperature, vesicles were separated from unbound toxin by centrifugation, and the pellet was washed once briefly with BBS/Tween. Subsequently, the pellets containing BBMV proteins (including bound, labelled toxin) were resuspended in 20 μl of 2% SDS in BBS. After heating at 100°C for 5 min, 200 μl of BBS was added to the sample and the whole volume was transferred on to a nitrocellulose membrane using a Minifold I Dot-Blot system (Schleicher & Schuell BioScience). Bound, labelled toxin on the blot was detected with streptavidin/peroxidase in a standard Western-blot protocol.

Oligomerization assays

Oligomerization assays were performed with small modifications of the method described by Rausell et al. [24]. Summarizing, 20 μl of solubilization buffer (50 mM sodium carbonate, pH 10.0) containing 10 pmol of protoxin was incubated with 10 μl of solubilization buffer containing different concentrations of BBMV (EGTA-free) from S. exigua or M. sexta for 15 or 30 min respectively. The incubation was stopped by the addition of 5 μl of a solution (10× concentrated) of Complete Protease Inhibitor Cocktail (Roche Diagnostics). Samples were then centrifuged at 12 000 g for 20 min. The supernatant was mixed with SDS/PAGE loading buffer and heated at 60°C for 10 min, and proteins in the sample were separated in an SDS/8%-PAGE gel. Monomeric and oligomeric forms of Cry1Ca were detected using antibodies raised against the Cry1Ca protoxin in a standard Western-blot protocol.

Toxin structure modelling

In order to obtain a three-dimensional model of Cry1Ca domain III, we used the Web-based modelling service SWISS-MODEL [25] with the structures of Cry3Aa (PDB code 1DLC) and Cry1Aa (1CIY) as templates. For this purpose, Cry1Ca was modelled in a first approach, and subsequently the hybrid G27 sequence (containing Cry1Ca domain III) was added to the sequence alignment, which was then edited and resubmitted for modelling. For visualization, a DeepView/Swiss-PdbViewer [26] generated script was used as input for POV-ray [Persistence of Vision Raytracer (version 3.6, 2004), Persistence of Vision Pty Ltd, Williamstown, Victoria, Australia].

RESULTS

Toxicity of Cry1Ca mutants

Cry1Ca amino acid residues were constructed in order to study the roles of different amino acids residues in the mode of action of the toxin. Mutant pPB19 was constructed to study the role of hypothetical loops 2 and 3 of domain II. Alanine substitutions of Gln 374 and Thr 440 were chosen based on previous work that showed a reduction in toxicity against S. exigua [15]. Mutant pPB20 was chosen to study the role of domain III by alanine substitution of residues Ser 541 to Val 544. The same mutations in the hybrid G27 (comprising domains I and II from Cry1Ca and domain III from Cry1Ca) reduced toxicity against S. exigua, but not against M. sexta [12]. Mutant pPB21 was generated by combination of the mutations of pPB19 and pPB20. All three mutants were highly expressed as inclusion bodies in E. coli, similar to wild-type Cry1Ca. The stability of the different toxins was compared by incubation for 24 h in the presence of S. exigua midgut juice; activation and degradation patterns similar to those of wild-type Cry1Ca toxin were observed (results not shown).

Bioassays of the wild-type and mutant toxins on neonate larvae of S. exigua revealed a biological effect of the mutations.

In agreement with previous results, where the above-described substitutions were shown to affect toxin activity, mutants pPB19 and pPB20 showed 35- and 30-fold decreases respectively in toxicity towards S. exigua (Table 1). A significantly stronger effect was detected when domain II and domain III substitutions were combined in the mutant pPB21: this mutant showed a 115-fold decrease in toxicity towards S. exigua (Table 1). No significant differences in toxicity among Cry1Ca and its mutants were detected when they were assayed against M. sexta larvae (Table 1). A decrease in the activity of the mutants was also detected for last-instar larvae of S. exigua, confirming that any change in the mode of action conferred by the mutations is maintained in the older larvae, used subsequently for molecular studies. Compared with the Cry1Ca wild-type toxin, GI expressed as percentage was reduced for all three mutants, with the pPB21 mutant having the lowest toxicity (Table 1).

Effects of mutations on binding to S. exigua BBMV

Competition of 125I-Cry1Ca with unlabelled Cry1Ca (‘homologous’) and its mutants (‘heterologous’) was performed in order to establish the effects of the mutations on binding to S. exigua BBMV. Binding parameters such as dissociation constant (Kd) and binding site concentration (Rt) were obtained for Cry1Ca and its mutants. Compared with Cry1Ca, the domain III mutants (pPB20 and pPB21) showed an approx. 8-fold decrease in affinity (higher Kd value) for the S. exigua binding sites (Table 2 and Figure 1A). The domain II mutant pPB19 showed a smaller, but statistically significant (t test; P = 0.0078), increase in Kd (lower affinity). An increase in the Kd value was also detected when comparing pPB21 with pPB20, although in this case it was not statistically significant (Table 2 and Figure 1A). These results indicate that the mutations introduced in domain III affected binding to S. exigua BBMV more drastically than those introduced in domain II.

| Table 1 | Toxicity of Cry1Ca and its mutant towards S. exigua and M. sexta larvae |
|-----------------|-----------------|-----------------|-----------------|
|                 | S. exigua | M. sexta |
| Toxin           | LC50 (ng/cm²) | GI (%) | LC50 (ng/cm²) |
| Cry1Ca          | 68 (47–88) | 110.8 ± 0.6 | 123 (94–152) |
| pPB19           | 2350 (1450–4101) | 27.2 ± 4.9 | 116 (90–145) |
| pPB20           | 1985 (1414–2693) | 51.0 ± 5.1 | 80 (61–100) |
| pPB21           | 7836 (5556–11 300) | 8.2 ± 6.5 | 85 (60–110) |

| Table 2 | Dissociation constant (Kd) and concentration of binding sites (Rt) estimated from competition experiments performed with 125I-labelled Cry1Ca and BBMV from S. exigua |
|-----------------|-----------------|-----------------|
|                 | Kd (nM) | Rt (pmol/mg of protein) |
| Cry1Ca          | 21.0 ± 1.9 | 18 ± 3 |
| pPB19           | 42 ± 4 | 13.1 ± 0.4 |
| pPB20           | 149 ± 16 | 14 ± 3 |
| pPB21           | 177 ± 4 | 15.6 ± 0.4 |
Figure 1  Effects of mutations in Cry1Ca on binding to S. exigua BBMV

Binding of labelled Cry1Ca to BBMV from Spodoptera exigua in the presence of increasing concentrations of unlabelled competitor is shown. (A) Competition of 125I-Cry1Ca with unlabelled Cry1Ca (■; thick solid line), pPB19 (○; broken line), pPB20 (▲; thin solid line) and pPB21 (●; dotted line). (B) Competition of biotin–Cry1Ca with increasing concentrations (1-, 10-, 100- and 1000-fold) of unlabelled Cry1Ca or Ppb19. (C) Competition of pPB19 for Cry1Ca binding sites was also detected semi-quantitatively using biotin-labelled Cry1Ca (Figure 1B). An excess of non-labelled competitor (Cry1Ca and pPB19) of 100× or 1000× reduced drastically the amount of biotinylated Cry1Ca that bound to S. exigua BBMV. As occurred in the competition experiments using 125I-Cry1Ca, in biotin-labelling experiments pPB19 showed a lower affinity for Cry1Ca binding sites than wild-type Cry1Ca. This is reflected by, at a competitor concentration of 10- or 100-fold excess, a higher intensity of the spots of biotinylated Cry1Ca in the competition with pPB19 compared with the competition with unlabelled Cry1Ca (Figure 1B).

Toxin oligomerization assays

Oligomerization of the Cry toxins prior to membrane insertion was described recently as an important step in the mode of action [3,24]. Additionally, the activation of protoxin in the presence of BBMV or in the presence of a peptide mimicking the cadherin-like protein promoted the formation of such a toxin oligomer [3,24,28,29]. We decided to study these phenomena in the Cry1Ca–S. exigua interaction and, if found, to check whether it is altered with the mutants under study.

In Figures 2(A) and 2(B) we show a comparison of the activation of Cry1Ca and pPB21 protoxins by incubation with different concentrations of S. exigua EGTA-free BBMV. Oligomeric forms with a molecular mass greater than 250 kDa were detected on immunoblots when the Cry1Ca protoxin was activated using BBMV from S. exigua (Figure 2B, lanes 1–3). Oligomeric forms, though present in the Coomassie Blue-stained gel, were not easily visible because of their low concentration and the lower sensitivity of Coomassie Blue staining compared with immunoblot detection (Figure 2A). Incubation of Cry1Ca protoxin with different amounts of S. exigua BBMV showed that the oligomer concentration decreased with increasing BBMV concentration, concurrent with increased levels of fully activated toxin. Under the experimental conditions used in this work, the activation of Cry1Ca protoxin with 1 µg of S. exigua BBMV generated the highest concentration of oligomeric forms (Figure 2B, lane 1). Incubation of Cry1Ca protoxin with 10 µg of BBMV was enough to fully activate most of the protoxin, reducing at the same time the amount of the oligomer (Figure 2B, lane 3).

In order to establish whether some of the studied mutants were affected with regard to toxin oligomerization, a similar experiment was performed using mutant pPB21. Coomassie Blue staining of proteins generated by activation of pPB21 protoxin in the presence of S. exigua BBMV showed the same pattern as obtained with Cry1Ca protoxin (Figure 2A). In contrast with results with Cry1Ca, immunoblots revealed the absence of the oligomeric form at all BBMV concentrations tested (Figure 2B, lanes 4–6). During the activation of pPB21, as well as during the activation of Cry1Ca, a molecule with an apparent molecular mass of 100 kDa was detected at lower BBMV concentrations.

Figure 2  Cry1Ca and pPB21 activation and oligomerization with S. exigua and M. sexta BBMV

Portions of 20 µl of solubilization buffer containing 10 pmol of Cry1Ca or pPB21 protoxin were incubated with 10 µl of solubilization buffer containing 1, 5 or 10 µg of BBMV proteins from S. exigua (A and B) or M. sexta (C and D) for 15 or 30 min respectively. Proteins in the sample were separated by SDS/8%-PAGE. Coomassie Brilliant Blue staining (A and C) and immunodetection of Cry1Ca on a Western blot (B and D) were performed.

© 2004 Biochemical Society
behaviour of the different mutants with that of wild-type Cry1Ca. These regions in determining insect specificity, we compared the toxicity against Spodoptera exigua (A and B) or M. sexta (C and D) for 15 or 30 min respectively. Proteins in the sample were separated by SDS/8%-PAGE. Coomassie Brilliant Blue staining (A and C) and immunodetection of Cry1Ca on a Western blot (B and D) were performed. Lanes 5 and 10 contain Cry1Ca treated under the same conditions but in the absence of BBMV.

Figure 3 Oligomer formation of Cry1Ca and its mutants by activation with S. exigua and M. sexta BBMV

Portions of 20 µl of solubilization buffer containing 10 pmol of Cry1Ca, pPB19, pPB20 or pPB21 protoxin were incubated with 10 µl of solubilization buffer containing 2 µg of BBMV from S. exigua (A and B) or M. sexta (C and D) for 15 or 30 min respectively. Proteins in the sample were separated by SDS/8%-PAGE. Coomassie Brilliant Blue staining (A and C) and immunodetection of Cry1Ca on a Western blot (B and D) were performed. Lanes 5 and 10 contain Cry1Ca treated under the same conditions but in the absence of BBMV.

In the present study we have focused on changes in the Cry1Ca primary structure that can lead to a change in insect specificity without completely abolishing insecticidal activity. We constructed Cry1Ca mutant proteins containing mutations in domains II and III and compared their toxicity against S. exigua and M. sexta. Bioassay results showed that although these mutations markedly reduced toxicity against S. exigua, they did not affect toxicity against M. sexta. For a better understanding of the role of these regions in determining insect specificity, we compared the behaviour of the different mutants with that of wild-type Cry1Ca toxin in three steps in the mode of action of Cry toxins: activation, receptor binding and toxin oligomerization.

The importance of domain II (specifically loops 2 and 3) in determining the toxicity against S. exigua has been documented previously. Yamamoto and Dean [15] reported that the double mutant Gln374Ala/Thr440Ala had reduced toxicity against S. exigua (although no toxicity values were given). Residue Gln253 (domain II) has also been shown to be important in determining the specific toxicity of Cry1Ca against Aedes aegypti larvae (Diptera). The mutation of Gln253 Asn reduced toxicity towards A. aegypti without affecting toxicity towards Spodoptera littoralis [30]. In contrast, the mutation Gln253Glu reduced the binding capacity and toxicity for both insect species.

We have shown that the reduced toxicity of pPB19 (domain II mutant) against S. exigua does not seem to be due directly to alterations in overall binding affinity, which was just 2-fold lower than that of Cry1Ca. This result is in contrast with that reported previously by Yamamoto and Dean [15], who showed a complete lack of competition of this mutant for Cry1Ca binding sites using biotin-labelled Cry1Ca. To discount the possibility that the differences in these results were caused by differences in the method employed, we also performed the competition experiment using biotin-labelled Cry1Ca, and obtained results that supported those obtained using 125I-Cry1Ca. We do not have a definitive explanation for these differences in the findings of the two studies. It is possible that the source of the S. exigua colonies employed in the respective assays could account for the discrepancy. Differences in affinity (Kd) of approx. 5-fold have been described for different populations of Helicoverpa armigera [31].

Regarding mutations affecting domain III, replacement by alanine of residues Ser541 to Val544 was based on previous results, where a hybrid toxin G27 (domains I and II from Cry1Ea and domain III from Cry1Ca) containing such substitutions showed a reduction in toxicity against S. exigua but not against M. sexta [12]. These changes in Cry1Ca, resulting in mutant pPB20, also had a marked effect on toxicity against S. exigua larvae, but not against M. sexta, revealing the importance of these residues in determining the specificity of Cry1Ca. Here, the decrease in toxicity was correlated with a much greater reduction (approx. 7-fold) in the affinity for the Cry1Ca binding sites present in BBMV from S. exigua. Our model of the three-dimensional structure of Cry1Ca domain III shows that residues 541–544 are probably part of a surface-exposed loop consisting of residues

DISCUSSION

In the present study we have focused on changes in the Cry1Ca primary structure that can lead to a change in insect specificity without completely abolishing insecticidal activity. We constructed Cry1Ca mutant proteins containing mutations in domains II and III and compared their toxicity against S. exigua and M. sexta. Bioassay results showed that although these mutations markedly reduced toxicity against S. exigua, they did not affect toxicity against M. sexta. For a better understanding of the role of these regions in determining insect specificity, we compared the behaviour of the different mutants with that of wild-type Cry1Ca
539–546 connecting two β-strands on the slightly concave outer β-sheet (Figure 4). The location of a tryptophan residue, which was shown previously to also be involved in specificity for S. exigua, is also indicated [12]. Thus we have shown that, similar to earlier results for binding of Cry1Ac to aminopeptidase N of M. sexta [14], this region of domain III is involved in receptor binding. Whereas the mutations in Cry1Ac had no apparent effect on toxicity against M. sexta in bioassays [14], the mutations reported here destroy activity against S. exigua. Activity against M. sexta is not affected, indicating that decreased stability of the mutant protein is not a likely explanation.

It has been described that activation of Cry1Ab with M. sexta BBMV or a peptide mimicking cadherin-like protein (BTR) promotes toxin oligomer formation, and that such oligomerization is dependent on the interaction with a domain II loop 2 region [29,32]. To test whether oligomerization could also be a step in the mode of action of Cry1Ca, and whether it could be impaired by some of the studied mutations, we first checked if Cry1Ca was able to form oligomers during interaction with BBMV. We found that, similarly to Cry1Ab and M. sexta BBMV, oligomer structures were also observed with Cry1Ca and S. exigua BBMV. However, pPB19 and pPB21 toxins (both with domain II mutations) were not able to form oligomers when activated with S. exigua BBMV, while pPB20 formed oligomers in a similar way to wild-type Cry1Ca. The importance of this phenomenon in the mode of action is reinforced by the observation that all mutants were equally toxic as Cry1Ca against M. sexta and that oligomer formation was not affected in any of them. Thus the mutations in pPB19 do not affect the capacity to form oligomers per se. From these results, we can conclude: (i) changes in domain II loops, but not in domain III, affect the interaction necessary for oligomer formation of Cry1Ca in S. exigua, and (ii) these same changes do not affect Cry1Ca oligomerization in M. sexta.

A previous study [33], in which residues in loop 2 of Cry1Ab toxin were replaced by alanine, showed that mutant Phe537Ala had reduced toxicity for M. sexta larvae. Competitions experiments revealed that this mutant had similar binding parameters as Cry1Ab, but dissociation binding experiments showed that the percentage of irreversible binding (membrane insertion) was lower. It would be interesting to determine whether this mutant is affected in oligomer formation in M. sexta.

In another study, domain II and domain III of Cry1C were expressed separately. Binding experiments in Spodoptera littoralis comparing the binding parameters of whole Cry1C and isolated domain II showed that, of the overall number of binding sites for Cry1C toxin, approx. 10 % are domain II binding sites [34]. According to those results, specific changes in domain II would hardly be reflected in overall binding, something that may be occurring with the pPB19 mutant and S. exigua BBMV. The occurrence of several binding sites for a Cry toxin can hide the lack of one binding site which, although present at a low concentrations, could be as important for the mode of action as other more abundant ones. Along the same lines, changes in these toxin binding sites in the insect would not be reflected in a large change in binding parameters. Such a situation may have occurred in strains of Plutella xylostella and S. exigua that are highly resistant to Cry1Ca, for which binding studies reported just a small change in binding capacity [17,35]. This theory is supported by the observation that lack of a cadherin-like receptor in a Cry1A-resistant strain of Heliothis virescens (YH2D) [36] hardly affects the detectable binding of Cry1Ab or Cry1Ac in the resistant strain [37]. Recently, the study of a strain of Pectinophora gossypiella (AZP-R) resistant to Cry1A toxins revealed that, although a cadherin-like receptor was mutated and the binding of Cry1Ab was altered, no differences were detected in the binding of Cry1Ac toxin [38,39].

In summary, in the present study we have shown how changes in the primary structure of Cry1Ca can be correlated with changes in different steps of its mode of action. We showed, for the first time, a clear correlation between toxin activity and the ability of the toxin to form oligomers after interaction with target membranes. Our data support a model where both domain II and domain III of Cry1Ca are involved in determining insect specificity through at least two different kinds of interaction: (i) the interaction of loops 2 and 3 of domain II with presumably a low-abundance binding site, and (ii) the interaction between residues 541–544 of domain III and a more abundant, second binding site.

We thank Dr Jules Beekwilder (PRI) for assistance with molecular graphics, and Dr A. Bravo for her helpful advice in the oligomerization assay protocol. R. d. M. was supported by Program subsidy 347 of the Dutch Ministry of Agriculture, Nature Management and Fisheries. S. H. was supported by a postdoctoral fellowship from the Spanish Ministry of Science and Technology and by a Marie Curie fellowship contract no. HPMF-CT-2002-01994 from the EU. The work performed in the University of Valencia was supported in part by the Spanish Ministry of Science and Technology and European FEDER funds (project no. AGL2003-05282-C03-01/AGR).

REFERENCES
Mutations affecting Cry1Ca specificity

20 Gonzalez-Cabrera, J., Escriche, B. and Ferrè, J. (2003) Binding of Bacillus thuringiensis Cry1Ab toxin specificity. Two structural determinants in the Manduca sexta Br-R1 receptor interact with loops alpha-8 and 2 in domain II of Cry1Ab toxin. Biochemistry 42, 10482–10489

2004 Biochemical Society