N-terminal Activation Is an Essential Early Step in the Mechanism of Action of the Bacillus thuringiensis Cry1Ac Insecticidal Toxin*

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A variant form of the Bacillus thuringiensis Cry1Ac toxin that is not cleaved at the N terminus during proteolytic activation with trypsin was found to be incapable of forming pores in Manduca sexta brush border membrane vesicles in vitro and had reduced insecticidal activity in vivo. Binding studies indicated an altered binding pattern of the mutant toxin in that bound toxin could not be fully displaced by a high molar excess of fully trypsin-activated toxin. These results suggest that proteolytic removal of the N-terminal peptide of Cry1Ac is an important step in toxin activation.

The entomocidal Cry1 toxins of Bacillus thuringiensis are synthesized as inactive protoxins of around 130 kDa within the bacterial cytoplasm (1). Upon ingestion by a susceptible insect the protoxin is activated through the proteolytic removal of an N-terminal peptide of 25–30 amino acids and approximately half of the remaining protein from the C terminus. The activated toxin then binds to specific sites on the brush border membrane of the midgut epithelium before inserting into the membrane and forming a pore. B. thuringiensis toxins produced in transgenic plants have often been expressed as proteins truncated at the C terminus because this has resulted in higher expression levels (2). These truncated forms are regularly described as the active form of the toxin despite having an intact N terminus. The role of the C-terminal extension to the active toxin is believed to be in the formation of crystalline inclusion bodies within the bacterium and is dispensable for toxicity (3). Little is known about the role of the N-terminal peptide and whether its removal is important in the mechanism of action of the toxin. It has previously been observed that if an engineered Cry1Ab or Cry1Ca toxin lacking the N-terminal peptide is expressed in Escherichia coli, growth of the E. coli culture is severely affected (4–6). It was speculated that the N-terminally truncated toxin was free to interact with the cell membrane of the E. coli host cell and that the first 28 amino acids prevented the Cry toxin from inserting into the membrane. When expressed in B. thuringiensis an N-terminally truncated Cry1Ac toxin was expressed at a much lower level than the full-length toxin, and the formation of crystals was repressed. Although this might have indicated a role of the N terminus in promoting crystallization, the low levels of expression and lack of crystals might also have been an effect of an inappropriate interaction between the toxin and the host cell (6). In this article we compare the toxicity, binding, and pore-forming abilities of activated Cry1Ac with a partially activated recombinant form of Cry1Ac in which the N terminus remains intact after treatment with trypsin.

EXPERIMENTAL PROCEDURES

Preparation of the Toxin Samples—Site-directed mutagenesis was used to remove the N-terminal tryptic cleavage site of Cry1Ac using methods described previously (7). Two amino acid substitutions were made, R28T and I29M. Both mutant and wild-type toxins were expressed in E. coli JM109, and crystalline material was purified from sonicated cell cultures by sucrose density centrifugation. The protoxin was solubilized in 50 mM Na2CO3, pH 11.4 (37 °C, 1 h) and then activated by incubation with trypsin (1 mg/ml; 37 °C, 1 h) in the same buffer. The trypsin-treated toxin was purified by Mono Q FPLC† and stored in buffer A: 100 mM methylglycine chloride, 10 mM 2-cyclohexylaminomethanesulfonic acid, pH 9.5 (Fig. 1).

Preparation of Brush Border Membrane Vesicles (BBMVs)—

Manduca sexta larvae were reared on artificial diet (Bio-Serve) at 23 °C and filtered through a 50-μm sieve. BBMVs from fifth instar larvae were prepared and analyzed as reported previously (8) except that neomycin sulfate (2.4 μg/ml) was included in the buffer (300 mM mannitol, 20 mM 2-mercaptoethanol, 5 mM EGTA, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 150 μM/ml pepstatin A, 100 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor, 10 μM HEPEs-HCl, pH 8.0). The vesicles were dialyzed overnight against 400 volumes of 150 mM KCl, 10 mM HEPEs-HCl, pH 7.5 (Sigma) and sonicated for six periods of 30 s each at 25 °C (BRANSON 1200 sonic bath, Danbury, CT) in the same solution. BBM enrichment was estimated according to the alkaline phosphatase (AP) and cytochrome-c oxidase activity relative to the initial homogenate (9.5-fold increase in AP/mg of protein). Vesicle orientation was determined from the AP activity in the presence and absence of 0.02% Triton X-100.

Fluorescence Measurements—Pore formation activity was assayed by monitoring changes in membrane potential with the fluorescent, positively charged dye 3,3′-dipropylthiodicarbocyanine (Di-C6-5), Molecular Probes, Eugene, OR) as described previously (9). Fluorescence was recorded at 620/670 nm in a Hansatech system (Norfolk, England). Hyperpolarization causes dye internalization into the BBMVs and a decrease in fluorescence; depolarization causes the opposite effect. BBMVs (10 μg) previously loaded with 150 mM KCl were suspended in 900 μl of 150 mM N-methyl-d-glucamine chloride, 10 mM HEPEs-HCl, pH 8 buffer. After equilibration of the dye (2 min), 50 nm toxin was added. Changes in membrane potential were monitored by successive additions of KCl to the BBMV suspension. Analyses of the slope (m) of ΔF (F) versus K+ equilibrium potential (Erev) (mV) curve are reported in this work. Erev was calculated with the Nernst equation.

Binding Assay—Binding analysis and homologous competition of wild-type and mutant toxins to M. sexta BBMVs were performed in a semiquantitative binding assay. Toxin was biotinylated by using biotin- N-hydroxysuccinimide ester (RNPS28, Amersham Biosciences) according to the manufacturer's instructions. Binding was performed in 100 μl of binding buffer (PBS, 0.1% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20, pH 7.6). 20 μg of BBMV protein were incubated with 10

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channel present in the BBMVs gave a similar response to the mutant C3-(5), were recorded as described under of a fluorescent dye sensitive to changes in membrane potential, Dis-

Changes in distribution obtained).

A was added instead of toxin (a trace similar to the mutant toxin was

contaminating procedure. A constant volume of the sample to be tested

was added to the assay mixture. The final volume was adjusted to 1.5 ml

with the same buffer. Finally the BBMVs were suspended in 20

samples were briefly centrifuged to remove insoluble material. 5-

were centrifuged at 10,000 rpm for 10 min)

was added. Samples were boiled for 3

min, electrophoresed in SDS-polyacrylamide gels, and electrotrans-

anol, 0.01% bromphenol blue) was added. Samples were boiled for 3

samples were centrifuged at 10,000 rpm for 10 min and then filtered through 0.2-

brush border membrane vesicles. Biotinylated, trypsin-treated wild-type (a) or mutant (b) toxin were incubated with the BBMVs in the absence (lanes 1) or presence of different -fold excesses of unlabeled, fully activated Cry1Ac toxin (lanes 2, 1000-fold; lanes 3, 500-fold; lanes 4, 100-fold, lanes 5, 50-fold. Lane M, molecular weight markers. c, binding of biotinylated toxin is expressed as percentage of the amount bound upon incubation with labeled toxin alone.

FIG. 3. Binding of wild-type and mutant Cry1Ac to M. sexta brush border membrane vesicles. Biotinylated, trypsin-treated wild-type (a) or mutant (b) toxin were incubated with the BBMVs in the absence (lanes 1) or presence of different -fold excesses of unlabeled, fully activated Cry1Ac toxin (lanes 2, 1000-fold; lanes 3, 500-fold; lanes 4, 100-fold, lanes 5, 50-fold. Lane M, molecular weight markers. c, binding of biotinylated toxin is expressed as percentage of the amount bound upon incubation with labeled toxin alone.

FIG. 4. Proteolytic digestion of wild-type and mutant Cry1Ac with M. sexta gut extract. Lanes 1–6, wild-type protoxin; lanes 4–6, mutant protoxin. Lanes 1 and 4, 1:10 dilution; lanes 2 and 5, 1:100 dilution; lanes 3 and 6, 1:1000 dilution of M. sexta gut extract.

RESULTS AND DISCUSSION

In vitro pore formation assays performed with either fully or partially activated Cry1Ac toxin clearly demonstrate that the partially activated toxin is incapable of forming pores in M. sexta BBMVs since no membrane polarization was observed upon addition of toxin (Fig. 2). To test whether this could have been due to reduced binding of the partially activated toxin, binding assays were performed (Fig. 3). The results show that initial binding of labeled, fully activated Cry1Ac toxin to M. sexta BBMVs is competitive and largely abolished in the presence of an excess of unlabeled toxin. In contrast, although the partially activated toxin binds to the BBMVs a significant proportion remains bound even in the presence of the large excess of unlabeled fully activated toxin. When the assay was repeated in the absence of BBMVs no labeled toxin was ob-

active dose estimates (50% lethal concentration of the toxins, LC50) were calculated using PROBIT analysis.

Midgut Juice Isolation and in Vitro Processing of Protoxins—Fifth instar M. sexta larvae were chilled for 10 min on ice, and midgut tissue was dissected. Midgut juice was separated from solid material by cen-

trifugation (5,000 rpm for 10 min) and filtered through 0.2-

samples were briefly centrifuged to remove insoluble material. 5-

samples were subjected to SDS-PAGE.
appreciable \textit{in vitro} pore formation activity could be observed
with the mutant toxin it was interesting to observe that the
mutant protoxin demonstrated \textit{in vivo} toxicity, albeit ~25-fold
lower than the wild-type toxin. A possible explanation for this
is that proteinases other than trypsin within the \textit{M. sexta} gut
were fully activating the mutant toxin. This was tested by
incubating wild-type and mutant toxins with varying concen-
trations of \textit{M. sexta} gut extract and monitoring activation by
SDS-PAGE. The results are shown in Fig. 4. At the highest
centration of gut extract used both protoxins appeared fully
activated, suggesting that alternative enzymes do indeed exist.
At higher dilutions of gut extract it can be seen that while the
wild-type protoxin was fully activated, the mutant protoxin
was only partially activated at the N terminus. N-terminal
sequencing has confirmed that the higher molecular weight
band has the same N terminus as the protoxin. The reduced
toxicity of the mutant protoxin could thus be explained by the
reduced rate of N-terminal activation. The fact that mutant
toxin, activated by endogenous proteinases, has insecticidal
activity eliminates the possibility that the lack of pore-forming
ability described earlier could have been due to a misfolded and
thus inactive protein.

It has previously been suggested that the presence of the
N-terminal peptide might prevent binding to non-target mem-
branes (4), suggesting that the pore-forming potential of do-
main I is only realized after proteolytic removal of this peptide.
In the homologous Cry1Ab protoxin a similar mutation that
prevented N-terminal processing also showed reduced toxicity
to \textit{M. sexta} larvae (4). Indirect evidence for a possible role of the
N-terminal fragment in modulating binding of the toxin has
come from the solution of the structure of the Cry2Aa toxin
(10). Unlike previously reported structures for the Cry3Aa and
Cry1Aa toxins, the Cry2Aa toxin had not been proteolytically
activated and therefore contained an uncleaved N-terminal
peptide. The structure revealed that the N-terminal region
masks a region of the toxin believed to be involved in the
interaction between the toxin and the brush border membrane
of the target insect. The N terminus of the Cry1 toxins might
similarly prevent an interaction between the toxin and the
target membrane, an important initial step in pore formation.
The altered binding properties of the mutant toxin could be due
to an additional binding activity between the N-terminal pep-
tide and the BBMVs following binding of the toxin to its recep-
tor. This additional binding affinity would be strong enough to
prevent full displacement of the toxin by an excess of competitor.

The conclusion to be drawn from this work is that N-terminal
cleavage of a Cry1 protoxin is required before the toxin can
properly be considered active. The reduced activity of the mu-
tant toxin \textit{in vivo} suggests that it might be possible to engineer
a toxin that would only be fully active in insects sharing a
specific combination of receptor and gut proteinase.

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