

MiniReview

Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action

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Abstract

The spore-forming bacterium *Bacillus thuringiensis* produces intracellular inclusions comprised of protoxins active on several orders of insects. These highly effective and specific toxins have great potential in agriculture and for the control of disease-related insect vectors. Inclusions ingested by larvae are solubilized and converted to active toxins in the midgut. There are two major classes, the cytolytic toxins and the δ -endotoxins. The former are produced by *B. thuringiensis* subspecies active on Diptera. The latter, which will be the focus of this review, are more prevalent and active on at least three orders of insects. They have a three-domain structure with extensive functional interactions among the domains. The initial reversible binding to receptors on larval midgut cells is largely dependent upon domains II and III. Subsequent steps involve toxin insertion into the membrane and aggregation, leading to the formation of gated, cation-selective channels. The channels are comprised of certain amphipathic helices in domain I, but the three processes of insertion, aggregation and the formation of functional channels are probably dependent upon all three domains. Lethality is believed to be due to destruction of the transmembrane potential, with the subsequent osmotic lysis of cells lining the midgut. In this review, the mode of action of these δ -endotoxins will be discussed with emphasis on unique features. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The spore-forming bacterium, *Bacillus thuringiensis* (*Bt*) is closely related to two other important spore-forming Bacilli, *Bacillus cereus* and *Bacillus anthracis*, and is differentiated largely on the basis of containing several plasmid-encoded protoxin genes. There are hundreds of *Bt* subspecies and most produce, primarily during sporulation, one or more parasporal inclusions, each comprised of either one or several related insecticidal protoxins, the so-called δ -endotoxins. Each of these toxins is active on a subset of insect larvae from at least three orders of insects [1]. Because of unique but overlapping specificity profiles, *Bt* subspecies are generally effective against a broad range of insects, usually within a particular order of insects. Many also produce, during growth, less well characterized insecticidal proteins, the so-called vegetative insecticidal proteins [2] as well as secreting other pathogenic factors [3].

Since a substantial fraction of the post-exponential metabolism of these bacteria is devoted to the synthesis of these highly specific insecticidal proteins, there must be some survival benefit to their production. While *Bt* strains have been isolated from many environments, some or all of the natural habitats must involve interactions with insect larvae. Epizootic infections are rare, however, so it is not likely that the primary reason for toxin production is to allow *Bt* to exploit nutrient-rich insect larvae. The bacterium is more likely to have a more subtle symbiotic interaction, perhaps with plants, to account for the extensive production of these highly specific and efficacious proteins. In fact, the commercial use of suspensions of spores and inclusions has been limited in part due to the need to spray at rather frequent intervals in order to sustain an effective level of the biopesticide. Not only are the δ -endotoxins readily inactivated, but the number of spores and/or vegetative cells decreases rather rapidly in the sprayed area. This problem has been circumvented by engineering plants (cotton, corn, potato, rice, etc.) to produce the toxin [1].

When insect larvae ingest inclusions (and the spores, which can be synergistic), the protoxins are solubilized and processed to toxins by midgut proteases. On the basis of *in vitro* studies, it is most likely that the toxin binds to receptors on cells lining the larval midgut, inserts into the cell membrane and forms ion channels (which are somewhat cation-selective) resulting in loss of the transmembrane potential which leads to osmotic cell lysis [4]. While this is the generally accepted mode of action, it should be noted that at sublethal concentrations of toxin, there are larval behavioral changes such as avoidance of the toxin during feeding and paralysis of feeding. These effects may be due to the formation of ion channels but a different or additional mechanism of toxin activity has not been excluded.

The mode of action of these toxins has been well reviewed [1,4–7], including a recent thorough presentation of the specificity of toxin-binding to receptors [1]. In this review, we shall discuss recent studies on the steps involved in toxin-binding, insertion and organization within the membrane which are unique features of these very effective biological insecticides.

2. An overview of toxin structure

Most of the research has been done with toxins active on Lepidoptera, designated CryI (see [8] for the terminology). The structures of a CryI toxin, CryIAa, and of a toxin active on Coleoptera, Cry3A, have been determined and are very similar despite only about 25–30% amino acid sequence identity [9,10]. There is another major group of toxins, designated Cry4 and CryIIb, which are active on Diptera. There may be additional toxins which are selective for other orders of insects as well as binary toxins including the dipteran-active toxin produced by *Bacillus sphaericus* [11]. The Cry3A and CryIAa toxins have a three-domain structure (Fig. 1) consisting of a seven α -helix bundle for domain I with six of the helices surrounding a central helix $\alpha 5$. Domains II and III are comprised of β -sheets but in different conformations, with domain III containing two antiparallel β -sheet sandwich structures. The helices in domain I are largely amphipathic, with the $\alpha 4$ -loop- $\alpha 5$ region being the most hydrophobic [10]. There are numerous studies, primarily mutagenesis, implicating domain I, especially helices $\alpha 4$ and $\alpha 5$, in the formation and function of the ion channel. Domains II and III have functions in specific binding to receptors and for domain III, in modulating ion channel activity [1].

The structure of domain I is very similar to pore-forming regions of the E1 group of colicins and the B subunit of diphtheria toxin, despite little sequence similarity among these toxins [12]. The monomeric form of colicin E1 may be sufficient to form ion channels in bacterial membranes. The state of oligomerization of the diphtheria

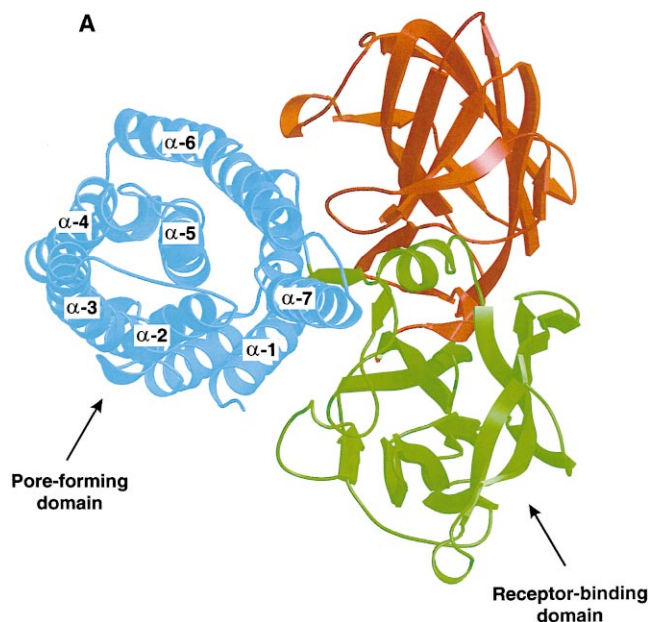


Fig. 1. Schematic ribbon representation of the Cry3A δ -endotoxin based on the structure of Li et al. [10]. The three domains are indicated with domain I (blue), comprised of seven α -helices, designated as the pore-forming domain. Domain II (green) is marked as a receptor-binding domain with certain loops being critical for this process [2]. The anti-parallel β -sheet sandwich structure of domain III is indicated in red. This domain functions both in binding to the receptor, probably via a lectin-binding pocket, and in modulating ion channel activity. Helix $\alpha 7$ is the one closest to domains II and III and may be responsible for the 'opening up' of domain I after a second step of toxin-binding to the receptor.

B subunit is not known and ion channels formed by δ endotoxins appear to be oligomers (see below). In contrast, toxins comprised of β -sheets, such as the pore-forming aerolysin and the α hemolysin of *Staphylococcus aureus*, form heptamers at the membrane surface prior to insertion [12].

3. Toxins initially bind reversibly to larval membrane receptors

Inclusions are solubilized by the high pH and reducing conditions in the midgut of lepidopteran and dipteran larvae. The protoxins of ca. 130 kDa are then proteolytically converted to active toxins of 55–65 kDa, primarily by removal of the carboxyl halves. Active CryI toxins bind reversibly and with high affinity to receptors on the surface of larval midgut cells [1]. In several insects, the receptors are aminopeptidase Ns. There appears to be more than one toxin-binding site on these molecules on the basis of kinetic studies [13–15] and peptidase digestion of the receptor [16]. A larger cadherin-like molecule has also been implicated as having other membrane proteins.

Binding has been measured by adding labeled toxins in the presence or absence of unlabelled toxin to membrane vesicles prepared from the midguts of insect larvae, so-

called brush border membrane vesicles (BBMVs). In this system, there is reversible binding to a receptor(s) as well as insertion of the toxin into the membrane (non-reversible binding), complicating the calculations of the rate constants. More accurate determinations of the binding constants have been obtained by anchoring purified toxin-binding proteins in a surface plasmon resonance system for the determination of the on and off rates [13–15]. The former would reflect the initial reversible binding and the latter provides a measure of the irreversible insertion into the membrane. More qualitative assessments of the initial binding, especially of the variety of proteins involved, have been obtained by immunoblotting.

In many cases, there is a good correlation between a so-called binding constant measured as described above with BBMV and toxicity. This value, which has been designated K_{com} , really reflects the ratio of the initial reversible binding and a second irreversible binding [1]. Even then, there are several exceptions to the correlation. The reason for high-affinity binding with low or no toxicity is not known. It has been suggested that the initial ‘on rate’ may not be indicative of the function of the receptor(s) in toxicity and that the membrane insertion step may be better correlated with toxicity [1]. There are also cases of several binding proteins in certain insects, some or all of which may function in toxin insertion into the membrane. In addition, there is evidence that the Cry1Ac toxin binds to more than one site on the aminopeptidase N receptor [13,15]. Site I is located within domain III and displays fast association and dissociation kinetics. Site II within Domain II possesses slower kinetics yet tighter affinity [15]. This dual binding may reflect a two-step process with an initial relatively non-specific reversible association followed, in cases of toxins active on the particular insect, by binding to a second site on the same receptor or on a receptor in close proximity. Binding at the second site may lead to a conformational change of the toxin necessary for membrane insertion. Alternatively, binding may be important for mobilizing toxin at the membrane surface, either for oligomerization or for a concentration-dependent insertion step. This possibility would probably depend upon the localization or mobilization of the toxin receptor(s) within the membrane which has yet to be established.

Despite the uncertainty of the function of the initial toxin–receptor interaction, there is evidence for its importance. In some cases, there was a correlation between resistance of an insect to a particular toxin and reduced binding (K_{com} value) of that toxin to vesicles prepared from BBMVs of the resistant insect [1]. The decreased binding may be due to an alteration of the receptor itself or of a membrane component other than the toxin receptor, which results in a change in the accessibility of the receptor for toxin-binding. There is evidence for a decreased fitness of toxin-resistant *Plutella xylostella* [1] which may reflect such pleiotropic membrane changes. In

several cases, a correlation between resistance and reduced toxin-binding was not found. Many of these insects were resistant to a number of toxins, including some to which they had never been exposed. The basis for resistance in these cases is not known but could involve such pleiotropic changes.

An important role for the receptor was also demonstrated in a variety of in vitro experiments. The concentration of toxin required to obtain ion channel activity in synthetic phospholipid vesicles was reduced at least 100-fold by the inclusion of toxin receptor [7]. Such experiments included the fusion of BBMVs with artificial phospholipid membranes and measurements of conductance by patch-clamping. The release from vesicles of voltage-dependent fluorescent dyes such as calcein [7] as well as the release of $^{86}\text{Rb}^+ - \text{K}^+$ from vesicles reconstituted with purified aminopeptidase N [17] have also been used to assay ion channel activity. The latter results imply that the only membrane protein required for ion channel formation by toxins is the aminopeptidase N receptor. However, the relation of the $^{86}\text{Rb}^+$ release activity to ion channel function in vivo or in BBMVs has not been established. As a result, interaction of the toxin with other membrane components is not excluded. In fact, there were significant differences between the conductance of the ion channels formed in synthetic phospholipid vesicles and in fusions to BBMV implying a role for some undefined membrane components in ion channel function.

Clearly, more studies of toxin–receptor interactions are needed. The genes encoding several receptors have been cloned so in vitro mutagenesis can be done in order to study receptor function in reconstituted vesicles. It should also be possible to compare the deduced sequences of receptors from resistant and sensitive insects to determine if such a change were the basis for resistance. This is obviously an important issue to resolve, especially for dealing with insect resistance.

It should be noted that, in general, there is surprisingly little known about the interaction of bacterial toxins with receptors. In contrast, the binding of δ -endotoxins to specific membrane receptors, especially aminopeptidase N, has been extensively analyzed. There is considerable potential, therefore, for developing relatively simple in vitro systems comprised of phospholipid vesicles containing one or a few membrane proteins (including the receptor) for exploring toxin-binding and the subsequent formation of ion channels. These systems could serve as a paradigm for understanding such interactions for other toxins.

4. δ -Endotoxins insert irreversibly into membranes

The so-called ‘irreversible binding step’, i.e. insertion of the toxin into the larval membrane, is not yet understood. Other bacterial pore-forming toxins utilize a variety of mechanisms for toxin-binding to and insertion into the

membrane. Pore-forming colicins and perhaps the B subunit of diphtheria toxin (both comprised of α -helices in the pore-forming region as are the δ -endotoxins) depend upon an acidic environment for a conformational change of the membrane insertion domain of the toxin resulting in a charge interaction of the toxin with membrane phospholipids [12]. Aerolysin and the *S. aureus* α -hemolysin are comprised of β -sheets in the pore-forming region. For these toxins, aggregation to heptamers at the membrane surface results in a conformational change exposing hydrophobic regions for membrane insertion [18,19].

The CryI toxins function in the alkaline environment of the larval midgut (at least for Lepidoptera and Diptera) which appears to be important for inclusion solubilization and conversion of the protoxin to toxin. There are indications, however, that a charge interaction with the membrane may be important at some stage for the insertion of the Cry toxins. A positive charge in the region of the loop connecting helices $\alpha 2$ and $\alpha 3$ in domain I is essential for activity [1]. A number of mutations of R93 in this region to non-charged or negatively charged residues or an A92D mutation resulted in toxins which still bound reversibly to BBMV but did not insert into the membrane. An alternative explanation for these results is that an intramolecular salt bridge, known to involve R93 in the CryIAa toxin [10], may be critical for toxin conformation and thus for insertion.

It is likely that prior to such a charge interaction, toxin insertion involves a conformational change, perhaps analogous to the conversion of toxins to a 'molten globule state' [12]. The initial reversible binding involves certain loops within domain II of the toxin which are in close proximity to helix $\alpha 7$ of domain I [1] (see Fig. 1). A swinging out of domain I with exposure of helix $\alpha 7$ to the membrane surface has been postulated on the basis of disulfide cross-linking of various domain I helices with each other and with loops within domain II [7,20]. Cross-linking resulted in the loss of activity which could be restored by reduction of the disulfide bonds. A reasonable explanation for these results is the requirement for a conformational change involving these helices.

Experiments with synthetic peptides of the domain I helices also support a role for helix $\alpha 7$ in enhancing the insertion of the critical pore-forming region, helix $\alpha 4$ -loop- $\alpha 5$, into the membrane. Among the seven synthetic peptides of the α -helices in domain I, peptide $\alpha 7$ bound most rapidly to phospholipid vesicles [21], consistent with its primary role in membrane insertion. This peptide also enhanced the penetration of peptides $\alpha 4$ and $\alpha 5$ into such vesicles. For example, synthetic peptides corresponding to helices $\alpha 5$ and $\alpha 7$ from two different α -endotoxins were incubated with phospholipid vesicles and only the former was found to permeate the membrane and oligomerize to form ion channels [21]. Furthermore, the $\alpha 5$ peptide specifically associated with the partially inserted $\alpha 7$ peptide as well as with itself in a parallel manner within the mem-

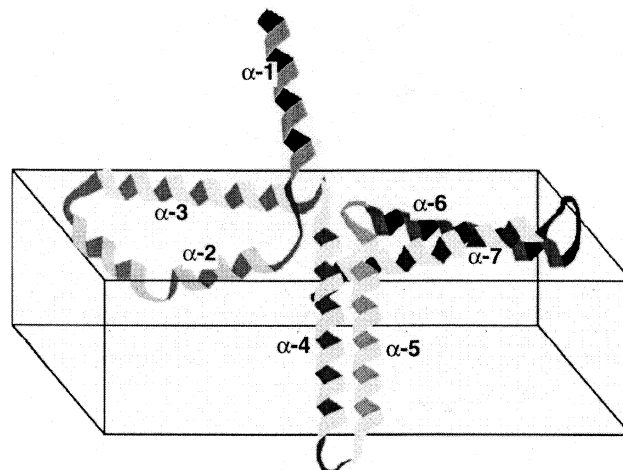


Fig. 2. A schematic presentation of a model for the interaction of domain I of a δ -endotoxin with a phospholipid membrane as suggested by Gazit et al. [21]. The structures of the individual helices were taken from the coordinates of the Cry3A structure [10]. The relative positions of the seven helices ($\alpha 1$ – $\alpha 7$) are based on the interaction of fluorescently labeled peptides with phospholipid vesicles. There was no evidence from these studies that the loop connecting helices $\alpha 4$ and $\alpha 5$ projects through the plane of the membrane (see the text for further discussion).

brane as expected if the pore were formed from several such helices.

In a more detailed study, peptides of the seven helices of the pore-forming domain of the Cry3A toxin were synthesized and compared as to their membrane interactions, structure within the membrane, orientation relative to the membrane plane and the network of peptide-peptide interactions in the membrane between all pairs of peptides [21]. Attenuated total reflectance-Fourier transform infrared spectroscopy was used to study the structure and orientation of the helices when bound or inserted into the membrane. All of the helices, except $\alpha 1$, interacted with the lipid membrane and only $\alpha 4$ and $\alpha 5$ were in a transmembrane orientation. Remarkably, a network of recognition of all the possible combinatorial pairs of membrane-bound helices showed that only $\alpha 4$ and $\alpha 5$ self-assembled within the membrane. Moreover, $\alpha 4$ and $\alpha 5$ coassembled in an antiparallel manner, an orientation expected if they had inserted as a hairpin.

Peptides of helices $\alpha 4$, $\alpha 5$ and $\alpha 6$ recognized $\alpha 7$ in the membrane-bound state. Since helix $\alpha 7$ is located at the interface between the pore-forming domain and the receptor-binding domains (Fig. 1), its ability to coassemble with $\alpha 5$ and $\alpha 6$ may assist the insertion of the $\alpha 4$ -loop- $\alpha 5$ hairpin into the membrane. Following the binding of the toxin to the receptor, the affinity of $\alpha 7$ for the membrane surface and for the other helices could lead to the unpacking of the pore-forming domain and facilitation of the insertion of helices $\alpha 4$ and $\alpha 5$ into the membrane. Overall, these results are consistent with a model in which only helices $\alpha 4$ and $\alpha 5$ insert into the membrane as a helical hairpin in an antiparallel manner, while the other helices lie on the membrane surface like ribs of an umbrella (the

‘umbrella model’) as proposed earlier by Li et al. [9] (Fig. 2). Alternatively, toxin monomers may aggregate at the membrane surface prior to insertion as do the pore-forming aerolysin and the *S. aureus* α -hemolysin. The resulting changes in toxin properties apparently enhance exposure of hydrophobic regions and thus toxin insertion.

In the case of the membrane-bound Cry3A toxin, there may be a proteolytic cleavage between helices $\alpha 3$ and $\alpha 4$ which could trigger a conformational change leading to the formation of functional channels [1]. The Cry4A toxin can also be cleaved between helices $\alpha 5$ and $\alpha 6$ to fragments of 20 and 45 kDa apparently without a substantial loss of activity for mosquito larvae [22]. This cleavage is not essential, however, since mutant toxins lacking the cleavage site were active. Deletion of the region encoding helix $\alpha 5$ from the smaller fragment did not alter toxicity when it was mixed with the 45-kDa proteolytic fragment whereas a further deletion into the region encoding helix $\alpha 4$ resulted in loss of toxicity of the reconstituted fragments. It appears that helix $\alpha 5$ but not $\alpha 4$ is dispensable for toxicity in reconstituted Cry4A toxin. It is likely that the structure of this toxin is very similar to that of the Cry1Aa and Cry3A toxins given the presence of conserved sequence blocks in all cases [1] and the extensive structural similarity of these two toxins despite limited sequence identity. If so, the Cry4A toxin probably has the same mode of action implying that helix $\alpha 5$ is not essential for ion channel formation, at least in such reconstitution experiments. Mutagenic studies of *cryI* toxin genes, however, clearly implicate helix $\alpha 5$ in toxicity [1] and as discussed below, probably in the aggregation of toxins within the membrane. The interaction of two proteolytic fragments of the Cry4A toxin may have resulted in the exposure of other regions of the toxin which compensated for the absence of helix $\alpha 5$.

To date, there have been no definitive reports of a conformational change of the toxin in part because of the difficulty of detecting a signal from a fluorescently labeled toxin (or from tryptophan residues) in the presence of BBMV. While toxins can form ion channels in synthetic phospholipid vesicles, the efficiency is very low and a large excess of toxin is required [7] so experiments to look for such changes have not been feasible. Definitive studies on toxin insertion may be possible with vesicles constructed from phospholipids (perhaps selected ones) containing the purified protein(s) (such as aminopeptidase N) required to mimic in vivo conditions. As previously mentioned, there is evidence that aminopeptidase N reconstituted into such vesicles may be sufficient [17] but further experiments are needed to compare the functional properties of ion channels formed in such vesicles to those due to toxin interaction with BBMV. Correlations of the behavior of various toxins in vivo and in such systems would be helpful as would the study of various mutant toxins. There is a large collection of Cry1 mutant toxins which could be exploited for this purpose [1].

5. Most of the toxin molecule is intimately associated with the membrane and forms aggregates therein

Whatever the mechanism for inserting δ -endotoxins into the membrane, most of the molecule is protected from digestion with protease K [23]. The only susceptible part is about 30 amino acids from the amino end which comprise helix $\alpha 1$. As mentioned above, a synthetic peptide of this helix is the only one of the seven fluorescently labeled peptides from domain I which did not bind to phospholipid vesicles [21]. In general, there is a good correlation between the membrane-binding and insertion properties of domain I synthetic peptides and those of the intact toxin. Synthetic peptides of helices $\alpha 4$ and $\alpha 5$ with specific amino acid changes known to inactivate the Cry1Ac1 toxin have altered membrane-binding and insertion properties.

In a random mutagenesis study of the regions encoding the domain I helices in the Cry1Ac toxin, the most significant loss of toxicity was due to mutations in helices $\alpha 4$ and $\alpha 5$ but not in the loop connecting these helices [1,24]. Among the synthetic peptides of the domain I helices only these two helices penetrated into the plane of the membrane [21,25]. Both helices are long enough to project through the membrane into the cytosol [9,10] and could thus potentially functionally interact with a cytoplasmic component. In order to examine whether any region of the toxin projected into the cytoplasm, BBMV were preloaded with protease K. Neither the Cry1Ab nor the Cry1Ac toxins were altered in size or function when inserted into these preloaded BBMV [26]. Most of the toxin molecule must remain within the plane of the membrane and function in such an environment. The helices within the membrane may be tilted or perhaps altered in conformation in order to enhance the interactions necessary for pore formation.

The retention of toxicity for *Manduca sexta* larvae by Cry1Ac toxins with mutations within the region encoding the loop connecting helices $\alpha 4$ and $\alpha 5$ [24] also indicates that a specific interaction between this region of the toxin and a cytoplasmic component is not essential for toxin activity. While this sort of functional interaction is unlikely, the overall orientation of the toxin within the membrane must be critical to its function. Fluorescent studies with domain I peptides indicate that specific domain I helices exist in different environments within or on the membrane. In particular, the helix $\alpha 4$ -loop- $\alpha 5$ region is buried within the membrane with a more superficial location for the other helices (Fig. 2) [21,25]. Domains II and III are intimately bound to the membrane (based on resistance to protease K) but their exact orientations are not known.

Aggregated forms of the Cry1A toxins could be extracted from BBMVs by employing relatively mild conditions for membrane solubilization [23]. Active Cry1Ab or Cry1Ac toxins formed aggregates, about the size expected

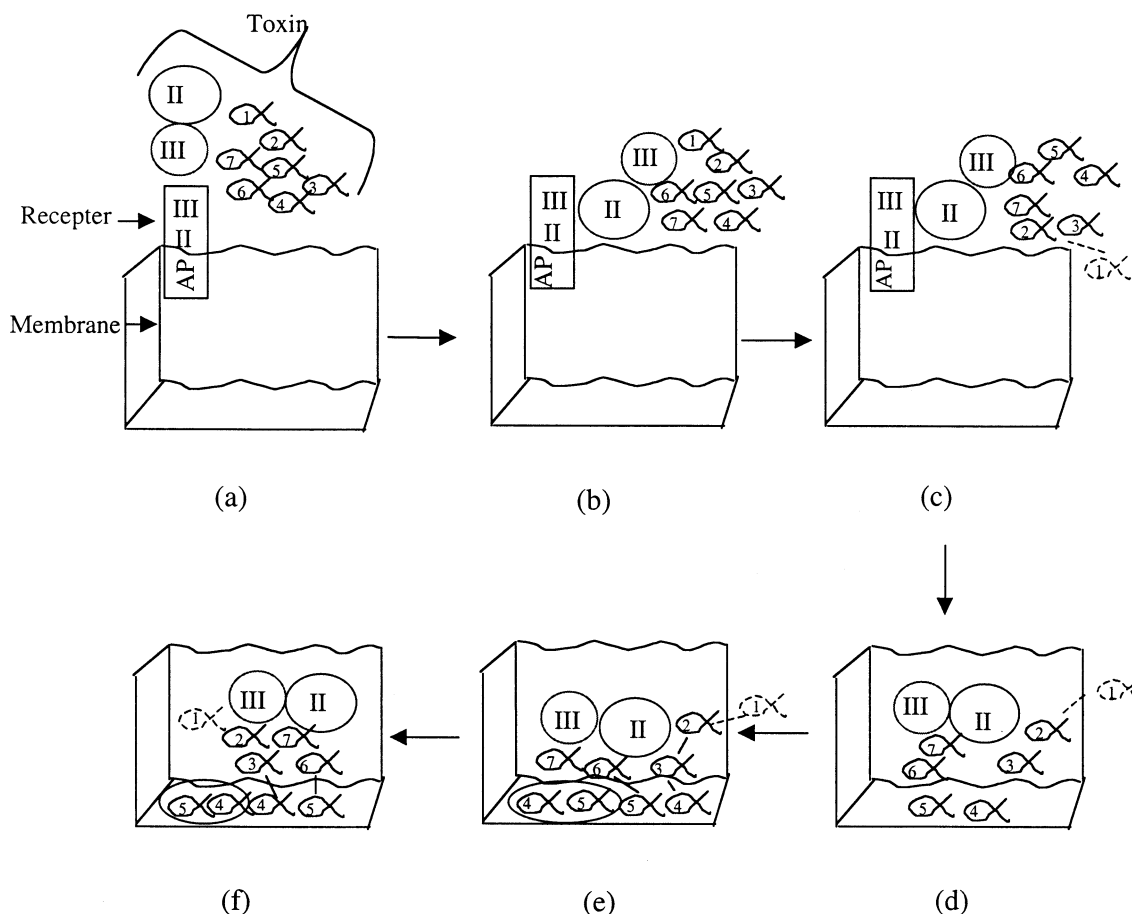


Fig. 3. Schematic of the steps proposed to account for the mechanism of action of δ -endotoxins: (a) initial reversible binding to a receptor (in this case to aminopeptidase N (AP), via a lectin-like cavity in domain III. This pocket in the CryIAc toxin is specific for *N*-acetyl galactosamine [15,16]. (b) A second step of binding to AP (perhaps the same molecule) via domain II loops brings domain I, especially helix α 7, close to the membrane. (c) A conformational change of the toxin results in the swinging out of domain I and the interaction of one face of domain I (especially the region between helices α 2 and α 3) with the membrane. (d) Interaction of the toxin (all except helix α 1) with the membrane with insertion of the hydrophobic helix α 4-loop- α 5 region into the membrane (see Fig. 2). (e) Aggregation of toxin monomers via the interaction of the α 5 helices (the number is not known but is probably > 3). (f) Organization of the α 4 helices to form the lumen of the ion channel.

for trimers, in *M. sexta* and *Heliothis virescens* BBMV. No such aggregates were formed by toxins which were inactive due to mutations either within helix α 5 or of the R93 residue implicated in an ionic interaction required for toxin insertion into the membrane. The aggregates did not contain the aminopeptidase N receptor although interactions with other membrane components have not been excluded. While possible trimers were detected with the extraction conditions employed, larger functional aggregates, perhaps even heptamers as found for the β -sheets in aerolysin [18] and the *S. aureus* α -hemolysin [19] may be present.

In contrast to the results with the helix α 5 mutants, inactive toxins with mutations encoding residues within helix α 4 did aggregate in the membrane, but did not form functional ion pores as determined by light scattering [24]. In comparable experiments, an inactive CryIAc toxin with a mutation in helix α 4 (N135Q) was examined by surface plasmon resonance for binding to purified aminopeptidase N from *M. sexta* anchored in a lipid environ-

ment [14]. The initial binding rate was the same as for the wild-type toxin but the off rate was more rapid suggesting a lack of interaction with the lipids by the mutant toxin. These results suggest that this particular α 4 mutant toxin may not be able to insert into the membrane and aggregate there. Differences in lipid environments and the presence or absence of membrane proteins in the two protocols may account for the apparent discrepancy. Alternatively, only a subset of inactive α 4 mutant toxins may be able to insert into the membrane and aggregate.

As discussed above, synthetic peptides of helices α 4 and α 5 were the only ones among the seven comprising domain I which inserted and oligomerized within the phospholipid bilayers [21]. A synthetic peptide of the α 4-loop- α 5 region was much more effective than either of these helices alone or as a mixture of the two helices in promoting the release of calcein from phospholipid vesicles [25]. While the α 5 but not the α 4 peptide aggregated in the membrane and formed a channel, it did so much less efficiently than the α 4-loop- α 5 peptide. These results and the

mutagenic studies of the *cryIAc* toxin gene form the basis for the models presented in Figs. 2 and 3 in which most of the toxin molecule is at or close to the surface and only helices $\alpha 4$ and $\alpha 5$ project into the plane of the membrane but not onto the cytoplasmic face. The exact relative locations of the helices and of domains II and III are not known. Since helix $\alpha 5$ is involved in aggregation, it must be available to interact with this helix from other toxin molecules in order to initiate ion channel formation.

This difference in aggregation properties of the mutants altered in $\alpha 4$ or $\alpha 5$ as well as the peptide results imply separate functions in ion channel formation for these two helices. Perhaps in analogy with the formation of the heptameric ion channel by the *S. aureus* α -hemolysin, there is a two-step process [19]. An initial aggregation due to interactions among the hydrophobic $\alpha 5$ helices would be followed by the formation of functional ion channels lined by the $\alpha 4$ helices. Separate functions for these two helices were also postulated on the basis of a different set of mutations of critical anionic residues within helix $\alpha 4$ [27]. In contrast, a synthetic peptide resembling helix $\alpha 5$ aggregated and formed calcein-releasing channels in phospholipid vesicles [21,25]. Caution must be exercised, however, in relating measurements of channel activity to the true function of a protein. In the case of the $\alpha 5$ peptide, channel activity was used as an additional indicator of oligomerization in the membrane rather than mimicking the function of this helix in the intact toxin. There are examples of proteins forming ion channels, such as the B subunit of the diphtheria toxin, which do not have a direct bearing on their primary function.

Aggregation may occur at the surface of the membrane or after integration of toxin monomers within the membrane. The results with fluorescently labeled synthetic peptides favor the latter possibility. The kinetics of fluorescent emission by peptide $\alpha 5$ were consistent with self-aggregation in the membrane in contrast to the binding kinetics of the other peptides of the domain I helices [21]. Since virtually the entire toxin molecule is intimately bound to the membrane, it is likely that the other helices of domain I, as well as domains II and III, are important for toxin function. For example, a highly conserved arginine cluster within domain III appears to be involved in the voltage-dependent regulation of the ion channels [1]. Mutation of the two outer R residues to K in this region resulted in lower insecticidal activity and reduced ion channel function as measured by light scattering or voltage clamping. This part of domain III is likely to be in close proximity to the ion channel within the membrane.

6. Conclusions and outlook

Some of the unique features of these δ -endotoxins as related to function have been discussed above but are worth emphasizing for future experiments. Despite a dis-

tinct three-domain structure of these toxins, there is considerable evidence for extensive functional interactions. (a) Specific toxin-binding to membrane receptors seems to involve residues in both domains II and III, perhaps in sequential steps leading to toxin insertion [13–15] (Fig. 3). The latter could require an induced conformational change for the insertion of toxin monomers or an enhancement of the concentration of the toxin at the membrane with the conformational change occurring during oligomerization. This step and the formation of ion channels could involve interactive functions among the three domains. (b) Regulation of the conductance properties of the ion channels almost certainly involves an interaction between domains I and III [1]. (c) A number of domain swapping experiments among *cryI* genes support such functional interactions for receptor-binding specificity and for the properties of the ion channels [28]. Some of the hybrid toxins had enhanced activity for certain insects which could be due to improved binding or perhaps more efficient insertion, aggregation or ion channel-forming processes.

Another consideration is that these toxins may have function(s) in addition to ion channel formation. Behavioral changes of larvae such as avoidance of the toxin and paralysis of feeding, may be more rapid than the formation of ion channels. If so, these behavioral changes could be due to effects of the toxin prior to ion channel formation, perhaps within the membrane but before there is extensive aggregation. Possibilities are alteration of the properties of pre-existing ion channels or of a membrane ATPase. These speculative modes of action may involve parts of the toxin not yet implicated in its function(s).

There are still uncertainties as to the role of receptor-binding, the process of insertion of the toxin into the membrane and the extent and mechanism of oligomerization to form ion channels. The progress in these areas has been considerable, however. There are now a number of experimental techniques and tools available to resolve many of the basic issues regarding the mode of action of these toxins. There is an extensive number of well characterized mutant toxins, especially the CryI class, purified and cloned toxin-binding proteins and the potential to develop relatively simple lipoprotein vesicles or preparations for investigating toxin insertion, ion channel formation and perhaps other functions of these toxins. There are indications that the mechanism of action of these δ -endotoxins, especially functional interactions among the domains, may be unique. Given the promising experimental systems and the wealth of information already available, novel insights into the mechanism of action of a multi-domain toxin should be forthcoming. With the refinement of the experimental systems for investigating their function(s), it should be possible to develop a complete picture of δ -endotoxin action. Such studies have very broad implications for dealing with resistance and for designing even more effective and specific biological control agents.

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