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The pre-pore from *Bacillus thuringiensis* Cry1Ab toxin is necessary to induce insect death in *Manduca sexta*

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Abstract

The insecticidal Cry toxins from *Bacillus thuringiensis* bacteria are pore-forming toxins that lyse midgut epithelial cells in insects. We have previously proposed that they form pre-pore oligomeric intermediates before membrane insertion.

For formation of these oligomers coiled-coil structures are important, and helix α -3 from Cry toxins could form coiled-coils. Our data shows that different mutations in helix α -3 are affected in pore formation and toxicity. Mutants affected in toxicity bind Bt-R₁ receptor with a similar K_D as the wild type toxin but do not form oligomers nor induce pore formation in planar lipid bilayers, indicating that the pre-pore oligomer is an obligate intermediate in the intoxication of Cry1Ab toxin and that interaction of monomeric Cry1Ab with Bt-R₁ is not enough to kill susceptible larvae.

1. Introduction

Pore forming toxins are biological weapons produced by a variety of living organisms, particularly bacteria but also some insects, reptiles and marine invertebrates [34]. These proteins affect the membrane of target cells, disrupting the permeability barrier, and leading eventually to cell death. The pore-forming toxins typically transform from soluble, monomeric proteins to oligomers that form transmembrane ionic channels [34]. The Cry toxins produced by *Bacillus thuringiensis* (Bt) are widely used as insecticides [10,35,39]. These proteins have been recognized as pore-forming toxins and their primary action is to lyse midgut epithelial cells in target insects by forming lytic pores in the apical microvilli membrane. It has been proposed that in order to exert their toxic effect, a pre-pore oligomeric intermediate is formed leading finally to membrane inserted oligomeric pores [3,35]. However, an alternative model proposed that the toxicity of Cry proteins is mainly due to the interaction of monomeric toxin with the Bt-R₁ receptor activating a PKA signaling pathway that results in cell death [45,35].

Numerous Bt strains have been isolated that show activity towards lepidopteran, dipteran or coleopteran insects. In addition, Bt strains active against Hymenoptera, Homoptera, Orthoptera and Mallophaga insect orders and to other non-insect organisms like nematodes, mites and protozoa have been reported [10,39]. The entomopathogenic activity of Bt is mainly due to Cry toxins. These toxins are highly specific to their target insect, innocuous to humans, vertebrates and plants, and completely biodegradable. Therefore, Bt is a viable alternative for

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the control of insect pests in agriculture and disease vectors of importance in public health. More than 150 different *cry* genes have been isolated. This constitutes an important arsenal for the control of a wide variety of insect pests [10].

The 3-D structures of several Cry toxins have been solved. The overall topology is similar within these toxins, suggesting similar mechanisms of action. The toxins are composed of three structural domains. Domain I is a seven α -helix bundle in which a central helix α -5 is surrounded by six outer helices. This domain has been implicated in formation of a membrane channel. Domain II consist of three anti-parallel β -sheets packed around a hydrophobic core in a “beta-prism”. This domain has been described as the specificity-determining domain. Finally, domain III is a β -sandwich of two antiparallel β -sheets. It is also implicated in insect specificity since it is involved in receptor binding [10,39]

Despite the fact that Cry toxins are widely used as insecticides, their mode of action is still not completely understood. In this review, we will summarize our current knowledge on the mode of action of Cry toxins that are active against lepidopteran insects and provide some novel information regarding the role of the toxin oligomeric structure in forming ionic channels in the targeted membrane and in the overall toxicity against insect larvae.

2. Models of the mode of action of Cry toxins in lepidopteran insects

Different models describing the mechanism of action of Cry toxins have been reported. For many years the Cry toxins have been recognized as pore-forming toxins that induce cell death by forming ionic pores [10,35,39]. Cry toxins insert into lipid rafts membranes present in the apical microvilli membrane of midgut cells of susceptible larvae, causing osmotic lysis and subsequent death of the larvae [3,10,39,46]. A second model proposed that these toxins activate a signal pathway through receptor interaction, resulting in insect cell death without the participation of lytic-pores [45]. It is important to note that this alternative model was proposed based on the effect of Cry1Ab toxin to cultured *Trichoplusia ni* H5 insect cells expressing the *Manduca sexta* toxin-receptor, Bt-R₁, not in insect larvae. Finally, a third model suggested that besides membrane disruption by Cry toxins, other midgut bacteria are necessary to produce septicemia that kill the larvae [4].

In the pore forming- and signal transduction-models, toxin receptor interaction is a key step that determines toxicity. In the case of Cry1A toxins, two receptors have been characterized in several lepidopteran species, cadherin-like proteins, (named Bt-R₁ in the case of *M. sexta*) [42], and the GPI-anchored proteins aminopeptidase-N (APN) and alkaline phosphatase [22, 25].

In the pore-forming model, it is proposed that Bt-R₁ and GPI-anchored receptors are important and participate in a sequential manner [3,23]. After proteolytic activation of Cry1A protoxin by insect midgut proteases, the activated toxin binds to the Bt-R₁ receptor, and this interaction facilitates additional cleavage of the N-terminal end of the toxin, eliminating helix α -1, resulting in the formation of a pre-pore oligomeric structure [13]. The oligomeric structure has been observed in several Cry toxins [1,13,18,26,30,33,36,37,40]. The pre-pore binds to a second receptor, the GPI-anchored aminopeptidase, due to its higher affinity to this receptor [3], facilitating the insertion of the oligomer into membrane lipid rafts and forming pores [3, 46]. Importantly, another GPI-anchored protein, an alkaline phosphatase, has been recognized as a Cry1Ac receptor in *Heliothis virescens* and *M. sexta* [22,23]. Alkaline phosphatase could also participate in facilitating the pre-pore membrane insertion into lipid rafts, explaining why Cry1Ac toxin mutations that were severely affected in APN binding are still active against *M. sexta* larvae [6,20]. In the pore forming model, the pre-pore oligomer is responsible for initiating cell death [3]. In contrast, in the signal transduction model [45], only the binding of the monomeric form of the Cry toxin to Bt-R₁ receptor is responsible for toxicity, and

interaction with the GPI-anchored receptors and pore formation are not relevant for Cry toxicity. It was proposed in the signal transduction model that binding of Cry toxin to Bt-R₁ receptor activates a G protein and adenylyl cyclase, which promotes production of intracellular cAMP. The increased levels of cAMP activate Protein Kinase A that causes cell death [45].

3. Role of coiled coil structures in oligomerization of different proteins

There are many proteins that form stable oligomeric structures and many of these oligomers are stabilized by α -helical coiled-coil structures. These coiled-coils constitute an important protein-folding motif formed in the interaction between two to five α -helices. They are involved in oligomerization of several proteins, forming specific oligomers with high thermodynamic stability [5,9]. The primary structure of coiled-coil structures is characterized by heptads of residues, $(abcdefg)_n$, with a unique pattern of internal a and d positions occupied mostly by apolar residues forming an hydrophobic core and positions g and e occupied by charged residues. The hydrophobic core of coiled coils is important for protein-protein interactions with a matching α -helix and mutagenesis of these positions affects the stability of this interaction [32]. It is important to note that the presence of charged residues at the core positions of coiled coils are correlated with proper alignment, orientation and selectivity of coiled-coils and contribute considerably to their stability [9,32].

3.1. Analysis of coiled-coil structures in Cry toxin family and their role in toxin oligomerization

Since domain I is a seven α -helices-bundle we hypothesized that coiled-coil structures of some α -helices might be involved in oligomer formation. We analyzed the possibility of the formation of coiled-coil structures by using the program COILS that compares a sequence to a database of known coiled-coils and derives a similarity score. Then by comparing this score to the distribution of scores in globular and coiled-coil proteins, this program calculates the probability that the sequence analyzed will adopt a coiled-coil conformation [28]. Analyses of protein sequences of some Cry toxins revealed that helix α -3 gave the highest score (0.4 for Cry1Ab, 0.97 for Cry2Aa, 0.99 for Cry3A, and 0.90 for Cry3Ba). Although the primary structure of helix α -3 is not conserved among these toxins, it is surprising that positions a and d of the predicted coiled-coil structure are highly conserved (Fig 1). We found that residues of helix α -3 in Cry1Ab at positions d (W117, Y110, L103 and A96) are present in all Cry1, Cry3, Cry7, Cry8, Cry9 toxins, while Cry4, Cry2 and Cry10 toxins show conservative substitutions. The same analysis showed that residues corresponding to positions a (R93, L100, Y107, F114) are also highly conserved among the Cry toxin family members, the first residue being a positive charged residue (R or K) while the three subsequent residues correspond to hydrophobic amino acids (Y, F, V or L). The high conservation in positions a and d in helix α -3 in the Cry toxin family, strongly suggests that a putative coiled-coil structure in this helix could have an important role in the mode of action of these toxins

Oligomerization assays performed in the presence of an 100 fold excess of the synthetic peptide corresponding to helix α -3 demonstrated that this helix inhibited formation of the 250 kDa oligomer. In contrast synthetic peptides corresponding to other helices of domain I did not affect oligomer formation [21].

3.2 Mutagenesis of helix α -3 of different Cry toxins

Some mutations in helix α -3 of Cry1Aa, Cry1Ab and Cry1Ac toxins have been characterized. The Cry1Aa toxin has been subjected to extensive mutagenesis of charged residues. Although none of the mutated residues corresponded to positions a or d of the predicted coiled-coil structure, most of the characterized Cry1Aa mutants were affected in the rate of pore-formation [41]. Specially, substitutions in residue R99 resulted in an extremely low toxicity to *M. sexta*

larvae and no-pore formation activity using a light scattering assay [41]. The observed changes in pore formation in these mutants could be explained if they were affected in oligomer formation, since this is important for channel structure.

The residue R99 of Cry1Aa is located in position *g* of the predicted coiled-coil structure. Recently, it was found that a similar change constructed in Cry1Ab toxin (mutant R99E) was severely affected in toxicity to *M. sexta* and in pore formation activity when analyzed in black lipid bilayers [21]. The low response in pore formation of mutant R99E correlated with the inability of this mutant to form the 250 kDa oligomeric structure [21]. In addition the binding characteristics of this mutant was analyzed, showing that mutant R99E was able to bind to BBMV with similar high affinity as the wild type, since binding competition of biotinylated-Cry1Ab toxin with unlabelled mutant protein was identical to that of the unlabelled wild type toxin [21]. Specifically the binding interaction with Bt-R₁ was not affected, since ligand blots and ELISA binding competition assay of the mutant R99E to a Bt-R₁ protein fragment that contains all the Cry1A binding sites characterized so far (CR7 to CR12) [14,15,19] showed that it bound to Bt-R₁ protein fragment with the same K_D as the wild type Cry1Ab toxin [21]. It is important to mention that the procedure of ELISA competition assay permits the determination of true association rate constants in solution, and several reports have shown agreement in the determination of K_D values by these assays and those obtained by conventional methods (immunoprecipitation of the radiolabeled antigen, fluorescence transfer or surface plasmon resonance) [11,12,17]. Other important mutations performed in helix α -3 of Cry1Ac toxin are those introduced in residues R93 and A92. These mutants were also affected toxicity, but only the conservative change R93K was fully active, while other changes of R93 (G, A, and S) showed 100-1000 fold reduced toxicity and many other changes resulted in complete loss of toxicity [8,44]. The authors of this study concluded that a positive charge in this position could participate in the formation of specific binding domain or that it could be important for the arrangement of α -helices in the amino terminal region of the toxin, and that the conformation of this domain could indirectly disrupt the specificity domain of the toxin [44]. The introduction of a negative charge in residue A92 supported this hypothesis since mutations A92E and A92D were severely affected in toxicity and correlated with loss of pore formation [8,44]. It was finally proposed that the irreversible insertion into the BBMV was affected in A92E and A92D mutants [8]. Residue R93 is located in position *a* of the predicted coiled coil structure of helix α -3 (Fig 1), and as stated above this is a highly conserved amino acid within the Cry toxin family. The presence of polar residues in the apolar interface has been documented for some coiled coil structures, it is correlated with proper alignment, orientation and selectivity of coiled-coils and contribute considerably to their stability [5,9]. In addition, it has been reported that a salt bridge within the α -helix that forms the coiled-coil stabilizes this structure and is important to trigger the coiled-coil [24]. The residue R93 of Cry1A toxins participate in a salt-bridged [16] suggesting that this helix could play an important role in toxin oligomerization.

Unfortunately the authors did not analyze oligomer formation or pore activity of R93 mutants. More recently we isolated a mutant R93F of Cry1Ab toxin, but this mutation resulted in formation of an unstable small crystal inclusion that was not analyzed further. However, we were able to isolate another mutant in Cry1Ab located in position *a* of the coiled coil structure of helix α -3, Y107E. This mutant was severely affected in toxicity to *M. sexta* with a $LC_{50} > 2000$ ng/cm². Oligomer formation was not observed with this mutant, and the pore formation activity was not present when analyzed in black lipid bilayers, suggesting an important role of this residue in oligomerization and channel formation [21]. It is important to mention that the binding affinity of this mutant to Bt-R₁ receptor was not affected and the K_D value obtained was similar to that of the wild type toxin [21].

4. Analysis of pore formation induced by oligomeric structures of different Cry toxins

Different approaches have been used to study pore formation activity. One approach analyses changes in ion permeability using a fluorescent dye that is sensitive to changes in membrane potential, 3,3'-dipropylthiodicarbocyanine iodide (diS-C₃(5)) [2,27,43]. Using this strategy it was reported that oligomeric Cry1Ab induced a dose-dependent K⁺ permeability and that the oligomeric Cry1Ab structure has a much higher pore-forming activity than the corresponding monomeric structure [30].

The oligomer and pore formation activities of other Cry toxins have also been reported (Cry1Aa, Cry1Ca, Cry1Da, Cry1Ea, and Cry1Fa) [30], showing that samples containing the oligomeric toxin showed higher pore-formation activity than samples activated with trypsin that only contain monomeric toxin [30]. This data shows that the oligomeric structure of Cry toxins, but not the monomer, is the intermediate that is responsible for insertion into the membrane and pore formation.

5. Final remarks

Most of the bacterial toxins known as pore forming toxins are secreted as water-soluble proteins and undergo conformational changes in order to insert into cell membrane of their target. These toxins are activated by host proteases after receptor binding inducing the formation of an oligomeric pre-pore structure that is insertion competent [34].

To determine the role of the pre-pore oligomer formation in the toxicity of Cry toxins some single-point mutations that affected pre-pore formation were isolated, showing that helix α -3, of domain I of Cry toxins is involved in oligomerization and mutations in some residues of this helix severely affected pore formation and toxicity without any effect in the binding interaction with Bt-R₁ receptor [21]. These data suggest that oligomer formation is an important step in the mechanism of action and that the pre-pore is playing a major role in toxicity in insect larvae.

Formation of the pre-pore oligomer has been observed in several Cry proteins with different insect specificity including lepidopteran specific, Cry1Ab [13,36,40] Cry1Ac [33]. Cry1Ca [18,30], Cry1Da, Cry1Ea, Cry1Fa [30]; coleopteran specific, Cry3Aa, Cry3Ba, Cry3Ca [37], and dipteran specific, Cry4Ba [26] and Cry11Aa (Pérez and Bravo, unpublished results). Protein samples containing Cry1Ab pre-pore oligomer were more toxic to *M. sexta* larvae than protein samples containing only monomeric Cry1Ab toxin [13]. In addition, Cry toxin samples containing oligomeric structures correlated with high pore activity in contrast to monomeric samples that showed marginal pore-formation activity [13,30,36]. The Cry1Ab pre-pore oligomer is membrane insertion competent in contrast to monomeric toxin that showed low membrane insertion rates [36]. The pre-pore oligomer induces high stable conductance in synthetic lipid bilayers suggesting insertion of a single conformation. In contrast insertion of the monomeric toxin induced multiple subconducting states showing unstable traces with current jumps of intermediate levels that are difficult to resolve [36]. All these data support the pore forming model, suggesting that the pre-pore oligomer is an obligate intermediate in the intoxication of Cry1Ab toxin and that the interaction of monomeric Cry1Ab with Bt-R₁ is not sufficient to kill susceptible larvae. However, we cannot, exclude the possibility that intracellular responses also have a role and synergize the effect of the toxin in insect larvae. In fact, we know that Cry toxins insert into lipid rafts membranes and it is important to mention that lipid rafts are detergent resistant membranes enriched in glycosphingolipids, cholesterol and GPI-anchored proteins and are involved in signal transduction, sorting and trafficking of plasma membrane proteins in mammalian cells [38]. Also, they function as pathogen portals

for different viruses, bacteria and toxins [7,38]. Recently, we demonstrated that the APNs in *M. sexta* and *H. virescens*, are located in lipid rafts, and that the integrity of these microdomains is essential for Cry1Ab pore activity [46]. These data could indicate that signal transduction induced by Cry toxin insertion into lipid rafts could be part of the mechanism of action of these toxins, but this remains to be demonstrated. It is important to mention that other pore forming toxins, such as the α -toxin from *Staphylococcus aureus* and aerolysin produced by *Aeromonas hydrophila*, induced cell death by two different mechanisms, pore-formation and apoptosis, depending on the cell type and on the dose of toxin [29,31]. Over-expression of antiapoptotic protein could block aerolysin-induced apoptosis, although this effect was overcome if higher toxin concentrations were used, where cells died quickly due to pore formation and apoptotic pathway was not triggered [31]. In the case of aerolysin it was demonstrated that apoptosis was not directly triggered by binding of the toxin to its receptor, but rather it was caused by the production of a small number of channels in the membrane [31] indicating that the intracellular downstream effects were triggered by pore formation and membrane depolarization. The data presented in this work also indicate that pore formation induced by oligomeric toxin is required for Cry1A toxicity *in vivo*.

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Figure 1. Analysis of coiled coil structures in Cry toxins

Panel A, Schematic representation of the putative coiled-coil structures of helix α -3 from different Cry toxins. The position of residues *a*, *b*, *c*, *d*, *e*, *f*, and *g* of the heptad and the conserved amino acid residues located in positions *a* and *d* are shown.

Panel B, Alignment of the amino acid sequence corresponding to helix α -3 of different Cry toxins. Green letters correspond to hydrophobic amino acid residues; blue to positively charged residues and pink to polar amino acids.