Proteolysis in the Gut of Mosquito Larvae Results in Further Activation of the Bacillus sphaericus Toxin

ANDREW H. BROADWELL and PAUL BAUMANN*
Department of Bacteriology, University of California, Davis, California 95616
Received 5 January 1987/Accepted 18 March 1987

Gut proteases from the larvae of the mosquito Culex pipiens convert the 43-kilodalton (kDa) toxin from Bacillus sphaericus 2362 to a 40-kDa peptide. The 50% lethal concentration of this peptide for tissue culture-grown cells of Culex quinquefasciatus was 1.0 μg/ml (as determined by the intracellular ATP assay), 54-fold less than that of the 43-kDa peptide. Gut proteases from Anopheles gambiae and Aedes aegypti, as well as bovine pancreatic trypsin, also converted the 43-kDa protein to a 40-kDa peptide which was indistinguishable from the peptide formed by the proteases from C. pipiens with respect to its toxicity to tissue culture-grown cells of C. quinquefasciatus. Evidence for the in vivo conversion of the 43-kDa protein to the 40-kDa peptide was also obtained from experiments in which larvae of C. pipiens, Anopheles gambiae, and Aedes aegypti were fed crystals from B. sphaericus 2362. By using the exclusion of trypan blue as an indication of cell viability, it was shown that chitobiase, chitotriose, N-acetylmuramic acid, and N-acetyleneuraminic acid decreased the toxicity of the 40-kDa peptide (from 100 to 50% mortality at about 10 mM concentrations of these sugars). Muramic acid, N-acetylglactosamine, and N-acetylgalactosamine were less effective, while several sugars had no effect, suggesting that the 40-kDa toxin binds to specific receptors on the cell membrane. The 40-kDa protein was less toxic to tissue culture-grown cells of Anopheles gambiae and Aedes dorsalis, and the same sugars which reduced the toxicity for cells of C. quinquefasciatus were also effective in reduction of toxicity for these cell lines. These results suggest that the three species of mosquito larval cells from the three species of mosquito have identical or similar receptors for the 40-kDa toxin. It therefore appears that differences in the susceptibility of species of mosquito larvae to the B. sphaericus toxin are not readily explained by the ability of the larvae to convert the 43-kDa toxin to the 40-kDa toxin or by differences in the nature of the receptor sites.

Bacillus sphaericus is an organism which in the course of sporulation produces a crystal protein (15, 20, 22). Ingestion of this protein causes destruction of the gut epithelial cells in the larvae of certain mosquito species, resulting in the death of the insect (17, 22). In general, the larvae of genus Culex are especially susceptible to this toxin, those of Anopheles spp. are moderately susceptible, and larvae of Aedes spp. are quite resistant (9, 17, 22). The larvicidal properties of the crystal have made B. sphaericus a useful agent for the biological control of mosquitoes (17, 22).

Cultures of B. sphaericus begin the synthesis of the crystal protein immediately upon the completion of exponential growth (6, 15). The initial crystal protein has a molecular mass of about 125 kilodaltons (kDa) (6). For strain 2362, the appearance of this protein is closely followed by the accumulation of 110-, 63-, and 43-kDa peptides (6). These three peptides have been purified, and it has been shown that the 110-kDa protein is a protoxin, the 43-kDa protein is a toxin, and the 63-kDa peptide has no toxic activity (2, 3, 6, 8). These results, as well as the results of immunological studies (3), suggest that the 125-kDa protein is the precursor of the lower-molecular-weight peptides.

In vivo experiments have shown that larvae of Culex pipiens fed crystals derived from 48-h cultures of B. sphaericus 2362 (which consist primarily of 110-, 63-, and 43-kDa peptides) rapidly degrade the two higher-molecular-weight proteins. The 43-kDa peptide is slowly degraded to a 40-kDa protein which remains in the gut (3). In this paper, we report the results of our investigations on the nature of this conversion and its significance with respect to the toxicity of the 40-kDa product.

* Corresponding author.

MATERIALS AND METHODS

The 43-kDa toxin, purified from B. sphaericus 2362 as previously described, was used throughout this study (3). Details of most of the methods have been previously described (3). These methods include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting of proteins from gels onto nitrocellulose paper, immunoblot assays with antisera to the 43- and the 63-kDa crystal proteins and the horseradish-peroxidase-based Immun-Blot Assay Kit (Bio-Rad Laboratories, Richmond, Calif.), N-terminal-sequence determination by automatic Edman degradation, crystal isolation from the crystal—spore complex of B. sphaericus 2362, dissection of midguts from instars 2 and 3 of larvae, preparation of crude cell extracts containing larval midgut proteases, and protease assays involving the substrate Azocoll (Sigma Chemical Co., St. Louis, Mo.). One unit of protease activity is defined as the amount of enzyme that releases 1 optical density unit at 520 nm from Azocoll per h at 28°C.

Preparation of the 40-kDa protein. Purified 43-kDa toxin (1 mg) was treated with 45 U of larval gut protease extract obtained from instars 2 and 3 of the larvae of C. pipiens. The reaction mixture containing 100 mM [3-(cyclohexylamino)]-1-propanesulfonic acid (CAPS) (pH 10) and 0.02% (wt/vol) sodium azide was incubated at 28°C for 24 h. The mixture was then passed through a Bio-Rad P-60 column (2.5 by 50 cm) with a flow rate of 15 to 18 ml/h. The elution buffer was 20 mM CAPS (pH 10) containing 10 mM β-mercaptoethanol and 80 mM NaCl. Fractions (0.5 ml) were collected, subjected to SDS-PAGE, and stained with Coomassie blue. The fractions in which the 40-kDa protein predominated were combined, concentrated by ultrafiltration through PM-30.

1333
filters (Amicon Corp., Lexington, Mass.), and passed through a 0.60 column again. Fractions containing only the 40-kDa protein were combined, concentrated, and used in all subsequent experiments. The amount of protein was determined by the method of Lowry et al. (18) with bovine serum albumin as the standard.

**Tissue culture assays.** The media and the conditions of cultivation have been previously described (6). In addition to the cell line of *Culex quinquefasciatus* used in our past study (6), we used cell lines from *Anopheles gambiae* MSQ55, *Anopheles stephensi* MSQ43 (gifts from L. Schneider, Walter Reed Army Research Institute, Washington, D. C.), and *Aedes dorsalis* (gift from J. L. Hardy, University of California, Berkeley). The trypan blue dye exclusion method and the assay for intracellular ATP were performed as previously described (6). The formula of Abbot (12) was used to correct the results for loss of viability in the controls, which ranged from 12 to 25% after an incubation of 240 min.

**Effect of sugars on the toxicity of the 40-kDa protein.** The experiments were performed by the trypan blue dye exclusion method to indicate cell viability. The sugar and the 40-kDa toxin were incubated for 1 h at 28°C in a solution containing 171 mM NaCl, 3.4 mM KCl, 10.1 mM NaH2PO4, and 1.8 mM K2HPO4 (pH 7.2) before they were added to cells suspended in the same solution. After a 2-h incubation, samples were removed and examined, and the results were corrected for loss of viability in the controls. D-Galactose, D-mannose, L-fucose, D-glucosamine, D-galactosamine, muramic acid, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetylmuramic acid, N,N'-diacetyltchitobiose, and N,N',N'-triacetyltchitotriose were obtained from Calbiochem-Behring, LaJolla, Calif. D-Glucose and N-acetylmuramic acid were obtained from Sigma. Colloidal chitin was prepared as described by Berger and Reynolds (4).

**Conversion of 43- to 40-kDa protein by proteases from different sources.** Crude cell extracts containing midgut proteases from instars 2 and 3 of the larvae of *C. pipiens, Anopheles gambiae*, and *Aedes aegypti* were prepared and assayed for protease activity as previously described (6). Samples (20 μg) of the purified 43-kDa protein were incubated with 2 protease units of each extract and 80 μg of bovine pancreatic trypsin (Sigma) for 16 h at 28°C. The toxicity of each preparation was tested for cells of *C. quinquefasciatus*, grown in tissue culture, at a concentration equivalent to 3.5 μg of the 40-kDa protein per ml.

**In vivo degradation of the crystal proteins.** One hundred larvae of *C. pipiens, Anopheles gambiae*, or *Aedes aegypti* (instars 2 and 3) were placed in plastic cups containing 20 ml of a crystal suspension (190 μg [dry weight] per ml) obtained from a 48-h culture of *B. sphaericus* 2362. At zero time, as well as at 2 and 4 h the larvae were harvested, homogenized, and centrifuged, and the supernatant and pellet fractions were subjected to SDS-PAGE, transferred to nitrocellulose paper, and reacted with antiserum to the crystal proteins as previously described (3).

**RESULTS**

**Properties of the 40-kDa protein.** Figure 1 shows the results of SDS-PAGE of the 43-kDa protein, which was the substrate for the larval gut proteases from *C. pipiens*, and the purified product, the 40-kDa peptide. No additional peptide bands were detected in either 8 or 14% (wt/vol) acrylamide gels. The sequence of the eight N-terminal residues of the 40-kDa peptide was determined, and by a comparison with the previously published sequence of the 43-kDa protein (3), it was established that the conversion of the 43- to the 40-kDa peptide involved, in part, the removal of six N-terminal amino acids. Approximately 20 amino acids had to be removed from the C terminus to account for the difference in the molecular masses.

When the effect of the 40-kDa protein on the intracellular ATP level of tissue culture-grown cells of *C. quinquefasciatus* was determined, the results shown in Fig. 2 were obtained. The concentration of the toxin which reduced the ATP level by 50% (LC50) was 1.0 μg/ml. This value compared with values determined for the 43-kDa protein by identical methods (6) indicates that the 40-kDa protein was

*FIG. 1. SDS-PAGE of the purified 43-kDa (lanes a and d) and 40-kDa (lanes b and c) proteins from *B. sphaericus* 2362 in 14% (lanes a and b) and 8% (lanes c and d) acrylamide gels. Each sample contained 35 μg of protein and the gels were stained with Coomassie blue.*

*FIG. 2. Effect of the purified 40-kDa toxin from *B. sphaericus* 2362 on the intracellular level of ATP in cells of *C. quinquefasciatus* after a 30-min incubation (left and lower axes, 100% was 8.84 × 10-12 mol of ATP in 4.4 × 106 cells). The data are also plotted as the logarithm of the amount (in micrograms) of protein versus probit (upper and right axes).*
54-fold more lethal to cells grown in tissue culture than the 43-kDa protein.

**Effect of sugars on toxicity.** The effect of various sugars on the toxicity of 1.75 μg of the 40-kDa protein per ml to cells of *C. quinquefasciatus* was determined (Fig. 3). About 10 mM N-acetylmuramic acid, N-acetyleneuraminic acid, chitobiose, or chitotriose resulted in a 50% decrease in the mortality of the cells. The former two compounds could not be tested at concentrations greater than 15 mM due to extensive clumping of the cells. Muramic acid, N-acetylgalactosamine, and N-acetylgalactosamine, at concentrations of approximately 25, 42, and 60 mM, respectively, also reduced mortality by 50%. D-Glucose, D-galactose, D-mannose, and D-glucoseamine, when tested at 200 mM, and D-galactosamine and L-fucose at 100 mM had no effect on the toxicity of the 40-kDa peptide. Preincubation of 60 μg of the 40-kDa toxin with 15 mg (dry weight) of colloidal chitin for 1 h at 28°C, followed by centrifugation and assay of the supernatant fraction, did not decrease toxicity, indicating that colloidal chitin was not able to bind the 40-kDa toxin.

**In vitro generation of the 40-kDa peptide.** Cell extracts of larval midguts from *C. pipiens, Aedes aegypti, and Anopheles gambiae* all contained proteases able to convert the 43-kDa protein to a peptide of 40 kDa with, in some cases, additional peptides at 26 to 28 kDa (Fig. 4, lanes a through d). A similar conversion was done by using bovine pancreatic trypsin (Fig. 4, lane e). These preparations had similar toxicities for tissue culture-grown cells of *C. quinquefasciatus* (Fig. 5), indicating that the 40-kDa toxin generated from the 43-kDa peptide by proteases from different sources was functionally equivalent.

**In vivo generation of the 40-kDa peptide.** Larvae of *C. pipiens, Anopheles gambiae, and Aedes aegypti* were incubated with crystals obtained from 48-h cultures of *B. sphaericus* 2362. After 2 h of feeding, larvae were homogenized and centrifuged, and SDS-PAGE of the supernatant fractions was done, followed by transfer of the proteins to nitrocellulose paper. Crystal proteins were detected by their reaction with specific antisera. All of the high-molecular-weight proteins were degraded, and only peptides of 43 and 40 kDa along with traces of 24 to 26-kDa peptides remained (Fig. 4 lanes g, h, and i). No material reacting with antisera to the crystal proteins was found in the pellet fraction (results not shown). Similar results were observed at 4 h.

**Toxicity of the 40-kDa protein for other cell lines.** Figure 6...
shows the effect of the 40-kDa toxin (tested at 14 and 56 μg/ml) on tissue culture-grown cells of Anopheles gambiae, Anopheles stephensi, and Aedes dorsalis. No loss of viability was detected when these cell lines were incubated with 3.5 μg of the 40-kDa toxin per ml, a concentration which rapidly killed the cells of C. quinquefasciatus (Fig. 5). As with C. quinquefasciatus, the addition of 50 mM chitobiase, 50 mM chitotriose, 150 mM muramic acid, 200 mM N-acetylgalactosamine, or 200 mM N-acetylmuratosomamine abolished the toxicity of 14 μg of the 40-kDa protein per ml for Anopheles gambiae and Aedes dorsalis (toxicity was not tested for Anopheles stephensi). When tested at 14 μg/ml, the 40-kDa toxin had no effect on cells of Spodoptera frugiperda and rat CSH2 cells grown in tissue culture.

DISCUSSION

In our previous study (6), it was shown that upon completion of exponential growth, B. sphaericus 2362 makes a 125-kDa crystal protein which is degraded during the course of sporulation to several peptides, of which the 43-kDa protein is toxic for tissue culture-grown cells of C. quinquefasciatus. Since the higher-molecular-weight peptides are nontoxic for tissue culture-grown cells, this sporulation-associated event consists of the conversion of a protoxin to a toxin. This study documents a supplemental activation which occurs in the midgut of larvae and is done by larval proteases. Evidence for this activation is obtained from in vitro experiments in which larval gut proteases were shown to convert the 43-kDa protein to a 40-kDa peptide (Fig. 1) with a concomitant 54-fold decrease in the LC50 for tissue culture-grown cells of C. quinquefasciatus (Fig. 2). This conversion also occurs in vivo, as is indicated by the accumulation of a 40-kDa peptide in mosquito larvae fed crystals of B. sphaericus (Fig. 4) (3). The persistence of the 40-kDa protein in the gut of mosquito larvae (Fig. 4, lanes g, h, and i) (3) as well as after extended treatment with proteolytic enzymes (Fig. 4, lanes b through e), suggests that this peptide is resistant to further proteolysis by gut enzymes.

It has been previously noted that there are considerable differences in the susceptibilities of species of mosquito larvae to the B. sphaericus toxin (9, 17, 22). Generally, larvae of the genus Culex appear to be the most susceptible, followed by those of the genera Anopheles and Aedes. Differences in susceptibility may involve a number of complex behavioral (21) as well as physiological attributes. In the latter category, susceptibility could be a function of the ability of larval gut proteases to convert the 43- to the 40-kDa protein. In this study, we have shown that larval gut proteases from three species of mosquito, as well as bovine trypsin, cause this conversion (Fig. 4, lane b through e) and that the resulting products are indistinguishable in their toxicity for cells of C. quinquefasciatus (Fig. 5). Aley et al. (1) have also recently shown that larval proteases from Aedes aegypti degrade the 43-kDa protein to a 40-kDa peptide. Since trypsin-liked enzymes are widespread among larvae (5, 13), it appears that the ability to activate the toxin is not a major factor determining differences in susceptibility.

In the case of many bacterial toxins, the initial interaction between the toxin and the susceptible cell involves attachment to specific carbohydrate receptors on the cell surface (10, 14, 19). These receptors may be distinct from the ultimate target site of the toxin. Evidence for a role for membrane receptors for the activated crystal toxin from Bacillus thuringiensis var. kurstaki has been recently presented (11, 16). In this system, the action of the toxin on susceptible cells in tissue culture is counteracted in a specific manner by a number of sugars (11). This inhibition is presumably due to the lectinlike ability of the toxin to bind specific carbohydrates, thereby blocking normal attachment of the toxin to the cell. Using tissue culture-grown cells of C. quinquefasciatus and purified 40-kDa toxin, we have shown that chitobiase, chitotriose, N-acetyl-D-muramic acid, and N-acetyleneuraminic acid are potent antagonists of the 40-kDa toxin (Fig. 3). These results, taken in conjunction with the lesser potency of muramic acid, N-acetylgalactosamine, and N-acetylglucosamine, do not readily lend themselves to a simple conclusion concerning the chemical nature of the receptor site. Butters and Hughes (7) have presented evidence indicating that the membrane glycoprotein of a cell line of Aedes aegypti contains chitobiase and N-acetylgalactosamine. It is possible that the potent antagonistic effect of chitobiase and chitotriose, as well as the greater antagonistic effect of N-acetylgalactosamine compared with N-acetylglucosamine, is an indication that this or a similar glycoprotein is the receptor of the 40-kDa protein. The inhibition by N-acetyleneuraminic acid may be fortuitous, since this compound is not found in the cell membrane of insects (7).

Indirect evidence that the 40-kDa protein attaches to specific carbohydrate receptor sites suggests that differences in the susceptibility of species of mosquito larvae may involve the presence or absence of specific receptors on the cell membrane recognized by the 40-kDa toxin. To examine this possibility, we have tested the effect of the 40-kDa toxin on several mosquito cell lines grown in tissue culture. The results of these experiments should be interpreted with some caution, since there are a number of problems involved with the extrapolation of results from tissue culture-grown cells to the gut of mosquito larvae. For the cell lines used in our

---

**FIG. 6.** Effect of the purified 40-kDa toxin on the mortality of cells of Anopheles gambiae (O and C), Aedes dorsalis (A and D), and Anopheles stephensi (M and D), tested at 56 (---) and 14 (----) μg of toxin per ml with the trypan blue dye exclusion technique. Points represent averages of three determinations.
studies, the source of the cells with respect to the tissue is in most cases unknown. Since the cells on which the toxin initially acts in vivo are those of the midgut epithelium, it is possible that cells derived from other tissues may not have the same receptor sites. In addition, the conditions of cultivation of the cells in tissue culture may influence the presence or absence of the receptors (14). Even if these possible problems, as well as the limited nature of our survey, are borne in mind, it nevertheless appears to be significant that the most susceptible cell line is from \textit{C. quinquefasciatus}, followed by lines from \textit{Anopheles gambiae}, \textit{Aedes dorsalis}, and \textit{Anopheles stephensi} (Fig. 6). A rough estimate of the LC$_{50}$ for the first three cell lines can be obtained from the mortality of the cells after 45 min of incubation (Fig. 5 and 6). These calculations indicated that the LC$_{50}$s for \textit{Anopheles gambiae} and \textit{Aedes dorsalis} were respectively, about 7 and 13 times higher than that for \textit{C. quinquefasciatus}. When various sugars which abolish the toxicity of the 40-kDa protein for cells of \textit{C. quinquefasciatus} were tested for their ability to overcome toxicity of the 40-kDa protein for cells of \textit{Anopheles gambiae} and \textit{Aedes dorsalis}, complete sparing was observed. These results suggest that in these cell lines, the toxin binds to similar receptors. The differences in susceptibility would therefore have to be explained by factors other than major differences in the chemical nature of the receptors. Cell lines may differ in the total number of receptors, in the efficiency of delivery of toxin to the target site, or in the difference in susceptibility of the target site. Since the biochemical mechanism of the action of this toxin on cells in unknown, these alternatives are conjectural.

This study and the previous studies (3, 6) on the crystal protein of \textit{B. sphaericus} 2362 and its interaction with larvae of \textit{C. pipiens} indicate a sequential two-step pathway for activation of the larvicide. Step 1 is associated with sporulation and involves the degradation of the 125-kDa protoxin to a 43-kDa toxin. Step 2 is associated with larvae and involves a further activation of the toxin by the degradation of the 43-kDa protein to the 40-kDa peptide. One major unanswered question concerns the pathway of degradation of the high-molecular-weight proteins (125 and 110 kDa) in larvae. Treatment of the purified 110-kDa peptide with larval gut proteases from \textit{C. pipiens} does not result in protein bands corresponding to either 43 or 40 kDa, nor is the resulting digested peptide toxic for cells of \textit{C. quinquefasciatus} (A. H. Broadwell, unpublished observations). Bioassays of the 110-kDa protein with larvae of \textit{C. pipiens} gave an LC$_{50}$ which on a molar basis is comparable to that obtained with the purified 43-kDa protein (6). These results indicate that the high-molecular-weight proteins in the crystal contribute to larval toxicity but that the pathway of their activation in larvae is not understood.

**ACKNOWLEDGMENTS**

We thank B. C. Campbell, E. Ferro, M. Privalsky, and S. Roberts for their assistance.

This investigation was supported by the University of California Mosquito Research Program, the University of California Agricultural Experiment Station, and the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Disease.

**LITERATURE CITED**


