Mechanism of Action of *Bacillus thuringiensis* Toxins

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The selectivity of Bacillus thuringiensis *toxins is determined both by the toxin structure and by factors inherent to the insect. These toxins contain distinct domains that appear to be functionally important in toxin binding to protein receptors in the midgut of susceptible insects, and the subsequent formation of a pore in the insect midgut epithelium. In this article features necessary for the insecticidal activity of these toxins are discussed. These include toxin structure, toxin processing in the insect midgut, the identification of toxin receptors in susceptible insects, and toxin pore formation in midgut cells. In addition a number of* B. thuringiensis *toxins act synergistically to exert their full insecticidal activity. This synergistic action is critical not only for expressing the insecticidal activity of these toxins, but could also play a role in delaying the onset of insect resistance.*

Key word: *Bacillus thuringiensis* - receptors - pore formation - synergism

The discovery *Bacillus thuringiensis* crystal toxicity by Heimpel and Angus (1959) laid the foundation for investigations during the last few decades on the mechanism of toxin action. A number of factors affect the mechanism of action and insecticidal activity of these *B. thuringiensis* toxins, including midgut structure and function, toxin diversity, toxin structure and processing, and finally, synergistic interactions between toxins.

FACTORS AFFECTING *BACILLUS THURINGIENSIS* TOXICITY

The insect midgut has numerous functions essential for normal insect homeostasis. Key among these functions is ion regulation. An understanding of insect midgut ion regulation is crucial for understanding *B. thuringiensis* toxin mode of action because a major consequence of toxicity is the disruption of midgut cellular osmotic balance. In addition, the midgut plays a key role in insect nutrition, and enzymes involved in food digestion, such as trypsin, chymotrypsin, also play a role in *B. thuringiensis* toxin processing. The midgut also plays an important role in detoxifying secondary plant products. The ability to detoxify these plant products can at times be affected by *B. thuringiensis* toxins, and hence affect the insects ability to metabolize these products. Finally, the midgut is an important site for insecticidal action. In addition to *B. thuringiensis* toxins, baculoviruses can also affect midgut function; therefore it is likely that future attempts to develop insecticidal compounds will increasingly use the insect midgut as a target organ.

Midgut structure and function - The insect midgut is made of a number of cell types; two cell types which predominate in a generalized lepidopteran midgut are the columnar and the goblet cells .

A consequence of insect feeding of plant material is a relatively high K^+ concentration in the midgut lumen compared to that in the cell and/or in the hemolymph (Harvey et al. 1983). This K^+ concentration drives a number of secondary midgut cellular processes. As an example, amino acid transport from the lumen into the cell through the columnar cell apical membrane amino acid symporter is aided by high lumen K^+ concentrations (Giordana et al. 1989, Hennigan et al. 1993). Similarly, transport into midgut cells of other nutrients, such as glucose, is also facilitated by high midgut lumen cation concentrations.

High columnar cell K^+ concentrations result from these transport processes. These columnar cell K^+ ions are then transported across the basement membrane potentially through K^+ channels or pumps; alternatively, K^+ can flow through gap junctions into the goblet cell. In this manner the uptake of amino acids and of other nutrients results in a relatively high insect hemolymph K^+ concentration (Bindokas & Adams 1988). However, for normal insect function it is essential that this K^+ hemolymph concentration be reduced. In lepidopteran larvae this reduction is achieved through the goblet cell; the K^+ excretion is, however, done through an indirect means. The process first involves proton ion extrusion from the goblet cell cytoplasm to the goblet cavity by

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means of a V-type ATPase (Wieczorek et al. 1989); which is structurally similar to other known V-type ATPases (Gill & Ross 1991, Graf et al. 1992). The goblet cell K^+ ions are then exchanged for H^+ ions, a process facilitated by a K^+/nH^+ antiport protein probably localized in the goblet cell apical membrane. In addition to this net pumping out of K^+ ions, the goblet cell is also known to exclude bicarbonate ions. This combination of bicarbonate and potassium ions excretion results in the formation of potassium carbonate/bicarbonate. It is thought that it is this formation of carbonate/bicarbonate ions that leads to a relatively alkaline midgut (Dow & Peacock 1989).

Therefore there is precise ionic regulation and osmotic balance in the insect gut. Insect ingestion of *B. thuringiensis* toxins, which act on the columnar cell apical membrane, disrupt this ion regulation, and as a consequence the midgut insect cells swell which results in a loss of osmotic balance and cell lysis. Upon treatment with an insecticidal *B. thuringiensis* toxin, the ionic regulation and osmotic balance is affected, and the columnar cell microvilli are observed to undergo disruption (Singh et al.1986). This osmotic imbalance and cell lysis results in insect death.

Selective toxicity - *B. thuringiensis* strains contain different toxins, and it is this toxin diversity that determines the selective insecticidal activity of each *B. thuringiensis* strain. These toxins are selective, not specific because although each toxin has its predominant activity towards a particular insect species, it usually also has very low insecticidal activity against other insects. For example, three toxins in the *B. thuringiensis* subsp. *kurstaki* strain HD-1, CryIA(a), CryIA(b), and CryIA(c), are toxic to lepidopteran insects, however, each of them have differing toxicity towards different lepidopteran species. Thus the CryIA(a) has higher toxicity towards *Bombyx mori* while the CryIA(c) has lower toxicity towards this insect species. The reverse is true for toxicity towards *Heliothis virescens* (Table I). One other toxin from this HD-1 strain, CryIIA, has toxicity towards both lepidopteran and dipteran insects. Similarly, *B. thuringiensis* subsp. *israelensis* has at least four different toxins, the CryIVA, CryIVB, CryIVD and CytA, each of which has differential toxicity to various mosquito species. The toxicity of each *B. thuringiensis* strain is therefore dependent on the toxins that are present in that strain. The selective insecticidal activity of each toxin is dependent on the concentration levels used against a particular insect species. Hence it is possible to obtain lepidopteran activity with toxins that are usually regarded as having only dipteran activity, and vice-versa.

TABLE I

Cry toxin binding to *Heliothis virescens* brush border membrane vesicles

	LC_{50} ng/ml	K_d nM	R_t pmol/mg
CryIA(a)	157	0.8	3.7
CryIA(b)	7	0.4	21
CryIA(c)	2	0.4	62

Data from van Rie et al. (1989)

Toxin structure and toxin processing - The CryIIIA crystal structure has been elucidated, and demonstrated to contain three domains (Li et al.1991). It is assumed that all *B. thuringiensis* Cry toxins have similar three-domain structures. Domain I, a seven α-helix bundle is thought to play a role in forming a transmembrane spanning region, with the fifth α-helix potentially forming a key component of the pore (Fig. 1). Domain II, a β-sheet rich region apparently plays an essential role in toxin selectivity (Ge et al. 1989), and is likely to be associated with the toxin receptor binding. Finally although structural data suggest that Domain III is involved in structural stabilization (Li et al. 1991) recent mutatagenesis data suggests that this domain may also be involved in modulating toxin activity (Chen et al. 1993).

The CryI, and CryIVA and CryIVB *B. thuringiensis* protoxins, which are relatively large proteins of approximately 130 kDa, when ingested by insects are solubilized in the alkaline midgut (Gill et al. 1992). The solubilized toxins are cleaved predominantly at the C-terminus by midgut proteases, for example trypsin, chymotrypsin, thermolysin, etc. (Fig. 1). Additionally there is N-terminal truncation; this, however, is relatively small usually of 7-30 amino acids. The naturally truncated CryII, CryIII and CryIVD toxins can also undergo limited proteolysis in the insect gut. With both the larger 130 kDa proteins and the naturally truncated proteins, the activated toxins are approximately around 60-70 kDa. These activated proteins retain apparently all the functional domains - domains I, II and III (Fig. 1).

Toxin processing is consequentially key in the formation of an activated toxin, and can affect the selectivity of a toxin. An example of this selectivity is the insecticidal activity of *B. thuringiensis* subsp. *kurstaki* CryIIA toxin to either mosquitoes or to lepidopteran insects if the toxin is first processed by either mosquito or lepidopteran midgut proteases, respectively (Widner & Whiteley 1989). Similarly Ellar and co-workers demonstrated that *B. thuringiensis* subsp. *aizawai*

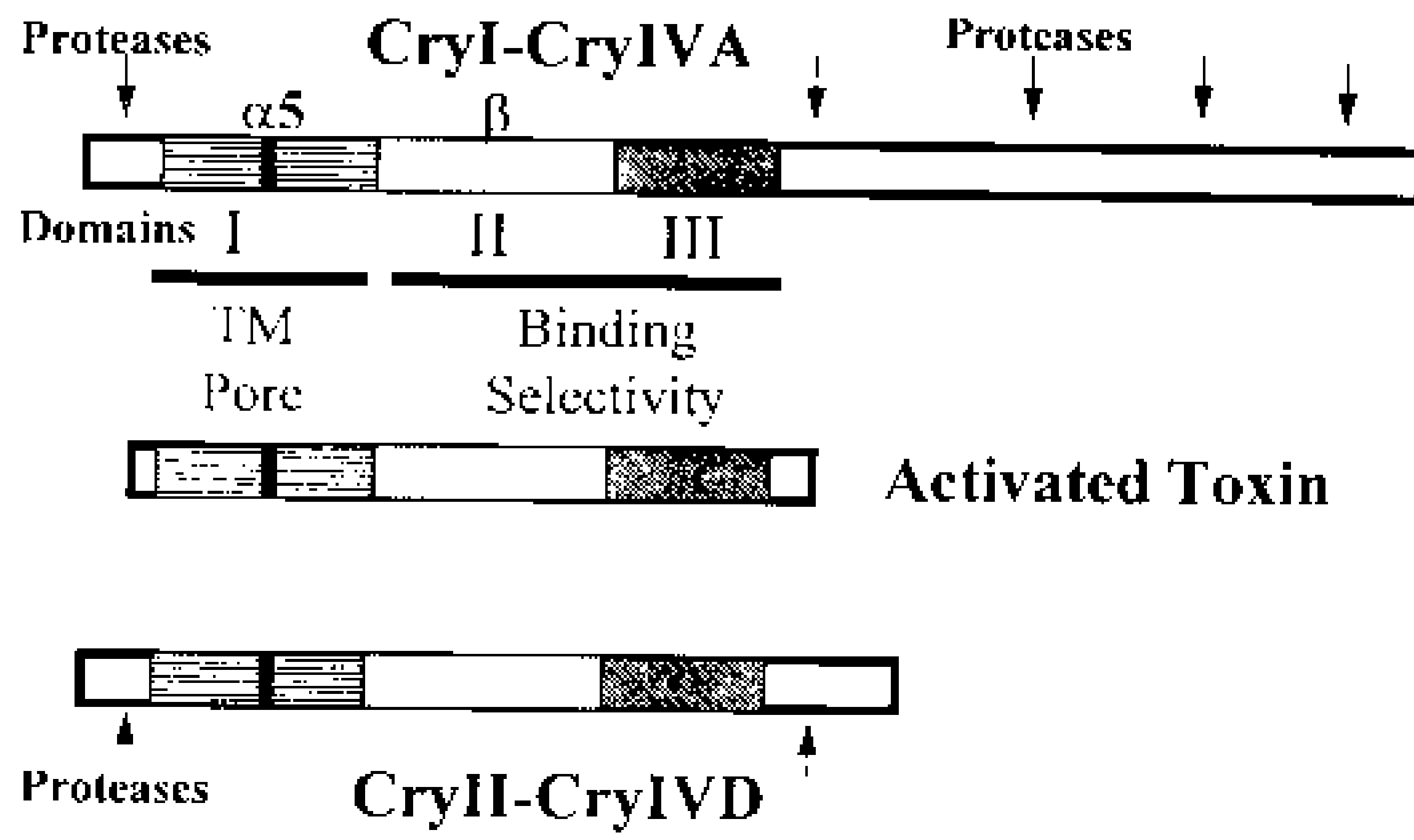

Fig. 1: schematic of Cry toxin structure and midgut processing. The 130kDa protoxins are activated by midgut proteases resulting in both N- and C-terminal cleavage. The resulting activated toxin is about 60-70kDa which contains the three domain structure elucidated for the CryIIIA toxin (Li et al.1991). The naturally truncated CryII, CryIII and CryIVD toxins undergo much less truncation.

toxicity to mosquito or to lepidopteran larvae is dependent on the toxin C-terminal processing (Haider et al. 1986). If a dipteran enzyme is involved a toxin more active towards dipterans is obtained, and similarly if processing is performed by lepidopteran proteases the toxins become active towards lepidopterans. These two examples demonstrate that toxin processing plays a much more important role in the selectivity of *B. thuringiensis* toxins than previously thought. In fact recent evidence suggests that differential toxin processing is observed in resistant *Plodia interpunctella* (Oppert et al. 1994).

A key feature in toxin selectivity is the interaction of toxins with receptor proteins in the insect midgut. It is clear that there is a great diversity of toxin receptors. For example, with the CryIA(c) toxin a number of receptors have been observed in some insects while in others only one major protein appears to be involved. In *Manduca sexta* a single protein binds the toxin (Knight et al. 1993, Sangadala et al. 1994) while in *Heliothis virescens* at least two proteins are involved (Garczynski et al. 1991). Similarly, there is preliminary evidence that in *B. thuringiensis* subsp. *israelensis* a number of different midgut proteins binds to different kinds of receptors. Consequently the selective toxicity of different *B. thuringiensis* proteins is determined by the receptors that are present in the insect midgut.

Synergism - A final factor that affects *B. thuringiensis* toxicity is synergism. Since a number of *B. thuringiensis* strains contain multiple toxins, these toxins at times do not act alone. The best example illustrating interactions between toxins are a number of mosquitocidal strains, best illustrated by *B. thuringiensis* subsp. *israelensis*. This isolate contains four toxins, the Cry IVA, CryIVB, CryIVD and the CytA, all essential for mosquitocidal toxicity. The mosquitocidal toxicity of the intact crystal is greater than the sum of the toxicity of each individual toxin. To illustrate this synergistic activity within two toxins, three constructs were made as illustrated in Fig. 2 using combinations of the CryIVD and the CytA toxins (Chang et al. 1993). When both these toxins are expressed, with 70% CytA and 30% CryIVD, the LC_{50} value obtained towards *Culex quinquifasciatus* is 26 ng/ ml. When only the CryIVD toxin is expressed the LC_{50} value is 37 ng/ml, and if only the CytA toxin is expressed the LC_{50} value is 1000 ng/ml. Although the LC_{50} value for the CryIVD/CytA combination is 26 ng/ml, and for CryIVD is 37 ng/ml. Most of the protein produced when both the CryIVD/CytA toxins are expressed together is the CytA toxin, and the CytA alone has a relatively low toxicity. Hence,there is a 4-5 fold synergism between the CryIVD and the CytA toxins (Chang et al. 1993) using the formula for evaluating synergism (Tabashnik 1992). Synergism has also been observed between the CryIVA and CryIVB and the CytA proteins, and synergism among other *B. thuringiensis* toxins is also likely.

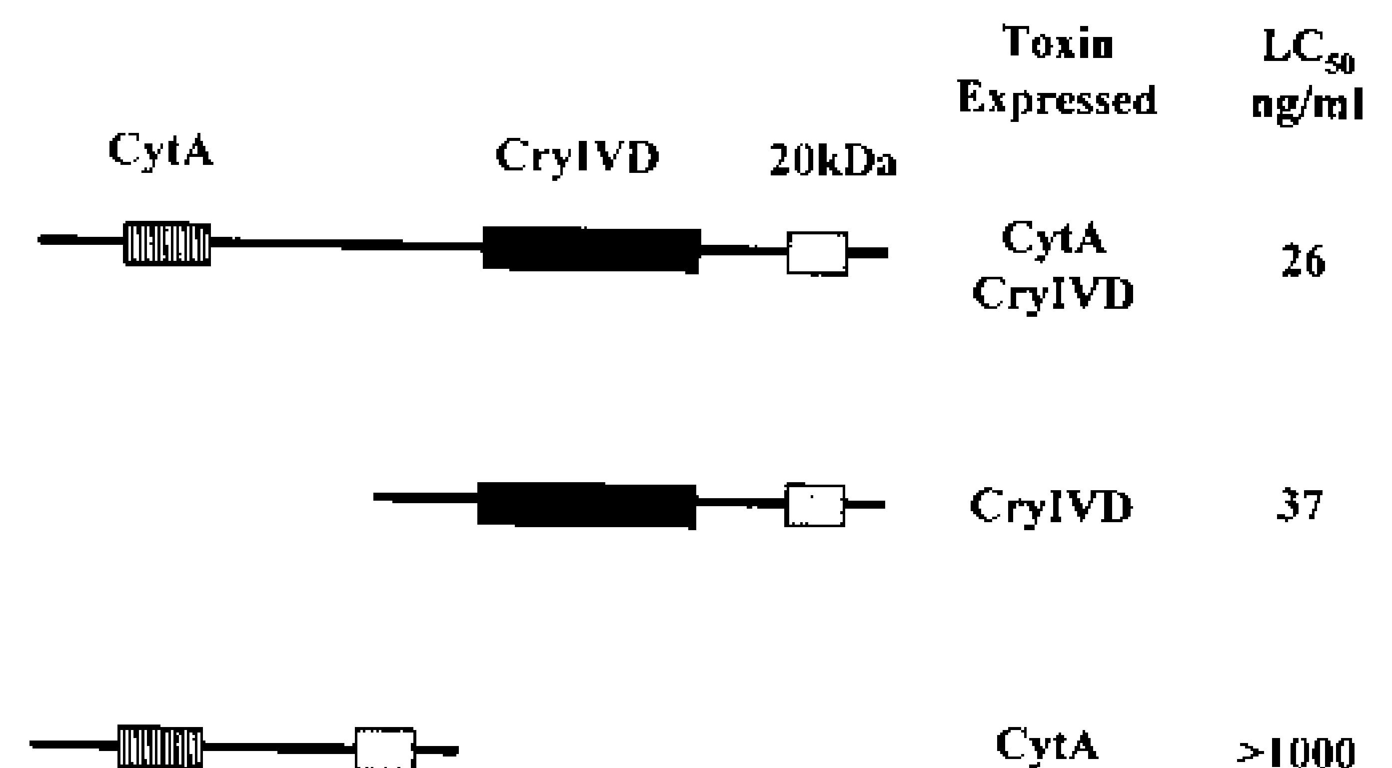

Fig. 2: synergistic interaction between the CryIVD and the CytA mosquitocidal toxins of *Bacillus thuringiensis* subsp. *israelensis*. The CytA toxin has low mosquitocidal activity, however, in its presence the CryIVD toxin shows higher level of toxicity.

MECHANISM OF ACTION OF *B. THURINGIENSIS* TOXINS

Cell membrane binding - In most cases when an insect ingests a *B. thuringiensis* crystal, the crystal is solubilized and an activated toxin obtained (Fig. 1). This toxin then binds in a competitive manner to a protein receptor on the columnar cell of the insect midgut. Elegant work initially done by a number of investigators (Hofmann et al.1988b; Hofmann et al. 1988a; Van Rie et al. 1989) shows that a receptor can be identified in the midgut of most insects. When radiolabelled activated *B. thuringiensis* toxin is incubated with an insect midgut brush border membrane vesicles it binds to it. This binding is

competed by increasing concentrations of cold toxin (Fig. 3). This competitive displacement, which indicates the presence of selective receptors for the toxin under investigation, has been used to identify the presence of toxin receptors in a variety of insect species. High affinity receptors are readily displaced with 100-fold cold toxin concentrations. In contrast higher concentrations of heterologous toxins with lower affinity to the receptor are required to displace the radiolabelled toxin; heterologous toxins with little affinity to the receptor are unable to displace any of the radiolabelled toxin (Fig. 3).

The selectivity observed with a number of *B. thuringiensis* toxins can in part be explained by the affinity of a toxin to midgut brush border membrane receptors of a particular insect. Hence, the greater CryIA(c) toxicity to *Heliothis virescens* is in large part due to the higher affinity and receptor concentration of this toxin than for the CryIA(a) toxin which has lower toxicity

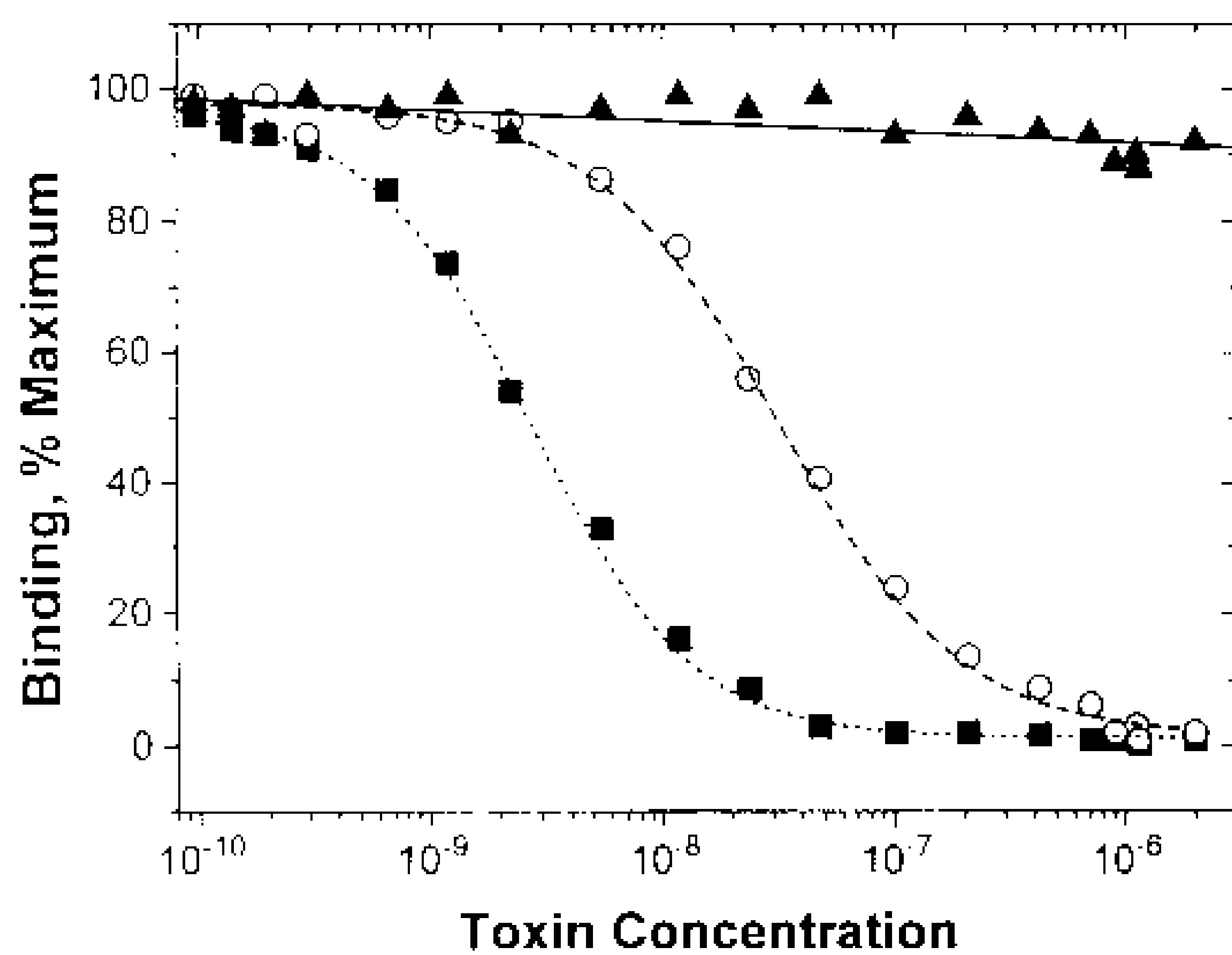

Fig. 3: hypothetical competitive toxin displacement graph. In the presence of a radiolabelled toxin increasing toxin concentrations can displace the labelled *Bacillus thuringiensis* toxin. High affinity toxin (■) require lower toxin concentration than lower affinity toxin (○) to compete with a known amount of labelled toxin. Toxins with little or no affinity (▲) are unable to compete.

(Table I). Consequently, when toxin affinity is observed in an insect that is resistant to a particular toxin, a change in these binding constants is usually observed (Pietrantonio et al. 1993). As an example, in *Plutella xyllostella* insects that are susceptible to CryIA(b) K_D and R_t values can be determined (Ferré et al. 1991). However, in resistant insects there is a lack of identifiable affinity constants (Table II). While there is low affinity for the CryIA(b) in resistant insects the affinity for another toxin, CryIC, is similar to that observed in susceptible insects (Table II). This data implies that although there is a change in resistant insect CryIA(b) affinity constants, there is little change in affinity for a different toxin. Therefore, different proteins are probably involved in the binding characteristics for these two different receptors.

Consequently there is significant interest in the identification of midgut receptor proteins that are involved in *B. thuringiensis* toxin binding. Recent evidence suggests that in *M. sexta* an aminopeptidase N plays an important role in the action of the CryIA(c) toxicity (Sangadala et al. 1994, Knight et al. 1993). In *H. virescens* three peaks with CryIA(c) toxin binding activity are observed when solubilized brush border membrane are separated using anion exchange chromatography. The affinity of these three fractions are different. It is likely that in most insects a number of proteins will bind *B. thuringiensis* toxins, however, the functional significance of each protein in relation to the mode of action of these toxins remains to be elucidated.

Toxin insertion and oligomerization - Subsequent to *B. thuringiensis* toxin binding insertion into the cell membrane occurs. However, there is little information on the precise steps that are required for this process to occur. It is likely nevertheless that a change in toxin conformation subsequent to binding facilitates domain I insertion into the cell membrane. Once the toxin is inserted, oligomerization of the toxin molecules apparently is needed. Although oligomerization could occur before toxin insertion it is more

TABLE II

Binding characteristics of CryIA(b) and CryIC toxins to resistant and susceptible *Plutella xylostella* brush border membrane vesicles

	CryIA(b)			CryIC		
	LC_{50} ng/cm2	K_d nM	R_t pmol/mg protein	LC_{50} ng/cm2	K_d nM	R pmol/mg protein
Susceptible	6.7	4.2	6	88.9	6.5	10.8
Resistant	>1350	NB	NB	46.5	7.6	2.9

Data from Ferré et al. (1991)

likely that oligomerization occurs in the cell membrane lipid bilayer. Limited studies performed with the CytA toxin support toxin oligomerization in the cell membrane rather than in solution (Chow et al. 1989, Maddrell et al. 1989). When CytA binding to cell membranes is monitored it is observed that there is increased binding both with time and increasing toxin concentration. Analysis of toxin in solution by sucrose density gradient analysis shows that in solution it exists as a monomer. Similar analysis of cell membrane bound toxin shows that at low toxin concentrations both monomeric and oligomeric structures are observed in the membrane (Chow et al.1989), while at high toxin concentrations the bound toxin exists essentially as an oligomeric structure. This oligomeric structure increases in size with increased toxin concentration, with an aggregate of approximately 300 kDa. The size of this aggregate, which includes both toxin molecules and cell membrane proteins, varies with the cell type. The actual number of toxin molecules that form the oligomeric structure are unclear but could be from 12-18 molecules per aggregate (Chow et al. 1989, Maddrell et al. 1989). Consequently toxin interaction with the cell membrane first involves binding followed by oligomerization. It is this oligomer that ultimately results in the toxicity of the *B. thuringiensis* toxin (Fig. 4). Although the CytA protein is structurally different from the Cry toxins, I opine that oligomerization is also a key component of Cry toxin action.

Pore formation, osmotic balance and cell lysis - There is increasing evidence that *B. thuringiensis* toxins insert into the plasma membrane to form a pore that is permeable to small ions and molecules (Knowles et al. 1989, Slatin et al. 1990, Schwartz et al. 1993). These studies using a variety techniques with or without insect cell membrane proteins show that pores formed by these toxins have the capacity to conduct ions. Most of the channels that were formed are voltage independent cation pores. To illustrate this further we have demonstrated that like other proteins the CryIVD can form cation channels. The conductance of the channel formed by CryIVD is relatively large. Subcloning of the N- and C-terminal domains individually and together followed by an analysis of their activity demonstrate that the N-terminal retains most of the pore forming ability, however, it does not have any mosquitocidal toxicity. In contrast with both the N and C terminal fragments together, pore forming capability and mosquitocidal activity are observed. These studies show that the N-terminal half by itself has pore forming ability. Nevertheless it is likely that other parts of the toxin can contribute to pore characteristics. The pore has been measure to be about 0.6 - 1.0nm in radius, which is large than the crystal radii of K^+ and Na^+. Since there is K^+ selectively for channels formed by Cry and Cyt *B. thuringiensis* toxins the basis for this ion selectivity is not known.

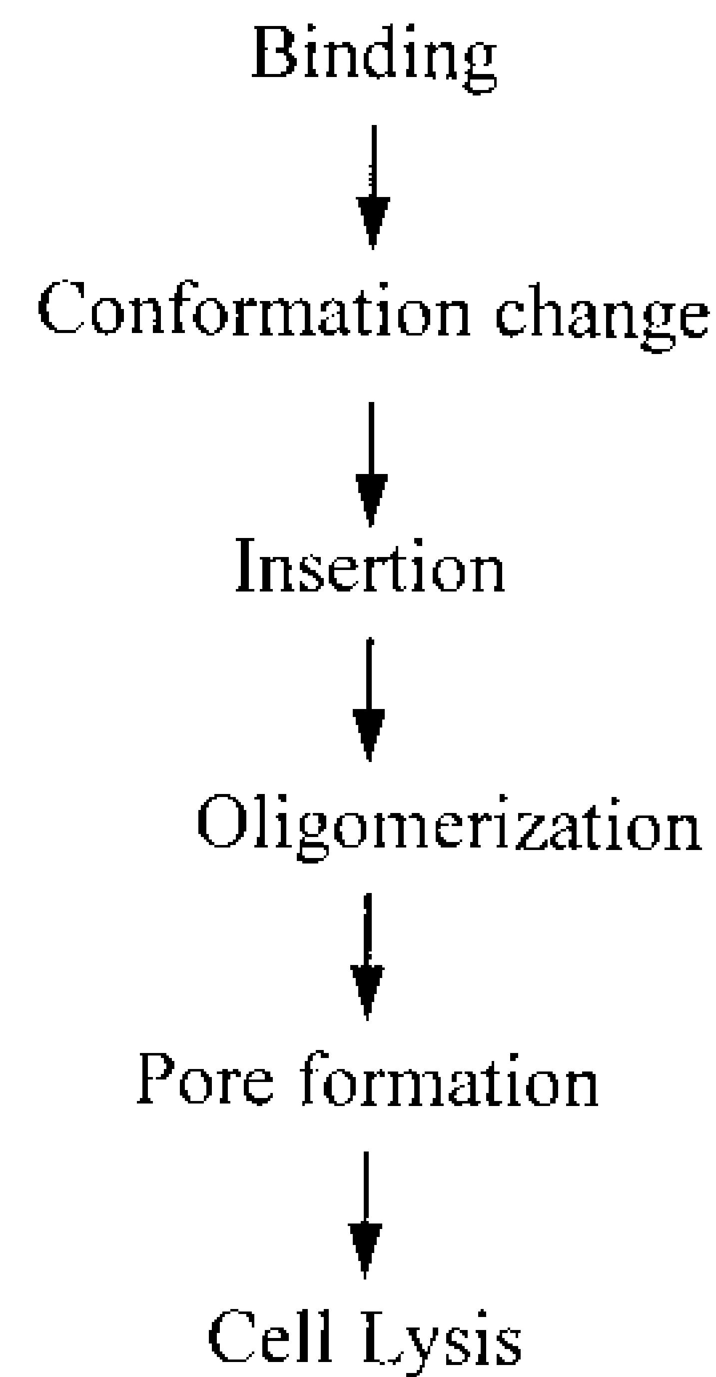

Fig. 4: characterization of the steps require for formation of toxin pores in cell membranes.

A consequence of pore formation is a disruption of columnar cell osmotic balance. Since the midgut cells are a very tightly regulated monolayer, any disruption of ion regulation in either the columnar cells or the goblet cells result in a loss of function, and ultimately that of the midgut. With *B. thuringiensis* toxins midgut microvilli ultrastructural integrity is disrupted early in the poisoning syndrome. This loss of integrity is due to loss of osmotic balance and subsequently results in loss of cell function, and ultimately results in cell lysis and insect death.

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