Role of DNA in the Activation of the Cry1A Insecticidal Crystal Protein from Bacillus thuringiensis*

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The Cry1A insecticidal crystal protein (protoxin) from six subspecies of Bacillus thuringiensis as well as the Cry1Aa, Cry1Ab, and Cry1Ac proteins cloned in Escherichia coli was found to contain 20-kilobase pair DNA. Only the N-terminal toxic moiety of the protoxin was found to interact with the DNA. Analysis of the crystal gave approximately 3 base pairs of DNA per molecule of protoxin, indicating that only a small region of the N-terminal toxic moiety interacts with the DNA. It is proposed that the DNA/protoxin complex is virus-like in structure with a central DNA core surrounded by protein interacting with the DNA with the peripheral ends of the C-terminal region extending outward. It is shown that this structure accounts for the unusual proteolysis observed in the generation of toxin in which it appears that peptides are removed by obligatory sequential cleavages starting from the C terminus of the protoxin. Activation of the protoxin by spruce budworm (Choristoneura fumiferana) gut juice is shown to proceed through intermediates consisting of protein-DNA complexes. Larval trypsin initially converts the 20-kilobase pair DNA-protoxin complex to a 20-kilobase pair DNA-toxin complex, which is subsequently converted to a 100-base pair DNA-toxin complex by a gut nuclease and ultimately to the DNA-free toxin.

Bacillus thuringiensis (Bt) deposits a proteinaceous crystal during sporulation (1). The major component of crystals toxic to lepidopteran larvae is a 130-kDa protein (protoxin) (2). On ingestion, the protoxin is acted on by a trypsin-like gut protease and converted to a 65-kDa toxin derived from the N-terminal region of the protein (3–5). The toxin binds to receptors on the brush border membrane (6–8) and is inserted into the membrane, leading to disruption of membrane function with subsequent larval death (9, 10). An unusual feature is that activation of the protoxin appears to occur by a sequential series of proteolytic cleavages, starting at the C terminus and proceeding toward the N terminus until the protease-stable toxin is generated (11, 12). As there is no known protein structural motif that would give rise to such a proteolytic process, this phenomenon is indicative of either a novel type of protein structure or the presence of an additional structural component that confers this property to the protein.

An unexpected finding was that a 20-kbp heterologous DNA fragment is intimately associated with the crystals from B. thuringiensis subsp. kurstaki HD73 (13). The DNA is not susceptible to nuclease attack by the protoxin or proteolyzed to toxin. The active toxin is not associated with DNA; however, evidence was obtained which indicated that the DNA was involved in the generation of toxin from the crystal protein. At present, the nature of the interaction of the Bt protein with DNA and the role of the DNA in the generation of toxin are unknown. The present investigation was undertaken to determine the role of the DNA in the structure and function of the Bt crystal protein.

EXPERIMENTAL PROCEDURES

Crystal Preparation—B. thuringiensis subsp. kurstaki HD73 was grown in half-strength trypticase broth. Harvesting of the cells for crystal purification was performed after 5–7 days based on examination of the cultures under a phase contrast microscope to confirm sporulation and crystal formation in a minimum of 50% of cells. The cells were then lysed in the presence of 1x NaCl and 0.1% Triton X-100 at 4 °C, and the crystals obtained were purified by Renografin gradient as described previously (14).

Cloned Cry1A Proteins—Inclusion bodies containing Cry1Aa, Cry1Ab, and Cry1Ac protoxins (15, 16) cloned in E. coli were obtained from Dr. Luke Masson of the Biotechnology Research Institute, Montreal, Quebec.

Amino Acid Analysis—Aliquots (20 µl) of purified crystal suspension were lyophilized and added to 1.00 ml of 6 N HCl containing 100 mmol of norleucine in a hydrolysis tube and hydrolyzed in vacuo for 24 h at 110 °C. Amino acid analysis was carried out on an Applied Biosystems model 420 amino acid analyzer equipped with an automated phenylisothiocyanate precolumn derivatization system.

Phosphate Analysis—Phosphate analyses were performed by the modified microprecedure of Bartlett (17). A standard phosphate solution was prepared by dissolving 1.097 g of KH₂PO₄ in 250 ml of deionized-distilled water such that a 1:100 dilution of the stock yielded a concentration of 10 µg/ml phosphate. Aliquots of crystals containing between 0.5 and 10 µg of phosphate (preliminary analyses were used to determine the volume of crystal suspension required) were freeze dried in digestion tubes. Similarly, aliquots (0.1–1.0 ml) of the standard phosphate solution were also freeze dried in digestion tubes. After lyophilization, 0.4 ml of perchloric acid was added to each tube, hydrolyzed for 4 min, and cooled. 4.2 ml of deionized-distilled H₂O, 0.2 ml of a 5% ammonium molybdate solution, and 0.2 ml of amido reagent (0.5 g amido in 50 ml of 20% sodium bisulfite solution and filtered) were added to each tube. The tubes were covered with aluminum foil and heated in a boiling water bath for 7 min, and the color was allowed to develop for 30 min. Absorbance of the molybdenum blue complex was measured at 830 nm (relative to a water blank) in quartz cuvettes (1.0 cm) using a Philips Pye Unicam PU 8800 UV-visible spectrophotometer. A standard curve was constructed using SigmaPlot 4.0 (with a 95% confidence interval).

Protein Quantification—For routine operations, protoxin and toxin concentrations were estimated from the absorbance at 280 nm (18). For quantification of the DNA/protein ratio, protoxin and toxin were quantified by dividing the amount of each amino acid obtained from amino
acid analysis by the number of residues predicted from the gene nucleotide sequence of the protoxin or toxin (18, 19).

Quantification of DNA/Protein Ratio—The DNA/protein ratio, expressed in base pairs of DNA/molecule of protein, was calculated as the ratio of the concentrations of phosphate and protein from a minimum of two independent assays for each crystal type. The mean error in the determinations was calculated from the standard errors in the phosphate and protein concentrations. Since the DNA is double-stranded, the value obtained for the amount of phosphate was halved to obtain a value expressed as base pairs of DNA/molecule of protein.

Preparation of Toxin-20-kbp DNA Complex—Purified crystals from B. thuringiensis subsp. kurstaki were solubilized in 100 mM CAPS, pH 10.5. The DNA was stained with ethidium bromide and the gel was photographed. Lane 1, E. coli-cloned Cry1Ac; lane 2, E. coli-cloned Cry1Ab; lane 3, E. coli-cloned Cry1Aa; lane 4, λ phage HindIII digested; lane 5, crystal from B. thuringiensis subsp. alesti; lane 6, crystal from B. thuringiensis subsp. entomocidus; lane 7, crystal from B. thuringiensis subsp. tolworthii; lane 8, crystal from B. thuringiensis subsp. galleria; lane 9, crystal from B. thuringiensis subsp. kurstaki HD73; lane 10, crystal from B. thuringiensis subsp. kurstaki HD1.

## RESULTS

Purified crystals containing Cry1A proteins from six different subspecies of Bt were solubilized, and electrophoresis was carried out on ethidium bromide-treated agarose gels (Fig. 1). All these crystals contained a DNA band of approximately 20 kbp. Purified inclusion bodies containing the cloned gene products corresponding to the Cry1Aa, Cry1Ab, and Cry1Ac protoxins were isolated from E. coli and examined for the presence of DNA. The protoxins cloned in E. coli also appear to be associated with 20-kbp DNA (Fig. 1).

To determine if toxin could be generated in the absence of nuclease activity, the crystal protein was solubilized in the presence of EDTA to inhibit the nuclease activity present in the bovine trypsin preparation used to proteolyze the protoxin to toxin. Fig. 2 shows that the 65-kDa toxin is readily generated in the absence of nucleases. Since the DNA is double-stranded, the value obtained for the amount of phosphate was halved to obtain a value expressed as base pairs of DNA/molecule of protein.

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Fig. 1. DNA associated with B. thuringiensis proteins from various sources. Samples were subjected to electrophoresis on a 0.9% (w/v) agarose gel in 100 mM CAPS, pH 10.5. The DNA was stained with ethidium bromide and the gel was photographed. Lane 1, E. coli-cloned Cry1Ac; lane 2, E. coli-cloned Cry1Ab; lane 3, E. coli-cloned Cry1Aa; lane 4, λ phage HindIII digest; lane 5, crystal from B. thuringiensis subsp. alesti; lane 6, crystal from B. thuringiensis subsp. entomocidus; lane 7, crystal from B. thuringiensis subsp. tolworthii; lane 8, crystal from B. thuringiensis subsp. galleria; lane 9, crystal from B. thuringiensis subsp. kurstaki HD73; lane 10, crystal from B. thuringiensis subsp. kurstaki HD1.

### Ion-exchange Chromatography—
Samples were chromatographed using a Bio-Rad HRLC MA7Q (50 x 3 mm) anion exchange column. Elution was carried out using a linear gradient of 0 to 1.0 M NaCl in 0.1 M CAPS buffer adjusted to pH 10.5. The flow rate was 2.5 ml/min, and the eluate was monitored by UV absorbance at 280 nm.

### Purification of Toxin-20-kbp DNA Complex—
Purified crystals from B. thuringiensis subsp. kurstaki were solubilized in 100 mM CAPS, pH 10.5, and ethidium bromide-treated for treatment of the DNA.

### Agarose Gel Electrophoresis—
Agarose gels (0.9%) were prepared using ultrapure agarose (Life Technologies, Inc.). Gels were prepared in 1 x CAPS buffer adjusted to pH 10.5. The digest was centrifuged to remove insoluble material and thoroughly dialyzed against distilled H2O/Gaetic acid (pH 5). The DNA and protein components were analyzed by agarose gel electrophoresis and SDS-PAGE.

### Polyacrylamide Gel Electrophoresis—
SDS-polyacrylamide gels (10%) were prepared from materials supplied by Bio-Rad, and electrophoresis was performed according to standard procedures from the manufacturer using the MiniProtean System (Bio-Rad). Protein detection was achieved by staining with Coomassie Brilliant Blue R-250.

### Extraction of DNA—
DNA was extracted from the DNA-protein complexes using a modified phenol/chloroform procedure as described previously (13).

### Agarose Gel Electrophoresis—
Agarose gels (0.9%) were prepared using ultrapure agarose (Life Technologies, Inc.). Gels were prepared in 0.1 x CAPS buffer (pH 10.5) and ethidium bromide-treated for treatment of the DNA.

### Ion-exchange Chromatography—
Samples were chromatographed using a Bio-Rad HRLE MA7Q (50 x 3.8 mm) anion exchange column. Elution was carried out using a linear gradient of 0 to 1.0 M NaCl in 0.1 M CAPS buffer adjusted to pH 10.5. The flow rate was 2.5 ml/min, and the eluate was monitored by UV absorbance at 280 nm.

### Spruce Budworm Gut Juice—
Sixth-instar larvae were forced to expectorate by teasing the mouth parts with a capillary. The gut juice collected in this manner was centrifuged at 12,000 g, and aliquots of the supernatant were stored at -20 °C as described previously (5).

### Gut Juice Activation of the Bt Crystal Protein—
Bt crystals were solubilized in 100 mM CAPS buffer (pH 10.5) and incubated at 37 °C with 0.5% (w/v) or 0.1% (w/v) spruce budworm gut juice. At various time intervals, aliquots were drawn and immediately frozen for analysis.

### RESULTS

Purified crystals containing Cry1A proteins from six different subspecies of Bt were solubilized, and electrophoresis was carried out on ethidium bromide-treated agarose gels (Fig. 1). All these crystals contained a DNA band of approximately 20 kbp. Purified inclusion bodies containing the cloned gene products corresponding to the Cry1Aa, Cry1Ab, and Cry1Ac protoxins were isolated from E. coli and examined for the presence of DNA. The protoxins cloned in E. coli also appear to be associated with 20-kbp DNA (Fig. 1).

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The fate of the DNA and the crystal protein during activation of solubilized crystal protein by spruce budworm gut juice was monitored by agarose gel electrophoresis and SDS-PAGE (Fig. 3). At the earliest time points (30 s and 15 min), most of the crystal protein was converted to toxin, while the 20-kbp DNA remained intact. The DNA is eventually broken down by the nuclease activity in the gut juice but after the toxin has been converted to toxin. Fig. 4 shows a time course for the same activation process with spruce budworm gut juice on an anion

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### FIG. 2. B. thuringiensis DNA-protein complexes. A, samples were subjected to electrophoresis on a 0.9% (w/v) agarose gel in 100 mM CAPS, pH 10.5, and stained with ethidium bromide. Lane 1, λ phage HindIII digest; lane 2, protoxin-DNA; lane 3, toxin-DNA; lane 4, DNA extracted from protoxin-DNA; lane 5, DNA extracted from toxin-DNA; lane 6, λ phage HindIII digest. B, samples were subjected to SDS-PAGE and stained with Coomassie Blue. Lane 1, molecular mass markers; lane 2, protoxin-DNA; lane 3, toxin-DNA; lane 4, molecular mass markers.

### FIG. 3. Activation of the solubilized protoxin by spruce budworm gut juice. Bt crystals were solubilized and treated with 0.1% gut juice and subjected to A, agarose gel electrophoresis and stained with ethidium bromide and B, SDS-PAGE and stained with Coomassie blue. Lanes 1 and 10 are A, λ phage HindIII digest and B, molecular mass markers; lane 2, 0 time; lane 3, 30 s; lane 4, 15 min; lane 5, 30 min; lane 6, 1 h; lane 7, 2 h; lane 8, 4 h; lane 9, 24 h.
exchange column (Bio-RAD MA7Q, 50 mm × 7.8 mm). The 100-bp DNA-toxin complex reported previously (13) elutes at 0.8 M NaCl and is the major product of the processing observed after 30 min. This peak decreases with time and elutes slightly earlier with a corresponding increase in the DNA-free toxin peak, eluting at 0.3 M NaCl. The other peaks eluting near the DNA-free toxin arise from other components of the spruce budworm gut juice.

The number of nucleotide base pairs of DNA per molecule of protein was determined for crystals, solubilized protoxin, and toxin-DNA complex (Table I). Quantification of the amount of protoxin or toxin was based on the amino acid composition determined from the gene nucleotide sequence (19). Samples to be analyzed were hydrolyzed in 6 N HCl, and each of the amino acids was quantified by amino acid analysis. The molar amount of protein was estimated by dividing moles of each amino acid by the number of residues of the amino acid in the protoxin. Phosphorus analysis was carried out on a corresponding aliquot of the sample, and the molar number of base pairs was half the molar phosphorus content. The data in Table I indicate there were from 2 to 5 nucleotide base pairs per molecule of protein.

**DISCUSSION**

The discovery that heterologous 20-kbp DNA was present in the insecticidal crystal of *B. thuringiensis* subsp. *kurstaki* HD73 (13) raised the question as to the role of this DNA. The evidence obtained indicated that the DNA was not an artifact of the crystal purification procedure, but was an integral component of the crystal that interacted specifically with the protoxin. The finding in the present study that 20-kbp DNA is present in crystals prepared from five other subspecies of Bt as well as the Cry1A gene products cloned in *E. coli* supports this conclusion. It was initially proposed (13) that the DNA interacted with the C-terminal region of the protoxin, and that nucleolytic and proteolytic co-processing of the DNA and protoxin gave rise to the sequential proteolysis observed in the generation of toxin. The finding that a 20-kbp-DNA-toxin complex could be generated by trypsin in the absence of nuclease activity indicates that this hypothesis is incorrect and that protoxin interacts with the DNA through its N-terminal toxic moiety.

The amount of protein relative to the DNA was quantified to obtain information that would provide clues as to the structure of the DNA-protoxin complex. An initial estimate of the amount of DNA relative to the protein in the solubilized protoxin was made at pH 10.5 using the absorbance at 260 and 280 nm (20) and found to be approximately 10 bp per molecule of protein (21). A great deal of confidence could not be attached to this estimate because of the many assumptions made in the spectroscopic approach, but it did indicate a large amount of protein relative to the DNA. The quantification procedure adopted in the present investigation was to quantify the protein by amino acid analysis and the DNA by phosphorous analysis. The value

![Fig. 4. Products of activation of the solubilized protoxin by spruce budworm gut juice.](image-url)

**TABLE I**

| Bt sample                  | DNA/molecule protein | bp  |
|----------------------------|----------------------|-----|
| *B. thuringiensis kurstaki*|                      |     |
| crystal HD73               | 3.5 ± 0.5            |     |
| crystal HD73               | 3.1 ± 0.4            |     |
| *B. thuringiensis kenya*   | 4.8 ± 1.5            |     |
| *B. thuringiensis sotto*   | 2.0 ± 0.5            |     |
| Solubilized HD73 protoxin  | 3.2 ± 1.1            |     |
| Solubilized HD73 protoxin  | 4.0 ± 1.4            |     |
| Cloned *E. coli* Cry1Ac protein | 2.3 ± 0.2        |     |
| HD73 toxin-20-kbp DNA      | 5.0 ± 1.0            |     |
| **Average:**               | 3.5 ± 1.0            |     |

* The values are given with the 95% confidence interval determined from the standard error in the estimate of the phosphorus content and the standard error in the estimate of the amount of protein from amino acid analysis.
of approximately 3 bp per molecule of protoxin obtained by this approach confirms that there is a large amount of protein relative to the DNA (Table I). If this value is correct, then only a very small region of an extended protein molecule must be involved in the interaction. Based on x-ray powder diffraction of Bt crystals, Holmes and Monro (22) concluded that the protoxin was an elongated ellipsoid molecule.

A model is proposed (Fig. 5) in which ellipsoid protoxin molecules are interacting with the DNA through the N-terminal toxic moiety while the C-terminal half of the molecule extends away from the central core. The molecules form a protein layer covering the DNA, which accounts for the observed protection of the DNA in the crystal from nuclease attack (13). The observation that the DNA isolated from the crystal is extensively digested by EcoRI and BamHI into small DNA fragments (13) indicates that the DNA is double-stranded. Furthermore, the observation that these DNA fragments can be cloned and fragmentary sequences obtained provides further evidence that the DNA is double-stranded.

The proposed nucleic acid–protein complex is virus-like in structure, and indeed, it has been reported that in tobacco mosaic virus one protein molecule interacts with three nucleotide bases (23). While the nucleic acid in the tobacco mosaic virus is single-stranded RNA, it does show that the proposed model is a plausible explanation of the experimental data. Moreover, the proposed structure accounts for the sequential proteolysis and processing of the DNA. The protoxin molecules are stacked together in such a way that only the C-terminal ends of the molecule are accessible to proteolytic enzymes. Thus, the cleavages must proceed in a sequential manner from the C terminus. Once the peripheral portion of the protoxin is removed, the DNA becomes susceptible to nuclease attack with the regions not interacting with the protein being processed more rapidly.

Fig. 6 is a proposed activation scheme for the conversion of the crystal protein to toxin in the larval gut. Spruce budworm gut juice has been found to contain a single trypsin-like protease (6) and a single type I DNase. On ingestion, the crystal is solubilized by cleavage of the disulfide bridges in the highly alkaline pH environment of the larval gut (25, 26). Cleavage of disulfide bridges has been shown to occur by a base-catalyzed β-elimination reaction with the formation of dehydroalanine and thiocystine residues (27). Gut juice from the spruce budworm acts rapidly to convert the crystal protein to toxin. However, by dilution of the gut juice it was possible to observe the relative rates of processing of the protein and the DNA (Fig. 3). The results obtained show that the 20-kbp DNA-protoxin is rapidly converted to 20-kbp DNA-toxin complex. Processing of the 20-kbp DNA-toxin complex proceeds rapidly to give a 100-bp DNA-toxin complex that can be observed to accumulate in the early stages of the activation process (Fig. 4). Further action of the larval gut DNase yields the DNA-free toxin. The relatively slow attack of the nuclease on the DNA in the 100-bp DNA toxin complex is indicative of a core structure in which the DNA is strongly associated with the protein.

Attempts to reconstitute the DNA-toxin complex from the DNA-free toxin and isolated 20-kbp DNA have been unsuccess-

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2 F. R. Clairmont, R. E. Milne, V. T. Pham, M. B. Carrière, and H. Kaplan, unpublished result.

3 H. Kaplan and J. P. Schernthaner, manuscript in preparation.
ful. This may be due to a failure of the reconstitution conditions. However, the endotherms of the protoxin and toxin obtained by differential scanning calorimetry indicate that the toxic moiety has different conformations in the protoxin and in the free toxin (24). The failure to reconstitute the DNA-toxin complex may be due to the fact that the toxic moiety undergoes a conformational change as a result of the processing of the DNA to give DNA-free toxin.

In conclusion, the present study provides further evidence that DNA is an integral component of the Bt crystal. The association of the protoxin with the DNA appears to be essential for crystal formation and probably facilitates the sequestering of the protein during sporulation. This association gives rise to a virus-like structure that is responsible for the unusual proteolysis observed during the activation process which involves co-processing of the protein and the DNA to give the active toxin.

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