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Study of the irreversible binding of *Bacillus thuringiensis* Cry1Aa to brush border membrane vesicles from *Bombyx mori* midgut

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Abstract

The binding of *Bacillus thuringiensis* δ-endotoxin to brush border membrane vesicles (BBMVs) from the target insect larval midgut comprises with not only a reversible but also an irreversible component. The irreversible binding of δ-endotoxin is thought to be a pathologically important factor. Here, we studied the irreversible binding of Cry1Aa to the BBMVs of *Bombyx mori*. The ^{125}I-labeled Cry1Aa bound to the solubilized brush border membrane (BBM) through rapid dissociation only, unlike the binding to BBMVs, indicating that the toxin bound to the solubilized BBM through only a reversible process. Low-temperature sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis revealed that the toxin bound irreversibly to BBMVs formed an oligomer of 220 kDa, whereas that bound reversibly to the solubilized BBM did not oligomeraize. When the ^{125}I-labeled Cry1Aa bound irreversibly to the BBMVs was digested by proteinase K, approximately 40% of the toxin observed to be resistant to proteinase K. The molecular mass of the toxin resistant to proteinase K was 60 kDa, suggesting that the irreversible binding comprise two forms. These results support the notion that the irreversible binding of the toxin to BBMVs is due to the insertion of the toxin into the lipid bilayers and oligomerization to form channels.

Keywords: *Bacillus thuringiensis*; Cry1Aa; BBMVs; *Bombyx mori*; Irreversible binding; Oligomerization; Proteinase K digestion

1. Introduction

Bacillus thuringiensis, which is a gram-positive spore-forming bacterium, produces a crystalline inclusions with insecticidal activity. These inclusions are composed of one or more δ-endotoxins in the form of inactive protoxins (Höfte and Whiteley, 1989). After ingestion by susceptible insect larvae, these protoxins are dissolved and undergo proteolytic digestion to form active toxins in the midguts (Haider et al., 1986; Ogiwara et al., 1992). The active toxins then bind to specific receptors, aminopeptidase N (Knight et al., 1994; Sangadala et al., 1994; Abdullah et al., 2006) or cadherin-like protein (CADR) (Vadlamudi et al., 1995; Ihara et al., 1998; Nagamatsu et al., 1998), on the midgut epithelial cell membranes. The binding of the δ-endotoxin to the midgut epithelial cell membranes appears to comprise of two components, a reversible and an irreversible component (Ihara et al., 1993; Chen et al., 1995; Liang et al., 1995; Rajamohan et al., 1995). Binding studies of Cry1Aa and Cry1Ab to brush border membrane vesicles (BBMVs) from *Bombyx mori* revealed that the proportion of these components was different between two toxins; the major component was irreversible for Cry1Aa and reversible for Cry1Ab (Ihara et al., 1993). Since Cry1Aa is more toxic to the insect than Cry1Ab, the irreversible binding has been suspected to be an important factor for their toxicity (Ihara et al., 1993). Later studies have also reported correlations between toxicity and irreversible binding (Chen et al., 1995; Liang et al., 1995; Rajamohan et al., 1995).

The three-dimensional structures of six Cry proteins have been determined (Li et al., 1991; Grochulski et al.,

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1995; Galitsky et al., 2001; Morse et al., 2001; Boonserm et al., 2005, 2006). These structures are extensively similar. A Cry protein molecule is composed of three domains connected by single linkers. Domain I (N-terminal domain) consists of seven amphipathic α-helices in which a central α-5 is hydrophobic and is encircled by the six other helices (Li et al., 1991; Grochulski et al., 1995; Galitsky et al., 2001; Morse et al., 2001; Boonserm et al., 2005, 2006). Study on synthetic peptides corresponding to α-5 of Cry3A revealed that the peptides have an ion channel activity and cytotoxicity against Sf-9 cells (Gazit et al., 1994). Synthesized α4–loop–α5 of Cry1Ac showed an activity of pore-formation (Gerber and Shai, 2000). Based on these results, post-binding events have been assumed to be an insertion into membrane and oligomerization to form pore. When Cry1Ab and Cry1Ac bound to the BBMVs of *Manduca sexta*, they formed large antigenic oligomers of 200 kDa, whereas inactive toxins with mutations in amphipathic helices (α-5) did not oligomerize (Aronson et al., 1999). Mutagenesis analysis revealed the residues involved in the oligomerization and degree of oligomerization correlated with toxicity (Aronson et al., 1999). Furthermore, recent studies demonstrated the formation of pre-pore oligomeric structure that is important for insertion into the membrane and for toxicity (Gómez et al., 2002; Rausell et al., 2004b). The pre-pore oligomer was formed by the incubation of Cry1Ab protoxin with the single chain antibody scFv73 that mimics the CADR or with the toxin-binding peptides of CADR, and treatment with *M. sexta* midgut juice (Gómez et al., 2002). Therefore it is assumed that, after binding of monomeric Cry toxin to CDAR, a conformational change is induced in the toxin to form the pre-pore oligomer (Bravo et al., 2007).

Irreversible binding of Cry toxin is thought to be a pathologically important factor. The irreversible binding in this case was assumed to be related with the post-binding events, such as membrane insertion, oligomer formation, and ion-channel formation. To date, only one study has dealt with the relationship between oligomerization and the irreversible binding of Cry1A toxins (Cry1Ab and Cry1Ac) to BBMVs of *M. sexta* and *Heliothis virescens* (Aronson et al., 1999). To gain a better understanding of this irreversible binding, we examined the binding of the Cry1Aa to *B. mori* BBMV or solubilized BBM, oligomerization, and protease digestion of the toxin bound irreversibly to the BBMVs.

2. Methods

2.1. Purification and iodination of activated δ-endotoxin

Cry1Aa was expressed and accumulated as inclusions in the recombinant *Escherichia coli* JM103 (Oeda et al., 1987). Cry1Aa was purified and activated as described previously (Ihara et al., 1993). The purified toxin was iodinated by the chloramine-T method as described previously (Ihara et al., 1993). The protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

2.2. Preparation and solubilization of BBMVs

The BBMVs from *B. mori* were isolated by the method of Wolfersberger (Wolfersberger et al., 1987). The final BBMV preparation was resuspended in TN buffer (20 mM Tris–HCl, 150 mM NaCl (pH 7.5)). The BBMVs were solubilized as described previously (Ihara et al., 1998). The BBMVs (5–10 mg/ml) were incubated in TN buffer containing 2% of cholic acid for 1 h at 4 °C. After centrifugation at 100,000*g* for 1 h, the resulting supernatant contained more than 80% of the binding activity of the toxin. Approximately 40% of the membrane protein was solubilized by this procedure.

2.3. Binding assay

The binding of the toxin to BBMV was assayed by the rapid filtration method as described previously (Ihara et al., 1993). For the binding of the toxin to solubilized BBM, the bound and free toxins were separated by gel filtration on Toyopearl HW-60 S (1.4 × 23 cm; Tosho, Tokyo, Japan). Solubilized BBM was incubated with ^{125}I-labeled toxin in TN buffer containing 0.1% of BSA and 1% cholic acid, for prevention of aggregation of membrane protein, at room temperature. For the binding of the toxin to BMMVs, BBMVs was incubated with ^{125}I-labeled toxin in TN buffer containing 0.1% of BSA at room temperature. Nonspecific binding was determined by incubation under the same conditions except for the presence of excess unlabeled toxin (0.8 μM). The sample was applied to the column, equilibrated with the same buffer, and eluted at a flow rate of 1 ml/min. Aliquots of 1 ml were fractionated and counted in a gamma counter (Packard COBRA).

2.4. Low-temperature sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Oligomerization of the toxin was analyzed by low-temperature sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Gómez et al., 2002). Samples were mixed with SDS-sample buffer (final concentration 62.5 mM Tris–HCl (pH 6.8), 5% glycerol, 4% 2-mercaptoethanol, 1% SDS, and 0.01% bromophenol blue) and incubated at 40, 60, 80, and 100 °C for 10 min. Samples were subjected to SDS–PAGE on conventional 5% gels and analyzed at 4 °C. Autoradiograms were obtained from the dried gels after exposure to Kodak X-OMAT AR film with enhancing screen for 1–3 days at −80 °C.

2.5. Protease digestion

The toxins bound irreversibly to the BBMVs were digested using several concentrations of proteinase K. The ^{125}I-labeled toxin and BBMVs to which it was bound