

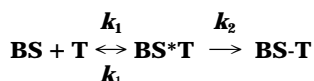
Irreversible Binding Kinetics of *Bacillus thuringiensis* CryIA δ -Endotoxins to Gypsy Moth Brush Border Membrane Vesicles Is Directly Correlated to Toxicity*

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To examine the binding of *Bacillus thuringiensis* δ -endotoxins, CryIAa, CryIAb, and CryIAc, to *Lymantria dispar* (gypsy moth) brush border membrane vesicles (BBMV), saturation kinetic analyses were conducted according to a two-step interaction scheme



for δ -endotoxin binding to BBMV, rather than the one-step reversible binding presented in prior reports.

The order of toxicity of the δ -endotoxins, as measured by the dose required for a 50% inhibition of weight gain (ID₅₀), was CryIAa (77.3 ng) > CryIAb (157 ng) > CryIAc (187 ng). While both the maximum extent of binding, B_{max} , and the half-maximum insertion rate concentration, $K_{1/2}$, was observed to be indirectly related to toxicity, the rate constant of irreversible binding, k_2 , was found to be directly correlated to toxicity.

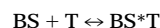
Bacillus thuringiensis synthesizes insecticidal crystalline inclusions containing various δ -endotoxins during sporulation. Due to the high specificity of the δ -endotoxins against target insects and its low persistence, *B. thuringiensis* has been an environmentally sound alternative in pest control for decades. The mode of action of the δ -endotoxin has now been elucidated in considerable detail by extensive studies in histopathology and biochemistry. Upon ingestion, the crystalline inclusions of δ -endotoxins are solubilized by the high pH environment of the midgut of the insect. The solubilized protoxins are converted to toxin by proteinases in midgut lumen. The toxins then bind to specific binding proteins in the microvilli of columnar cells and insert into the membrane of the microvilli. Pore formation by the toxins in the membrane causes the leakage of ions and probably other contents of the cell and finally lyses the cell (1, 2).

Binding of toxin to the microvillar membrane is one of the most intensively studied steps in its mode of action. An *in vitro* system with brush border membrane vesicles (BBMV)¹ estab-

lished by Wolfersberger *et al.* (3) has made it possible to study binding at the molecular level. Hofmann *et al.* (4) were the first to identify specific binding sites of toxins in the BBMV of susceptible insects by binding assays. Now the binding assay has become a standard tool in studies of mechanism of specificity of wild type toxins and their mutants (5–13), as well as the mechanism of resistance development in insects (14–16).

Gypsy moth (*Lymantria dispar*) is a major forest pest in the United States, as well as in other parts of the world. Members of the CryIA class of *B. thuringiensis* δ -endotoxins show different toxicities against gypsy moth. Interestingly, an inverse relationship between toxicities against gypsy moth larvae and binding properties to BBMV from the larvae has been observed with CryIAb and CryIAc (13). While CryIAb, a stronger toxin, showed lower affinity in the binding assay, CryIAc, a weaker toxin, showed higher affinity (13). As with other published binding studies, the binding properties of the toxins in this assay was analyzed by modified Scatchard equations (17).

So far, all published binding studies known to us have used the Scatchard equation or the Hill equation (18), which analyzes binding parameters assuming a one-step reversible interaction (Reaction 1).



REACTION 1

Here BS is the binding site on the membrane, T is the toxin, and BS**T* is the dissociable complex formed by binding site and toxin. However, a large body of evidence indicates that the binding between toxin and BBMV quickly becomes irreversible (4, 6, 7, 19, 20). Irreversibility of the toxin-BBMV interaction was observed in early studies (19) and has been considered briefly in most binding studies mentioned above. The irreversible binding might reflect insertion of toxin into brush border membrane (19, 20). A two-step interaction would describe toxin-BBMV interaction better than Reaction 1.



REACTION 2

Here BS-T is the toxin irreversibly bound to the membrane.

The observation of irreversible binding should immediately disqualify the application of the Scatchard equation or the Hill equation in analysis of binding parameters of toxin-BBMV interaction (21, 22). However, the effect of irreversible interaction on the binding parameters has not been included in the calculations of binding parameters (4–16), even in a recent paper in which irreversibility of the toxin-BBMV interaction has been proposed as a determinant in toxicity of two toxins, CryIA(a) and CryIA(b), against *Bombyx mori* (20).

The purpose of this study is to apply saturation kinetics of irreversible binding, rather than competition binding assays (4) to study binding parameters based on Reaction 2 and use this method to explore the relationship between the toxicity of

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¹ The abbreviations are: BBMV, brush border membrane vesicles; CryIAa, CryIAb, and CryIAc, the three insecticidal crystal δ -endotoxins in class IA; BS, binding site for toxin; T, toxin; BS**T*, reversible binding complex of binding site and toxin; BS-T, irreversible binding complex of binding site and toxin.

CryIA toxins against gypsy moth larvae and their binding properties to BBMV from the larvae.

MATERIALS AND METHODS

Preparation of Toxins—The constructions of the plasmid pOS4101 (CryIAa), pOS4201 (CryIAb), and pOS4301 (CryIAb), the expressions of toxin genes, the purifications of crystal inclusions, solubilization of protoxins, and activation of toxins were described elsewhere (11).

Bioassays—*L. dispar* larvae free of abnormal performance syndrome (23)² were supplied by N. R. Dubois, United States Department of Agriculture, Hamden, CT. Bioassays of Cry toxins with *L. dispar* larvae have been described in detail elsewhere (24). Briefly, 4th instar larvae were dosed orally with various amounts of toxin. After 5 days on untreated artificial diet, the weight gain of the larvae was determined. The dose resulting in 50% inhibition, ID₅₀, was calculated as the indicator of toxicity.

Preparation of BBMV—BBMV were prepared by the differential magnesium precipitation method of Wolfersberger *et al.* (3) from 5th instar *L. dispar*. Alkaline phosphatase, a brush border membrane marker enzyme, was enriched 10 times relative to the homogenate of the midgut from *L. dispar* larval.

Iodination of Toxin—Toxins were iodinated using the Iodo-Beads technique (35). Twenty μ g of toxin was labeled using 1 μ Ci of ¹²⁵I and one Iodo-Bead (Pierce) in a final volume of 0.15 ml of 50 mM sodium carbonate buffer, pH 9.5. Free iodine was separated from toxin using a prepacked Excellulose GF-5 column (Pierce).

Determination of Protein Concentrations—Protein concentrations of purified toxins and BBMV were determined by the Bradford method (25).

Determination of Labeling Specific Activity—0.02 pmol of labeled toxin was titrated by increasing amount of BBMV. Total binding at saturating amount of BBMV was taken as radioactivity of the labeled toxin. Radioautography showed that all input toxin precipitate with the saturating amount of BBMV in assay (no toxin band left in the supernatant after centrifugation) and toxin band was the only band showed in the precipitate. The specific activity of the labeled toxins (*i.e.* bound toxins) was determined as 5.8×10^4 cpm/pmol, 6.3×10^4 cpm/pmol, and 4.5×10^4 cpm/pmol for CryIAa, CryIAb, and CryIAb, respectively.

Binding Assays—BBMV were incubated with ¹²⁵I-labeled toxin in 200 μ l of binding buffer (11). The bound toxin was separated from free toxin by the centrifugation method (11). Nonspecific binding was measured in the presence of 1000-fold excess of unlabeled toxin. In all assays, the nonspecific binding of CryIAa is $\leq 12\%$ of total binding, CryIAb $\leq 27\%$, and CryIAb $\leq 17\%$.

For the time course of specific binding, 400 μ g/ml BBMV was incubated with 0.1 nM or 10 nM labeled toxin in the presence or absence of 1,000-fold excess of unlabeled toxin for various times before centrifugation. The specific binding of the toxins to the BBMV was calculated by subtracting the nonspecific binding (the binding with excess of unlabeled toxins) from the total binding (the binding without unlabeled toxins).

For the time course of irreversible binding, 400 μ g/ml BBMV was incubated with 0.1 or 10 nM labeled toxin for various times, after which 1 ml of binding buffer with at least 1,000-fold excess of cold toxin was added to the tube. The incubation was continued for 1 h before centrifugation. The nonspecific binding was determined from the tubes in which labeled toxin and 1,000-fold excess of unlabeled toxin were added to BBMV at the same time.

For the saturation assay of specific binding, various concentrations of BBMV were incubated with 0.1 nM labeled toxin in the presence or absence of 1,000-fold excess of unlabeled toxin for 3 h before centrifugation. The specific binding of toxins to BBMV was calculated by subtracting the nonspecific binding (the binding with excess of unlabeled toxins) from the total binding (the binding without addition of unlabeled toxins).

For the saturation assay of irreversible binding, the saturation assay of specific binding was performed allowing 3 h of incubation. Subsequently, binding buffer (1 ml) with or without 1000 fold excess of unlabeled toxin was added to the specific binding assay tubes and nonspecific binding assay tubes, respectively. Incubation was continued for another hour before centrifugation.

For the saturation kinetics assay, irreversible binding time courses were measured with 0.1 nM toxin and various concentrations of BBMV. k_{obs} from these time courses were plotted against concentration of binding sites in BBMV.

TABLE I
Toxicity and binding parameters of CryIA toxins

Toxins ^{a,b}	ID ₅₀	$K_{1/2}$	k_2
	ng	nM	1/min
CryIAa	77.3 (61.7–95.9)	0.973 \pm 0.015	0.299 \pm 0.028
CryIAb	157 (128–193)	0.326 \pm 0.087	0.190 \pm 0.022
CryIAb	187 (154–229)	0.306 \pm 0.077	0.151 \pm 0.014

^a ID₅₀, dose of toxins that causes growth inhibition in 50% of larvae assayed. 95% confidence interval is in parentheses.

^b $K_{1/2}$, half-maximum insertion rate concentration; k_2 , insertion rate constant. Both are followed by their standard errors.

Calculation of B_{max} of Irreversible Binding—The presaturation portion of the curve from the data of saturation assay of irreversible binding (see "Results") was fitted using SigmaPlot software (Jandel Scientific Co.) by Equation 1

$$f = B_{max} \cdot x + a \quad (\text{Eq. 1})$$

Here x is the amount of BBMV, and f is fitted to the amount of irreversibly bound toxin; B_{max} is equal to the slope of the curve, which is the maximum binding of given amount of BBMV; a is the interception of the curve on the y axis, which is close to zero.

Calculation of k_{obs} of Specific or Irreversible Binding—The time course of irreversible binding was fitted by Sigma Plot (Jandel Scientific Co.) using either a single exponential fit or a double exponential fit:

$$f = [T] \cdot (1 - \exp(-k_{obs} \cdot x)) + a \quad (\text{Eq. 2})$$

or

$$f = [T]_1 \cdot (1 - \exp(-k_{obs1} \cdot x)) + [T]_2 \cdot (1 - \exp(-k_{obs2} \cdot x)) + a \quad (\text{Eq. 3})$$

Here x is the time, f is fitted to the concentration of bound toxin, and $[T]$ is total specific binding or total irreversible binding; k_{obs} is the observed first-order rate constant; a is the correction factor, which is close to zero.

Calculation of $K_{1/2}$ and k_2 of Irreversible Binding—The saturation kinetic data were fitted to the following equation.

$$f = k_2 \cdot x / (K_{1/2} + x) \quad (\text{Eq. 4})$$

Here x is the concentration of the binding site; f is fitted to the observed first-order rate constant, k_{obs} ; k_2 is the irreversible binding rate constant; $K_{1/2}$ is the concentration of the binding sites giving half-maximum insertion rate of irreversible binding.

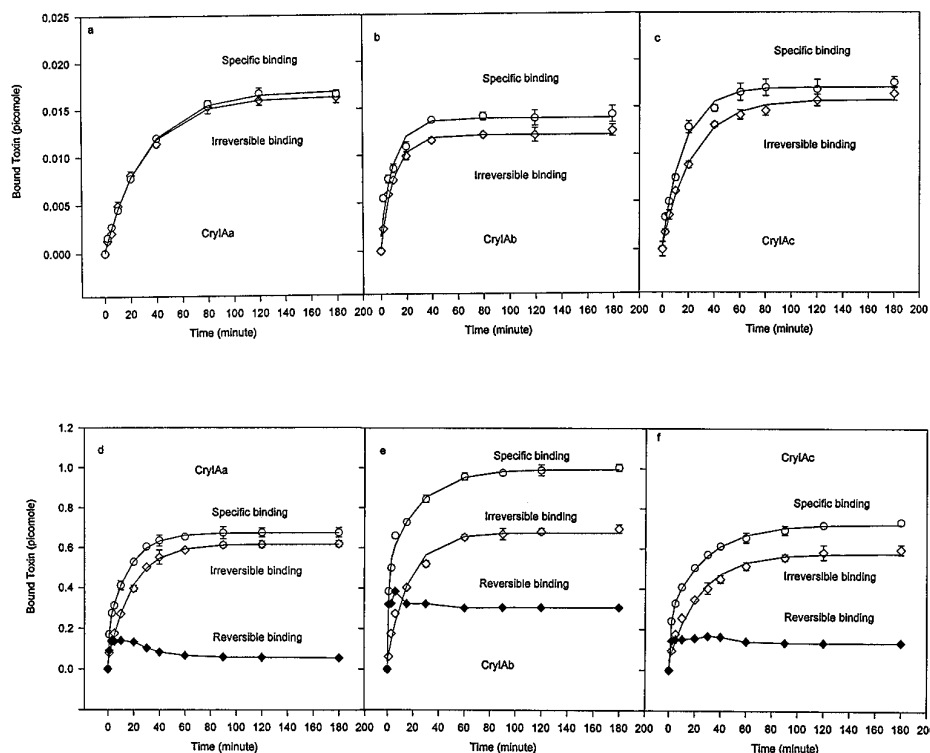
RESULTS

Bioassay of CryIA Toxins with *L. dispar*—Table I shows toxicity of CryIA toxins to *L. dispar* larvae. The order of the potency from strongest to weakest is CryIAa, CryIAb, and CryIAb. These data agree qualitatively with those of Wolfersberger (13) and van Frankenhuyzen *et al.* (26) with respect to the order of toxicities but differ in the level of activity of the CryIAb toxin. A more dramatic difference (400 times) in potency between CryIAb and CryIAb was observed by Wolfersberger (13) and van Frankenhuyzen *et al.* (26). We only observed a slight difference in potency between the two toxins (Table I). The difference between our data and that of Wolfersberger (13) and van Frankenhuyzen *et al.* (26) might result from difference in source of insect, stage of larvae assayed, method used, index measured, or presence of abnormal performance syndrome.

Specific Binding and Irreversible Binding—Specific binding is the total amount of bound toxin minus nonspecific binding to BBMV (labeled toxin bound in the presence of excess unlabeled toxin). We find that specific binding consists of a reversible binding component, which could be chased off by an excess amount of unlabeled toxin, and an irreversible binding component, which could not be chased off. Fig. 1 shows the time

² T. M. O'Dell, personal communication.

FIG. 1. Time course of specific binding and irreversible binding of CryIA toxins to *L. dispar* BBMV. *a* and *d*, CryIAa; *b* and *e*, CryIAb; *c* and *f*, CryIAc. *a-c*, 0.1 nM toxin to 400 μ g/ml BBMV; *d-f*, 10 nM toxin to 400 μ g/ml BBMV. Open circle, specific binding; open diamond, irreversible binding; filled diamond, reversible binding. Standard errors are represented by error bars. Single exponential fits for irreversible binding curves in *d-f* give 0.06, 0.05, and 0.04 min^{-1} for rate constants, respectively. Two exponential fits for specific binding curves in *d-f* give 1.4, 1.1, and 0.7 min^{-1} for the fast phase, respectively, and 0.06, 0.05, and 0.04 min^{-1} for the slow phase, respectively.



course of specific binding and irreversible binding of all three CryIA toxins to *L. dispar* BBMV. A fixed concentration of BBMV (400 μ g/ml) was incubated with either a similar concentration of labeled toxins (0.1 nM in Fig. 1, *a-c*) or a higher concentration (10 nM in Fig. 1, *d-f*). When the amount of labeled toxin input was similar to the amount of irreversible binding sites available, almost all of the labeled toxin became irreversibly bound (Fig. 1, *a-c*). However, when the amount of labeled toxin input was greater than the irreversible binding sites available, a small difference between specific binding and irreversible binding was observed in CryIAa (Fig. 1*d*), and a larger difference was observed in CryIAb and CryIAc (Fig. 1, *e* and *f*). Since the difference between specific binding and irreversible binding is equal to reversible binding, the results suggest that the reversible binding is greater when toxins are in excess.

These data also indicate that at low concentrations of toxin, the rate of irreversible binding ($k_{\text{obs}} = 0.03 \text{ min}^{-1}$ for CryIAa) is comparable with the rate of specific binding ($k_{\text{obs}} = 0.03 \text{ min}^{-1}$ for CryIAa). Accumulation of reversible complex could not be detected by chasing with cold toxins. Thus, initial binding is the rate-determining step in the overall binding process when concentration of toxin is low. At high concentrations of toxin, the kinetics of specific binding are biphasic and fit to a fast rate constant of 1.4 min^{-1} and a slower rate constant of 0.06 min^{-1} (for CryIAa). The kinetics of irreversible binding at high toxin concentration, however, fit to a single exponential with a rate constant of 0.06 min^{-1} (for CryIAa), which is the same as the rate of the slower phase during specific binding. These kinetics indicate that the reversible complex accumulates with a bimolecular rate constant k_1 of about $1.4 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$ ($k_1 = k_{\text{obs}}/[T]$) when toxin is in excess. Under these conditions, the irreversible binding is limited by the rate of reversible complex changing to the irreversible complex.

Reversible Binding—The difference between the time course of specific binding and that of irreversible binding yielded the reversible binding time course (Fig. 1, *d-f*). The concentration of the reversible binding complex increased quickly, remained at steady state for about 20 min, and then decreased to a low

steady state level (Fig. 1, *d-f*). This remaining level of reversible binding complex probably indicates that there must be a small population of reversible bound toxin that does not proceed to an irreversible stage at high toxin to binding site ratios. This may be because there is a small percentage of reversible binding sites distinct from the irreversible binding sites.

Determination of B_{max} —The presence of a certain number of reversible binding sites on BBMV gives rise to two different B_{max} concepts: maximum specific binding and maximum irreversible binding. To investigate the two concepts of B_{max} , saturation binding curves for both specific and irreversible binding were measured. These two curves were similar for CryIAa and CryIAc (Fig. 2, *a* and *c*). However, they were different for CryIAb (Fig. 2*b*), exhibiting a significant difference between specific B_{max} and irreversible B_{max} .

The saturation binding curves (Fig. 2) had two characteristics: a sharp inflection point at saturation and linear portions of the curve both before and after the inflection point. The same characteristic curve can be found in most of previously reported saturation binding results (7–8, 11, 15). The stoichiometric nature of the irreversible binding curve indicates that the slope of the first straight line in the curve is equal to the irreversible B_{max} . Since specific binding consists of irreversible and reversible binding components, the stoichiometric nature of the specific binding curve indicates that the reversible binding is tight under the conditions of the assay. The slope of the first line in the specific binding would be close to specific B_{max} . The B_{max} calculated from CryIAa, CryIAb, and CryIAc saturation curves (Fig. 2) are shown in Table II. A significant difference between specific B_{max} and irreversible B_{max} is observed only in CryIAb. Since the toxicity of Cry toxins is assumed to depend on their ability to insert into the membrane, the irreversible B_{max} would be of primary concern. So " B_{max} " in the later part of this paper refers to the irreversible B_{max} .

Saturable Kinetics of the Irreversible Binding—Fig. 1 is consistent with the reversibly bound complex of CryIAa being an intermediate in the pathway that accumulated for a short period. These kinetics are characteristic of toxin binding to BS in BBMV by a two-step mechanism,

FIG. 2. Saturation binding of CryIA toxins to *L. dispar* BBMV. a, CryIAa; b, CryIAb; c, CryIAc. Open circle, specific binding; open diamond, irreversible binding. Standard errors are represented by error bars. 0.1 nM labeled toxins were incubated with increasing concentration of BBMV.

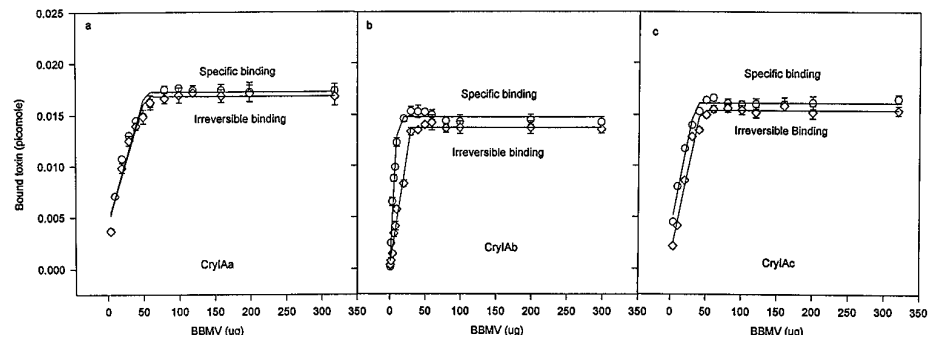


TABLE II
Maximum bindings of CryIA toxins

	CryIAa ^a	CryIAb	CryIAc
	pmol/mg	pmol/mg	pmol/mg
Specific B_{\max}	0.241 ± 0.028	0.872 ± 0.065	0.360 ± 0.028
Irreversible B_{\max}	0.237 ± 0.022	0.459 ± 0.034	0.339 ± 0.025

^a B_{\max} values are followed by their standard errors.



REACTION 3

where k_1 is the bimolecular association rate constant for BS*T complex, k_{-1} is the dissociation rate constant for BS*T complex, and k_2 is the rate constant for irreversible binding.

If binding of toxin to BBMV is a two-step process as shown above, then the rate of irreversible complex formation, k_{obs} , is related to BS concentration by the following relationship (27),

$$k_{\text{obs}} = k_2 \cdot [\text{BS}] / (K_{1/2} + [\text{BS}]), \quad (\text{Eq. 5})$$

which predicts that the plot of k_{obs} versus [BS] should be saturable. The maximum rate will be equal to k_2 and the BS concentration at half the maximum rate is the $K_{1/2}$, which is equal to $(k_2 + k_{-1})/k_1$. It represents the stability of the reversible binding complex, when k_2 is comparable to k_{-1} , or $k_2 \gg k_{-1}$. It is equal to K_d , the dissociation constant of the reversible binding complex, when the $k_{-1} \gg k_2$. On the other hand, if binding of toxin was a one-step process, as shown in Reaction 1, then the rate, k_{obs} would increase linearly with BS concentration.

Saturable k_{obs} was observed in time course of irreversible binding assays of all three CryIA toxins with increasing concentration of binding site, as shown in Fig. 3. CryIAa appeared to have a saturated k_{obs} at a higher concentration of binding site than CryIAb and CryIAc (Fig. 3). The reaction constants, k_2 and $K_{1/2}$, calculated from Fig. 3 for all three toxins are listed in Table I.

DISCUSSION

Channel or pore formation in the epithelium plasma membrane of the insect larvae midgut is believed to be critical to the mechanism of insecticidal activity of *B. thuringiensis* toxins. The first two steps in the channel formation process for an activated toxin are binding to the membrane and insertion into the membrane (1, 2). Thus, characterization of these events is essential for a detailed understanding of the overall channel formation process. Specific binding sites for *B. thuringiensis* toxins have been successfully identified in many insect species by binding assays (4). However, the role of binding studies now becomes more important and more complicated when the focus shifts to cloning and characterization of binding proteins (receptors) and extensive mutagenesis in all three domains of the

toxins. For example, it may be necessary to determine whether a particular mutation affects only the initial binding step or the irreversible binding step.

Here we propose a two-step kinetic model for interaction between *B. thuringiensis* toxin and BBMV, as shown in Reaction 2, based on the following observations. 1) A large body of evidence, including results presented in this paper, has shown that the binding of *B. thuringiensis* toxin to BBMV is mainly irreversible (4, 6, 7, 19, 20); 2) accumulation of reversible binding complex at early times of incubation (Fig. 1); 3) k_{obs} of irreversible binding was saturable at high concentration of BBMV (Fig. 2). Neither a one-step reversible binding model nor a one-step irreversible binding model could explain these observations.

This paper utilizes saturation kinetics to analyze binding data and to derive binding parameters based on the two-step interaction model between the toxins and BBMV. The method used by this paper enables us for the first time to study separately the first two steps in channel formation, initial binding and irreversible binding, by analyzing the affinity of the initial binding and the rate constant of irreversible binding.

We observe that specific binding of toxin to BBMV involves two types of binding sites: a site that could develop into an irreversible stage (irreversible binding site) and a site that remains reversible (reversible binding site) (Figs. 1 and 2, Table II). Thus, it is important to compare the irreversible B_{\max} values of different toxins rather than the specific B_{\max} values, since irreversible binding is thought to reflect insertion and, consequently, is more directly related to toxicities.

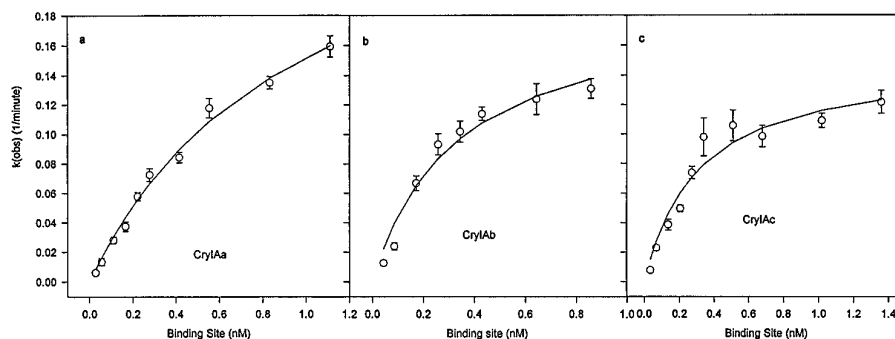
To simplify the analysis of saturation kinetics, the reversible binding sites have not been considered in Reaction 3. Despite extensive studies, there are also many uncertain steps in the channel formation process. For example, it is not clear whether insertion is facilitated by the binding proteins or whether the binding proteins are recyclable after the toxins insert. Thus the Reaction 3 should be considered as a minimum reasonable mechanism, which accounts for the experimental results.

Among the three wild type CryIA toxins, CryIAb and CryIAc show a similar binding affinity, which is 3 times higher than that of CryIAa (Table I). However, CryIAa has a faster irreversible binding rate. The order of the irreversible binding rate constant of the three toxins is CryIAa > CryIAb > CryIAc, which is directly correlated to the order of toxicity of the three toxins (Table I).

CryIAb has more irreversible binding sites than CryIAc and almost twice of that of CryIAa (Table II). Several reports have shown that CryIAb toxin and CryIAa toxin bind to a same binding protein in Western blots with BBMV proteins from various species of insects (28, 29), including *L. dispar*.³ It would

³ Valaitis, A. P., Lee, M. K., Rajamohan, F., and Dean, D. H. (1995) *Insect Biochem. Mol. Biol.*, in press.

FIG. 3. Saturation kinetics in the binding of CryIA toxins to *L. dispar* BBMV. a, CryIAa; b, CryIAb; c, CryIAc. k_{obs} of irreversible binding of 0.1 nM labeled toxin to increasing concentration of binding site was plotted against concentration of binding site. Standard errors are represented by error bars.

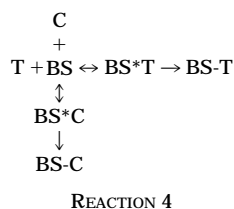


be tempting to speculate that the large CryIAa-CryIAb-binding protein, which is 210 kDa as a monomer,³ could have twice the amount of binding sites for CryIAb relative to CryIAa.

Toxicity of a toxin can be represented by its efficacy and potency. Efficacy is determined by the number of binding sites for the toxin in the target larva. When sufficient toxins are administered to the larvae, maximum growth inhibition could be observed in all three toxins (data not shown). This indicates that a specific number of CryIAa or CryIAc binding sites is sufficient to induce a maximum efficacy in growth inhibition and an increase in the amount of binding sites of CryIAb would not increase its efficacy.

The potency of a toxin is believed to depend on the mechanism of its action. For example, if the effect of a drug is produced through the reversible binding between the drug and its receptor, the affinity of binding usually determines the potency of the drug. We have demonstrated that the potency of the CryIA toxins is directly related to their insertion rate constant, k_2 . Quicker insertion brings a stronger potency to a toxin. It would not be too difficult to imagine that *L. dispar* larvae could have a self-defense mechanism when it is attacked by CryIA toxins. For example, aminopeptidase N, identified as CryIAc-binding protein in some insect species (30, 31), is attached to the plasma membrane of the epithelium cell by a glycosyl phosphatidylinositol anchor (32). Conformational change of the aminopeptidase N upon binding of the toxin could lead it to be susceptible to some membrane bound phospholipases and cleaved from the membrane. Consequently, the toxin could lose its activity in channel formation because of the cleavage of the aminopeptidase N. Therefore, quicker insertion of a toxin would leave less time for phospholipases to cleave its binding protein, thus leading to a stronger potency.

It is worth noting that although the parameters derived from the Scatchard equation would be misleading, the data obtained from the competition assays, the most popular assay in previous literature, are still informative if the assay is conducted in an appropriate way. The competition assay could be represented by the following reaction (Reaction 4).



Here C is the competitor, BS^{*}C is the reversible complex formed between competitor and binding site, and BS-C is the irreversible complex. Assume that the irreversible binding sites are predominant to the reversible binding sites, and the incubation time is long enough for all binding complexes at irreversible binding sites to become irreversible. When the amount of binding sites is not greater than that of labeled

toxin, the decrease in the radioactivity detected with the BBMV would be determined by the competition between rate of BS-T formation and rate of BS-C formation. The rate of the irreversible complex formation depends on both the concentration of reversible complex and the rate constant for irreversible binding, k_2 , for each toxin, rate = $k_2 \cdot [BS^*T]$. The [BS^{*}T] depends on the stability of the reversible complex, $K_{1/2}$ (or K_d). Thus, the competition assay reflects the combination of the effect of k_2 and $K_{1/2}$ (or K_d) in the whole integration process of the toxin to membrane. A different competition binding curve indicates a difference in the whole integration process. However, it could not differentiate whether the difference comes from the binding affinity ($K_{1/2}$, or K_d) or from the irreversible binding rate constant (k_2). On the other hand, if there is no difference between the competition binding curves, one may not assume that the two toxins have the same binding affinity and the same irreversible binding rate constant. A higher affinity might be offset by a smaller irreversible binding rate constant. Indeed, Chen *et al.* (33) have identified such a mutant, CryIAa A92E. Keeping this in mind, the competition assay is still a useful method to screen mutants with different binding properties since it is a much simpler assay than the method presented in this paper.

To quantitatively represent the difference in competition binding curves, the concentration of the competitor that causes 50% inhibition in specific binding of labeled toxin (IC_{50}), would be useful in comparison. However, the physiological meaning of the IC_{50} should not be considered same as the K_d in the Scatchard equation; instead it reflects the combination of $K_{1/2}$ (or K_d) and k_2 , as discussed above.

A second binding assay, the dissociation assay, has been more frequently used since researchers have become aware of the role of irreversible step in the determination of toxicity. The percentage of dissociation, or the amount of toxin that is chased off from specifically bound toxins, has been compared for different toxins (20). However, because of the possible existence of a reversible binding site, the percentage of the dissociation is not very informative in understanding the mechanism of toxicity. To compare irreversible binding of two different toxins, a time course of irreversible binding as shown in this paper (Fig. 1) would be more informative than the dissociation binding assay. Not only would the absolute level of the irreversible binding be detected, the rate of irreversible binding would also be viewed through the time course of irreversible binding.

In this report we find a direct relationship between toxicity of the CryIA toxins and the irreversible binding rate constant, k_2 , to *L. dispar* BBMV. This indicates that this step, which we assume is primarily the insertion of toxin into the apical membrane, is an important step in determining the activity of a toxin. This observation apparently solves the paradox posed by the paper of Wolfersberger (13) and is consistent with the observations of Wolfersberger (34) that inhibition of K⁺ gradient-driven amino acid transport by δ -endotoxins CryIAb and

CryIAC on *L. dispar* BBMV is directly related to their larvicidal activity. This of course does not exclude the importance of other events in determining toxicity. Further investigation of post-insertion events, such as oligomerization of inserted toxins in the membrane, will be needed to elucidate the mechanism of channel formation of the toxin in the membrane.

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