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The *Bacillus thuringiensis* Insecticidal Toxin Binds Biotin-Containing Proteins

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Brush border membrane vesicles from larvae of the tobacco hornworm, *Manduca sexta*, contain protein bands of 85 and 120 kDa which react directly with streptavidin conjugated to alkaline phosphatase. The binding could be prevented either by including 10 μM biotin in the reaction mixture or by prior incubation of the brush border membrane vesicles with an activated 60- to 65-kDa toxin from *Bacillus thuringiensis* HD-73. The ability of *B. thuringiensis* toxins to recognize biotin-containing proteins was confirmed by their binding to pyruvate carboxylase, a biotin-containing enzyme, as well as to biotinylated ovalbumin and biotinylated bovine serum albumin but not to their nonbiotinylated counterparts. Activated HD-73 toxin also inhibited the enzymatic activity of pyruvate carboxylase. The biotin binding site is likely contained in domain III of the toxin. Two highly conserved regions within domain III are similar in sequence to the biotin binding sites of avidin, streptavidin, and a biotin-specific monoclonal antibody. In particular, block 4 of the *B. thuringiensis* toxin contains the YAS biotin-specific motif. On the basis of its N-terminal amino acid sequence, the 120-kDa biotin-containing protein is totally distinct from the 120-kDa aminopeptidase N reported to be a receptor for Cry1Ac toxin.

During sporulation, the bacterium *Bacillus thuringiensis* produces parasporal protein inclusions which may be toxic to the larval stage of lepidopteran, dipteran, and coleopteran insects (18, 24, 26, 27, 34). After ingestion, the crystals are solubilized in the larval midgut, releasing 130- to 135-kDa protein chains called protoxins which are then activated by gut proteases into mature toxins of ca. 60 to 65 kDa (18, 24, 26). These activated toxins bind to specific receptors present in the larval midgut epithelia, leading to membrane disruption and insect death.

These receptors are of intense interest because their binding specificity was often correlated with the insect host specificity of *B. thuringiensis*. Susceptible insects had toxin-binding receptors in their brush border membrane vesicles (BBMVs), whereas nonsusceptible insects did not (22, 53). Furthermore, in some studies, the development of insect resistance to *B. thuringiensis* (38, 47) was shown to be due to changes in these receptors. With regard to Cry1Ab resistance, the resistant *Plutella xylostella* had no detectable Cry1Ab receptors (16) while the resistant *Plodia interpunctella* had an equivalent number of receptors, but those receptors had decreased toxin affinity (54). High-affinity, saturable toxin-binding sites (receptors) have also been identified in the tobacco hornworm, *Manduca sexta* (17, 25, 35, 52); the cabbage butterfly, *Pieris brassicae* (22); the tobacco budworm, *Heliothis virescens* (17, 19, 32, 41); the gypsy moth, *Lymantria dispar* (28); the cotton leafworm, *Spodoptera littoralis* (43); *Spodoptera frugiperda* (17); and the cotton bollworm, *Helicoverpa zea* (17). For *M. sexta*, the Cry1Ac and Cry1Ab receptors have been reported to be a 120-kDa glycoprotein (17, 25) and a 210-kDa glycoprotein (35, 52), respectively. Also, two groups (25, 44) have suggested that this 120-kDa receptor has a normal function in insect nutrition as an aminopeptidase. This assignment was based on assays for aminopeptidase activity and partial amino acid sequence comparisons between the 120-kDa receptor and the aminopeptidase N family of proteases (25, 44). These studies suggest that proteins with normal functions in insect physiology are utilized as toxin receptors and that the prevalence and properties of these receptors determine both host specificity and resistance.

However, there are multiple toxin-binding sites, and the actual story is likely more complicated. For instance, Wolfersberger (56) found that the actual dissociation constants for Cry1Ab and Cry1Ac binding in gypsy moth larvae were opposite what would have been predicted from their observed potencies versus those larvae. Also, Garczynski et al. (17) studied the binding of ^{125}I-Cry1Ac to BBMVs from four insects which differed greatly in their sensitivity to the Cry1Ac toxin. They found high-affinity, saturable binding sites in all four of the insects tested, including the highly resistant *S. frugiperda* larvae. Similarly, Feldmann et al. (15) detected binding of Cry11A toxin to six proteins ranging from 48 to 110 kDa from *M. sexta*, an insect for which Cry11A is not toxic, while Masson et al. (36) compared the kinetics of toxin binding to BBMVs from susceptible and resistant *Plutella xylostella* larvae. The binding kinetics for Cry1Ac did not differ significantly between susceptible and resistant larvae, and it was concluded that factors other than binding were altered in the resistant insect (36, 48).

A further source of confusion arises from the large number of potential receptors detected in some cases, even in studies which used seemingly appropriate precautions against proteolysis artifacts. Protein blots of BBMVs detected six Cry1Ac receptors in *Heliothis virescens* larvae (17), five Cry1Ac receptors in *Helicoverpa zea* larvae (17), and nine Cry1Ab receptors in *M. sexta* larvae (15). Do all of these binding proteins represent actual receptors participating in pathogenesis? As a partial resolution of this dilemma, we now present evidence that the activated 65-kDa Cry1Ac toxin exhibits a general affinity for biotinylated proteins which is reflected in its ability to bind biotin-containing proteins in BBMVs from *M. sexta* larvae. On

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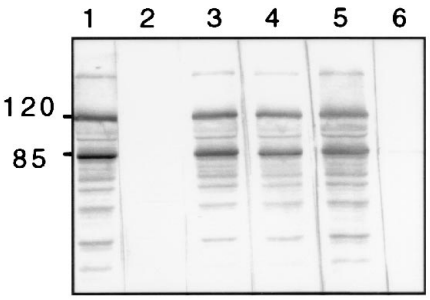

FIG. 1. Western blot showing binding of Str-AP to *M. sexta* BBMV. BBMVs were separated by SDS–10% PAGE, transferred to nitrocellulose membranes, incubated with the indicated reagent, and stained for alkaline phosphatase activity. Lane 1, Str-AP; lane 2, AP substrate only (no Str-AP); lane 3, Str-AP plus 300 μM RGDS; lane 4, Str-AP plus 300 μM HPQN; lane 5, Str-AP with delayed addition of biotin to a final concentration of 10 μM; lane 6, Str-AP plus 10 μM biotin. Molecular mass markers are on the left. The visible band with the highest molecular mass is ca. 170 kDa.

the basis of amino acid sequence similarities (24, 30), it seems likely that all Cry1 and Cry3 toxins and most Cry4 toxins will be found to have affinity for biotinylated proteins. These observations should help clarify those cases in which there is no clear correlation between insect pathogenicity and ligand blot affinity data.

MATERIALS AND METHODS

Activated toxin. *B. thuringiensis* HD-73 (Cry1Ac) was grown on a glucose-yeast extract-salts medium (40) until sporulation had occurred. The insecticidal crystals were purified on sodium bromide gradients (3) and then solubilized in 0.1 M Na_2CO_3 (pH 10) with 50 mM β-mercaptoethanol at room temperature for 1 h. Any insoluble particles were removed by centrifugation at 10,000 × *g*. Trypsin was added to ca. 15% by weight of the total protoxin. After overnight digestion at room temperature, another batch of trypsin was added for an additional 2 to 4 h of digestion. This solution was added to a Sephadex G-150 column, which was subsequently eluted with 100 mM NaCl–20 mM sodium phosphate, pH 7.6. Protein fractions were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and those containing the purified 65-kDa toxin were pooled and lyopholized. All protein assays were performed by the method of Lowry et al. (31).

Biotinylation. d-Biotin-*N*-hydroxysuccinimide ester (BNHS; Sigma, St. Louis, Mo.) was used to modify proteins through their lysine ε-amino groups. Our methods followed those recommended by Bayer and Wilchek (5). For biotinylation of bovine serum albumin (BSA) and ovalbumin (both from Sigma), the proteins were dissolved in 0.2 M borate buffer (pH 8.6) at 3 mg/ml while the BNHS was dissolved in dimethyl formamide. BSA-to-BNHS molar reaction ratios ranged from 1:7 to 1:100, and the reactions were conducted for 1 h at room temperature without shaking. Free reagent was then removed by dialysis against phosphate-buffered saline (PBS) at 4°C. Note that these biotinylation ratios refer to the biotin-to-protein ratios during biotinylation rather than to the number of biotins actually attached. Biotinylation of activated HD-73 toxin followed the conditions of Denolf et al. (10) with a reaction ratio of 1:7. The 50% lethal concentration of the Cry1Ab crystals for *M. sexta* larvae did not change following biotinylation (10).

BBMVs. BBMV preparation followed the protocol of Wolfersberger et al. (55) using fresh fourth- to fifth-instar *Manduca sexta* larvae. The midgut suspension solution included a protease inhibitor cocktail consisting of aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin A (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 5 mM]. Final BBMVs were suspended in a solution containing 150 mM NaCl, 2.7 mM KCl, 9.5 mM sodium phosphate (pH 7.5) (52), and the inhibitor cocktail. The BBMVs were either used immediately or stored at −70°C.

Electrophoresis and blotting. All electrophoresis (SDS–10% PAGE) and electrotransfer procedures followed the manufacturer's guidelines (Bio-Rad, Richmond, Calif.). For the detection of insect gut receptors, 20 μl of BBMV preparation (containing 20 to 40 μg of protein) was mixed with 10 μl of cracking buffer and heated at 95°C for 5 min. Electrotransfer to nitrocellulose membranes was performed by either the 1-h or overnight procedure, whereupon the membranes were washed three times with TBST (10 mM Tris, 100 mM NaCl, 0.25% Tween 20; pH 7.5), blocked with 3% BSA in TBST for 1 h, and analyzed. In some cases, the membrane was incubated directly with a 2-μg/ml solution of streptavidin conjugated to alkaline phosphatase (Str-AP; Pierce, Rockford, Ill.) in TBST for 1 h, and in other cases, the membrane was incubated first with unlabeled or biotinylated toxin (20 μg/ml in TBST plus 3-mg/ml BSA), washed, and then incubated with Str-AP. Color was developed with phosphatase substrate (Kit II; Vector Laboratory, Burlingame, Calif.). Identical results were obtained with nylon and polyvinylidene difluoride (PVDF) membranes when 3% BSA and 5% dried milk were used as blocking reagents. For the experiments to test the biotin binding ability of *B. thuringiensis* toxins, activated HD-73 toxin was subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with biotinylated carrier protein (BSA or ovalbumin; 3 μg/ml) followed by Str-AP.

Purification of the 120-kDa biotin-containing protein. BBMV suspensions were solubilized in a 5-mg/ml solution of deoxycholic acid, and the supernatant was dialyzed against PBS overnight at 4°C. The 120-kDa biotin-containing protein was affinity purified by adding 100 μl of streptavidin beads (1% solids, high load, 0.93-μm diameter; Seradyn, Indianapolis, Ind.) and incubating the suspension on ice for 1 h in 0.4 M NaCl–20 mM sodium phosphate, pH 7.3. The beads were centrifuged to remove the supernatant and washed three times with PBS. The bound biotin-containing protein was released by heating the beads at 95°C for 5 min in 50 μl of SDS sample buffer containing 5 mM free biotin. The resultant supernatant was subjected to SDS-PAGE. The procedure for electroblotting onto PVDF membranes followed that of Matsudaira (37). Briefly, after electrophoresis, the gel was soaked in an electroblotting buffer (10 mM CAPS [pH 11], 10% methanol) for 5 min at room temperature and then transferred to a PVDF membrane at 90 V (300 mA) for 20 min at room temperature. The membrane was rinsed with deionized water, stained with 0.1% Coomassie blue R-250 in 50% methanol for 1 min, destained at room temperature three times with 50% methanol–10% acetic acid and three times with deionized water, and air dried.

N-terminal amino acid sequence. The 120-kDa band was cut from the membrane, and its sequence was determined by automated peptide sequencing on an Applied Biosystems 477A sequencer with an on-line 120A high-performance liquid chromatography analyzer. Two independently generated protein samples were sequenced, and they gave identical results.

Use of streptavidin-coated beads to purify activated HD-73 toxin. This sandwich protocol is based on the fact that both streptavidin and activated *B. thuringiensis* toxin bind to biotinylated ovalbumin. Streptavidin beads (50 μl, 1% solids, medium load, 0.92-μm diameter; Seradyn) were incubated with 100 μl of PBS and 50 μl of biotinylated ovalbumin (1:50 biotinylation reaction ratio; 1.8 mg/ml in PBS) on ice for 2 h and then washed twice with PBS. The beads were incubated on ice for 2 h with 200 μl of ovalbumin (1.8 mg/ml in PBS) to block nonspecific binding, washed two more times with PBS, resuspended in 200 μl of PBS, and divided into four equal aliquots. Each of the four toxin samples contained 30 μl of toxin (120 μg/ml in 0.1 M Na_2CO_3 at pH 9.7 with ovalbumin at 1.2 mg/ml) premixed on ice for 30 min with 10 μl of PBS, 10 μl of biotin (1.2 mg/ml in PBS), 10 μl of biocytin (1.2 mg/ml in PBS), or 10 μl of biotinylated ovalbumin (1.8 mg/ml in PBS). The beads and toxins were mixed on ice for 2 h, centrifuged, and washed twice with PBS. The pellets were mixed with 20 μl of SDS sample buffer and then heated at 95°C for 3 min. The proteins released were separated by SDS–10% PAGE, transferred to PVDF membranes, and detected with primary antibodies against activated HD-73 toxin followed by a secondary antibody-AP conjugate.

Pyruvate carboxylase. The spectrophotometric enzyme assay for pyruvate carboxylase was conducted as described by Scrutton et al. (46). This assay converts pyruvate to oxalacetate, which acts as a substrate for malate dehydrogenase, with the conversion of NADH to NAD. The disappearance of NADH was followed at 340 nm. Chicken liver pyruvate carboxylase, malate dehydrogenase, and acetyl coenzyme A (acetyl-CoA) (activator) were purchased from Sigma.

Bioassays. Bioassays for determination of the toxicity of *B. thuringiensis* crystals, using *M. sexta* larvae, were conducted as described by Du et al. (14).

RESULTS

Biotin-containing proteins in BBMVs. The use of biotinylation as a nonradioactive means of labelling proteins is now a common practice, and biotinylation of *B. thuringiensis* Cry toxins has been used to screen for toxin receptors in insects (10). As part of the controls for a similar study, we noted that two major bands appeared whenever the insect BBMV preparations were incubated directly with Str-AP (Fig. 1, lane 1) or avidin-AP (13). The presumptive biotin-containing proteins were ca. 120 and 85 kDa in size, and as determined by Coomassie blue staining, they were not among the major proteins present in BBMVs.

Because AP is used as a marker enzyme in the preparation of BBMVs (55), we ran a control (Fig. 1, lane 2) in which no Str-AP was added. Both the 120- and 85-kDa bands disappeared; there was no endogenous AP activity. Thus, the AP activity detected (lane 1) was from the Str-AP rather than from the BBMVs themselves. This result was expected from our use of denaturing gels to separate the BBMV proteins (Fig. 1).

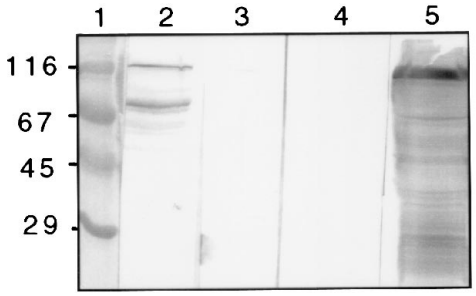

FIG. 2. Effect of activated *B. thuringiensis* toxin on the binding of Str-AP to *M. sexta* BBMV. BBMVs were separated by SDS-PAGE, transferred to nitrocellulose membranes, and stained as in Fig. 1. Lane 1, molecular mass markers at 116, 67, 45, and 29 kDa; lane 2, Str-AP only; lane 3, blotted with activated HD-73 toxin (20 μg/ml; pH 7.4) and then with Str-AP; lane 4, blotted with activated HD-73 toxin (20 μg/ml; pH 9.4) and then with Str-AP; lane 5, blotted with biotinylated activated HD-73 toxin (20 μg/ml; pH 7.4) and then with Str-AP.

Two other nonspecific (i.e., non-biotin-mediated) mechanisms by which streptavidin could bind to denatured BBMV proteins would be if those proteins contained either RGDS (2) or HPQN (11) sequences. However, the presence of these tetrapeptides during Western blotting (immunoblotting) with Str-AP left the 120- and 85-kDa bands intact (Fig. 1, lanes 3 and 4). Confirmation that the Str-AP binding observed was biotin mediated was provided by the ability of free biotin (lane 6) or biocytin (13) to prevent Str-AP attachment. Both the 120- and 85-kDa bands disappeared (lane 6). We conclude that the 120- and 85-kDa bands are due to biotin-containing proteins.

Although it does appear in most BBMV preparations (Fig. 1), the upper band at ca. 170 kDa is found primarily in the supernatant fraction rather than the pellet fraction (13). Thus, it is likely to be a cytoplasmic protein rather than a membrane component. We have not examined any of the other, less intense bands further.

Binding of toxin to the biotin-containing BBMV proteins. We next sought to determine to what extent these biotin proteins were involved with the insecticidal activity of *B. thuringiensis* and whether they coincided with any of the Cry receptors in BBMVs. In this light, we found that the activated 60- to 65-kDa toxin was able to bind the biotin proteins from BBMVs (see Fig. 2) and to protect them from digestion by trypsin (see Fig. 3). Furthermore, it was able to bind biotinylated ovalbumin (see Fig. 4) and pyruvate carboxylase (see Fig. 6). We will first examine the binding of this toxin to BBMV proteins. The 120- and 85-kDa bands in the BBMVs were detected with Str-AP alone (Fig. 2, lane 2). However, when the BBMVs were blotted with the activated 60- to 65-kDa toxin, washed, and then incubated with Str-AP, the 120- and 85-kDa bands were not detected at either pH 7.4 (lane 3) or pH 9.4 (lane 4). The pH 9.4 incubation was chosen to simulate the alkaline conditions present in the larval midgut (12, 20). Presumably, prior blotting with the activated toxin blocked subsequent binding by Str-AP. This scenario was confirmed by using biotinylated toxin instead of unlabeled toxin (lane 5). In this case, the intensity of the 120-kDa band was increased dramatically while that of the 85-kDa band was decreased somewhat (lane 5). The reason for this difference probably reflects an increased intensity of the 120-kDa band due to binding of toxin to aminopeptidase N (25, 44) as well as to the 120-kDa biotin protein.

Binding to the activated toxin also protected the solubilized biotin proteins from digestion by trypsin (Fig. 3). After 15 min (lane 7), the 120-kDa protein was completely digested and the 85-kDa protein was partially digested. In contrast, in the presence of activated toxin (lane 9), both biotin proteins were partially protected from trypsin digestion. The intensity of the 120-kDa band following 15 min of digestion with toxin (lane 9) was roughly equivalent to that following 4 min of digestion without toxin (lane 3). BSA (lane 8) provided no protection.

Binding of *B. thuringiensis* toxin to biotinylated ovalbumin and BSA. We next examined whether *B. thuringiensis* toxin could bind to other biotin-containing proteins as well as to those present in BBMVs. Biotinylated ovalbumin was used as a model protein (Fig. 4). Two *B. thuringiensis* toxin preparations were separated by SDS-PAGE. They were the purified activated 65-kDa toxin (Fig. 4A, lane 2) and a partially activated protoxin (Fig. 4A, lane 3). When examined by Western blotting, no color developed after direct incubation with Str-AP (Fig. 4B, lane 1). However, when they were first blotted with biotinylated ovalbumin, an Str-AP band appeared at ca. 65 kDa (Fig. 4B, lanes 2 to 4), corresponding to the activated toxin at 65 kDa (Fig. 4A, lane 2). This procedure requires two or more biotins per ovalbumin, one to be bound by the toxin and one to be recognized by the Str-AP (21). The binding of biotinylated ovalbumin to the toxin was equally strong in the presence (Fig. 4B, lane 3) and absence (lane 2) of a 1,000-fold excess of unlabeled ovalbumin and in the presence (lane 4) and absence (lane 2) of free biotin. Virtually identical results were obtained when biotinylated BSA was used instead of biotiny-

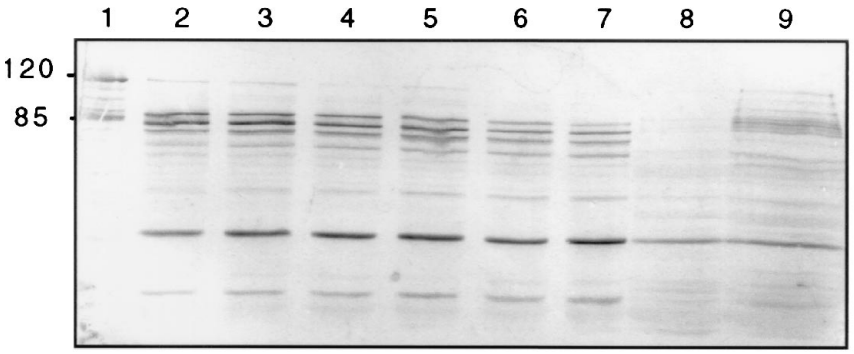

FIG. 3. Trypsin digestion of the biotin-containing proteins from *M. sexta* BBMVs. Deoxycholate-solubilized BBMV preparations were mixed with trypsin (final concentration, 140 μg/ml) and incubated on ice for the indicated periods of time. After digestion, the samples were mixed with SDS sample buffer containing the protease inhibitor cocktail and analyzed immediately by SDS-PAGE and Western blotting with Str-AP. Lanes 1 to 7, BBMV preparations digested for 0, 2, 4, 6, 8, 10, and 15 min, respectively; lanes 8 and 9, solubilized BBMVs premixed with 375-μg/ml BSA (lane 8) or activated HD-73 toxin (lane 9) and then digested with trypsin for 15 min.

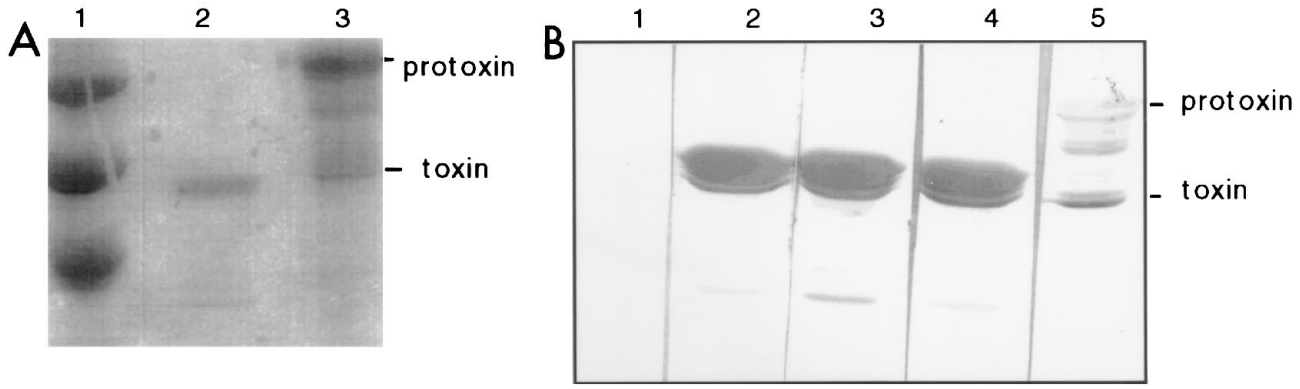

FIG. 4. Effect of biotinylated ovalbumin on the binding of Str-AP to *B. thuringiensis* toxins. (A) Preparation subjected to SDS-PAGE and resultant gels stained with Coomassie blue. Lane 1, molecular mass markers at 116, 67, and 45 kDa; lane 2, purified activated HD-73 toxin; lane 3, HD-73 protoxin. (B) Preparation subjected to SDS-PAGE and resultant gel stained for AP activity as described in the legend to Fig. 1. Lane 1, activated toxin blotted with Str-AP; lane 2, activated toxin blotted with biotinylated ovalbumin (3 μg/ml) and then with Str-AP; lane 3, activated toxin blotted with a mixture of biotinylated ovalbumin (3 μg/ml) and ovalbumin (3 mg/ml) and then with Str-AP; lane 4, activated toxin blotted with a mixture of biotinylated ovalbumin (3 μg/ml) and free biotin (200 μg/ml; 0.8 mM) and then with Str-AP; lane 5, *B. thuringiensis* protoxin blotted with biotinylated ovalbumin (3 μg/ml) and then with Str-AP.

lated ovalbumin (13). Interestingly, the 132-kDa protoxin exhibited comparatively weak binding to biotinylated ovalbumin (Fig. 4B, lane 5). Use of a protoxin preparation which had been partially activated (Fig. 4A, lane 3) showed that the activated 65-kDa toxin exhibited far stronger binding to biotinylated ovalbumin, even though it was only a minor portion of the protein present (compare lane 3 of Fig. 4A and lane 5 of Fig. 4B).

This affinity of activated toxin for biotinylated ovalbumin can be turned around and used in a sandwich mechanism to purify *B. thuringiensis* toxins (Fig. 5). Activated toxin (65 kDa) bound to beads coated with both streptavidin and biotinylated ovalbumin (lane 3), even in the presence of free biotin (lane 4) or biocytin (lane 5). However, the toxin did not bind to those beads in the presence of excess biotinylated ovalbumin (lane 6) or to beads coated with streptavidin alone (lane 2).

Binding to biotinylated proteins versus binding to free biotin. The *B. thuringiensis* toxin appears to bind biotinylated proteins but not free biotin. The inability of free biotin to prevent the binding of *B. thuringiensis* toxin to biotinylated ovalbumin (Fig. 4B, lane 4; Fig. 5, lane 4) is fully consistent with the findings of Dakshinamurti et al. (8). They characterized four antibiotin monoclonal antibodies (MAbs). The affinities of three of them for biotinylated BSA were unaffected by free biotin even at concentrations as high as 10 mM. The affinity of the fourth MAb for biotinylated BSA was reduced by 50% when biotin was at 0.1 mM. Thus, biotin-specific MAbs, unlike avidin and streptavidin, exhibit far greater affinity for protein-bound biotin than for free biotin. Presumably, *B. thuringiensis* toxins also fall in this category. Their preference for biotin-containing proteins is consistent with the negligible effect of free biotin on *B. thuringiensis* toxicity. In bioassays with *M. sexta* larvae, HD-73 crystals exhibited identical 50% lethal concentration values (40 ng/cm^2) both with and without free biotin (20 μg/cm^2).

Pyruvate carboxylase. Biotin accessibility may differ depending on whether the biotin had been added in vitro (e.g., biotinylated ovalbumin) or in vivo. However, the distinction did not seem to matter; activated toxin was able to detect both biotinylated ovalbumin (Fig. 4 and 5) and pyruvate carboxylase (Fig. 6 and 7). In Western blots (Fig. 6), pyruvate carboxylase was detected by both streptavidin (lane 1) and activated *B. thuringiensis* toxin (lanes 5 to 7). Furthermore, the *B. thuringiensis* toxin bound native, enzymatically active pyruvate carboxylase (Fig. 7) as well as the denatured enzyme. Like avidin, the *B. thuringiensis* toxin inhibited pyruvate carboxylase activity whereas a control protein (BSA) did not (Fig. 7). Furthermore, the inhibition by *B. thuringiensis* toxin was reversed by anti-*B. thuringiensis* antibodies (Fig. 7), and the degree of inhibition increased as the toxin/enzyme molar ratio was increased from 1:1 to 22:1 (13).

N-terminal amino acid sequence of the 120-kDa protein. The most intense of the Str-AP-reactive bands from BBMVs was located at 120 kDa (Fig. 1), and this band was intensified when blotted with biotinylated Cry1Ac toxin (Fig. 2). However, aminopeptidase N, the presumptive Cry1Ac receptor of *M. sexta*, is also located at 120 kDa (25, 44). Are these proteins identical or related, or is it merely a coincidence that both are 120 kDa in size? We believe it is a coincidence; they are distinct proteins. First, the 120-kDa biotin protein and aminopeptidase N could be separated when subjected to SDS–5% PAGE rather than SDS–10% PAGE. On 5% gels, the streptavidin-reactive biotin protein shifted to ca. 125 to 130 kDa. The

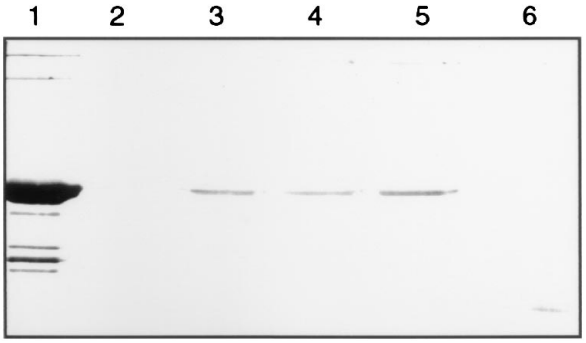

FIG. 5. Use of streptavidin-coated affinity beads to purify activated HD-73 toxin. Commercial streptavidin beads were coated with biotinylated ovalbumin and then incubated with the indicated toxin preparations. After washing, the proteins were released from the beads with SDS sample buffer and analyzed by SDS-PAGE and Western blotting using primary antibodies against activated HD-73 toxin and secondary antibody-AP conjugates. Lane 1, toxin was added directly to SDS-PAGE, no beads; lane 2, control omitting the biotinylated ovalbumin; lane 3, toxin with biotinylated-ovalbumin-coated streptavidin beads; lanes 4 to 6, same as for lane 3 except that the activated toxin was premixed with either free biotin (lane 4), biocytin (lane 5), or excess biotinylated ovalbumin (lane 6).

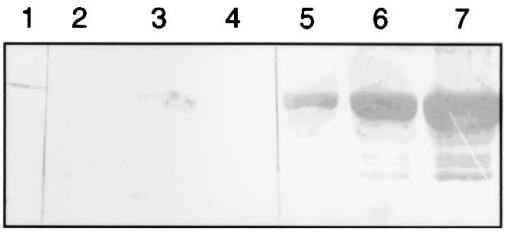

FIG. 6. Binding of activated *B. thuringiensis* toxin to pyruvate carboxylase. Bovine liver pyruvate carboxylase (Sigma) was separated by SDS–10% PAGE, transferred to nitrocellulose membranes, incubated with the indicated reagents, and stained for alkaline phosphatase activity. Lane 1, 10 ng of pyruvate carboxylase, blotted with Str-AP; lanes 2 to 4 (no-toxin controls), 1, 3, and 10 μg of pyruvate carboxylase, respectively, blotted with primary antibodies against activated HD-73 toxin and secondary antibody-AP conjugate; lanes 5 to 7, 1, 3, and 10 μg of pyruvate carboxylase, respectively, blotted with activated 65-kDa HD-73 toxin, primary antibodies against activated HD-73 toxin, and secondary antibody-AP conjugate.

band remaining at 115 to 120 kDa, presumably aminopeptidase N, did not bind streptavidin but did strongly bind the 65-kDa Cry1Ac toxin (13). Our data on the biotin binding ability of Cry1Ac toxin are not in conflict with the conclusion (25, 44) that aminopeptidase N is the dominant receptor for that toxin. Second, the 120-kDa biotin protein was purified on the basis of its affinity for streptavidin-coated latex beads and subjected to automated N-terminal amino acid sequence analysis. The first 25 amino acids were AATDS KIVEY KPIRT TNVAQ EXXIQ; the identities of the first 15 amino acids were unambiguous, while those of the latter 10 were more tentative.

This 25-amino-acid N-terminal sequence was examined by TFastA analysis (Genetics Computer Group). It did not reveal strong identity with any protein sequence in the GenBank-EMBL database. The protein was an unknown. In particular, it did not show homology with either the 120-kDa or the 172-kDa receptor recently cloned and sequenced from *M. sexta* (25, 52). This result is not surprising. Neither the 120-kDa aminopeptidase N (25) nor the 172-kDa receptor (210 kDa with glycosylation) cloned and sequenced by Vadlamudi et al. (52) contains the AMKM biotin attachment motif (42, 49), and thus they are unlikely to be biotin-containing proteins. The N-terminal sequence did, however, exhibit 37% identity with mouse pyruvate carboxylase (EC 6.4.1.1) and 35% identity with the proton-transporting ATPase (EC 3.6.1.35) from *M. sexta.*

Conserved amino acid sequences. We believe that domain III of the *B. thuringiensis* toxins contains a biotin binding site because there are pronounced sequence similarities between domain III and known biotin-binding proteins. Figure 8 shows those portions of avidin (30), streptavidin (30), and an antibiotin MAb (4) that participate in biotin binding. For avidin, the location of the biotin binding site was defined by comparison of X-ray crystallographic structures of avidin and the avidin-biotin complex (30). Sixteen amino acids participate in the biotin binding site on avidin (30). However, YXS/T was identified as the biotin binding motif (21) because peptides containing this motif could bind biotin by themselves (21). This YXS/T motif, shown in boldface in Fig. 8, recognizes the ureido group of biotin, with the tyrosine of YXS/T forming a critical hydrogen bond with the ureido oxygen in biotin (21, 30). This motif is also present in the Cry1, Cry3, and Cry4 sequences, but it is not present in the 27-kDa general cytolytic toxin cyt1A. In the Cry proteins, YXS/T is located at the end of the conserved block 4 region (Fig. 8). Similarly, the conserved VXXD sequence at the start of block 5 (Fig. 8) corresponds to the VXXD/N sequence of avidin and streptavidin; the D/N side chains interact directly with biotin by forming a hydrogen bond with the ureido ring of biotin (30).

Avidin contains eight antiparallel β-strands (30), while the Cry3A toxin contains 23 β-strands (29). Fig. 8 also shows that the positions of the β-17 and β-23 strands in domain III of the *B. thuringiensis* toxin correspond to β-3 and β-8 of avidin (Fig. 8). Finally, in the Cry3A toxin, two critical amino acid residues Y-567 and D-636 are adjacent to one another (29).

DISCUSSION

We have shown that BBMVs from the tobacco hornworm, *M. sexta*, contain two prominent streptavidin-binding proteins which are ca. 120 and 85 kDa in size. These proteins are probably biotin-containing proteins, since the streptavidin-BBMV binding was blocked by free biotin but not by the RGDS or HPQN tetrapeptide. These controls were necessary because streptavidin is known to have affinity for both RGDS (2) and HPQN (10) sequences. A similar phenomenon was noted by Ziegler et al. (58) in neurosecretory cells and brain cells from *M. sexta*. For these cell types, they found that Str-AP reacted with proteins which were ca. 250, 130, and 85 kDa in size.

The 120- and 85-kDa biotin proteins in BBMV preparations are of physiological interest because activated *B. thuringiensis* HD-73 toxin binds to them, as well as to pyruvate carboxylase, biotinylated ovalbumin, and biotinylated BSA. The sequence similarities between Cry1Ac toxin and both avidin and streptavidin (Fig. 8) suggest that biotin binding is localized in blocks 4 and 5 of domain III, while the highly conserved nature of blocks 4 and 5 (24) suggests that biotin binding is a common feature of Cry1, Cry3, and Cry4 toxins. The realization that the Cry1 proteins are biotin-binding proteins impacts our understanding of toxins and receptors in at least five ways, as indicated below.

(i) Minimum toxin size. During pathogenesis, the protoxin is processed to an activated toxin which is ca. 65 kDa in size. However, there is a minimum size below which toxicity is lost. By using deletion analysis of Cry1Ab, Höfte et al. (23) found that the shortest toxic fragment was localized between amino acids 29 and 607; removal of a further 4 residues from the C terminus completely abolished toxicity (23). Similar observations have been made for the Cry1Aa and Cry1Ac genes (1, 45). In each case, the minimum size coincides with the bound-

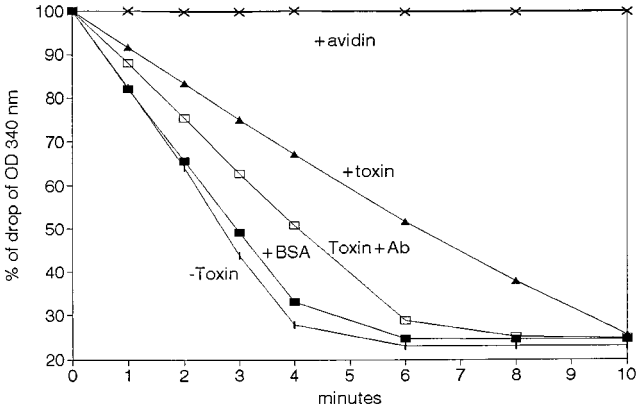

FIG. 7. Inhibition of pyruvate carboxylase by activated *B. thuringiensis* toxin. Symbols: ǀ, no additions; ■, plus BSA (60 μg/ml); ×, plus avidin (20 μg/ml); ▲, plus activated Cry1Ac toxin (20 μg/ml; toxin/enzyme molar ratio, 11:1); □, plus toxin (20 μg/ml) premixed for 20 min at room temperature with 10 μl of polyclonal antibodies against the activated toxin.

		Block 4	Block 5
Bt toxins	CryIAa	521 RYRVRIR**YAS**	596 VYIDRIEFVPAE
	CryIAb	522 RYRVRIR**YAS**	597 VYIDRIEFVPAE
	CryIAc	525 RYRVRVR**YAS**	598 VIIDRFEFIPVT
	CryIB	529 RYRIGFR**YAS**	604 VYIDKIEIIPVT
	CryIC	518 RYRLRFR**YAS**	605 LYIDKIEIILAD
	CryID	511 SYYIRFR**YAS**	581 VYIDRIEFIPVT
	CryIIIA	560 KYRARIH**YAS**	633 VYIDKIEFIPVN
		β-17	β-23
	CryIVA	585 SYFIRIR**YAS**	667 VLIDKIEFLPIT
	CryIVC	558 QYQVRIR**YAT**	623 VIIDRIEIIPIT
Streptavidin		40 TGT**YES**	125 VGHDTFTKVKPS
Avidin		30 TGTYTT	115 VGINIFTRLRTQ
		β-3	β-8
Anti-biotin mAb CDR2		56 RTN**YNS**	

FIG. 8. Alignment of amino acid sequences showing the similarities between nine *B. thuringiensis* (Bt) Cry toxins and three biotin-binding proteins. The *B. thuringiensis* sequences are from Höfte and Whiteley (24). The avidin and streptavidin sequences are from Livnah et al. (30), and the antibiotin MAb sequence is from Bagci et al. (4). The number to the left of each protein indicates the position of the initial amino acid. Letters in boldfaced type represent amino acids known to interact with biotin (30) as well as the presumptive biotin-binding portions of the Cry sequences. Underlined amino acids show the location of the β-strand regions in avidin (30) and the coleopteran-specific Cry3A toxin (29) as determined from their three-dimensional structures.

aries of block 5. We suggest that block 5 is needed for biotin binding, which in turn is needed for toxicity.

(ii) Western blots. Toxin receptors are commonly studied by Western blotting of BBMV proteins that had been separated by SDS-PAGE (35). Thus, these proteins had been denatured by SDS prior to their recognition by the Cry toxins, and the fact that they are recognized could mean, among several possibilities, that the receptor proteins had been refolded correctly or that they were still denatured but were recognized because of their attached biotin. The data presented here suggest that Cry toxins should have some affinity for all biotin-containing proteins, regardless of their physiological function or whether they are native or denatured. This affinity for biotinylated proteins may be involved in cases where there is no clear correlation between ligand blot data and insect pathogenicity. In this regard, because of the mitochondrial location of pyruvate carboxylase (51), it is important that even the best BBMV preparations typically contain up to 4% mitochondria as a contaminant (55).

(iii) An essential tyrosine. Which amino acid side chains are required for toxicity? Yan and McCarthy (57) used tetranitromethane to modify tyrosine residues in toxin from *B. thuringiensis* subsp. *thuringiensis*. Cytolytic activity towards a lepidopteran cell line was decreased 66% following nitration of 12 of the 24 tyrosines available. Later, Cummings and Ellar (6) showed that modifying 7 of the 27 tyrosine residues in Cry1Ac toxin with either tetranitromethane or *p*-nitrobenzenesulfonyl fluoride caused a dramatic loss of toxicity whereas modifying only 5 or 6 of the tyrosines did not cause any loss of toxicity. These data suggest that a single tyrosine is essential for toxicity. In the conserved amino acid sequences identified by Höfte and Whiteley (24), there are two tyrosines in block 1, seven in block 2, and two in block 4, one of which is part of the YAS biotin binding motif. We suggest that the YAS tyrosine is the one which is essential for toxicity. This point is significant for binding studies using radiolabeled toxin because both the iodogen and chloramine T methods iodinate tyrosine residues; consequently, Cry proteins in which the YAS tyrosine is iodinated may no longer bind biotin. Thus, iodination of the tyrosine in YAS could explain the apparent contradictions between receptor binding studies using different detection methods (35). In *M. sexta*, for instance, two groups using ^{125}I-Cry1Ab (35, 52) found a single 210-kDa binding protein whereas another group using immunological detection of unlabeled Cry1Ab found nine binding proteins (15).

(iv) High- and low-affinity receptors. Two categories of receptors are generally recognized, depending on whether they exhibit high or low affinity for the Cry toxins (17, 54). This distinction may also be influenced by the biotin binding ability of domain III. It is generally thought that domain II mediates specific attachment of toxin to the insect receptors (29). However, two groups (9, 28) have recently shown that domain III also participates in receptor binding and host specificity. The concerted action of domains II and III could be necessary for high-affinity binding sites, whereas the action of domain III alone could result in an apparent low-affinity binding site. Thus, some of the low-affinity receptors could be products of the detection system chosen rather than actual in vivo targets of the Cry toxins.

(v) Avidin toxicity. There are comparatively few biotin-containing enzymes in animals. Vertebrates contain only four (42), and the number present in insects (50) is presumably similar. The four biotin enzymes in mammals are pyruvate carboxylase, propionyl-CoA carboxylase, acetyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase. Since Cry1Ac toxin binds to denatured pyruvate carboxylase (Fig. 6) and inhibits pyruvate carboxylase activity (Fig. 7), the idea that pyruvate carboxylase is a target for the toxin is attractive and provocative. However, there is as yet no evidence that the two molecules even contact one another during insect pathogenesis, since pyruvate carboxylase is thought to be a mitochondrial enzyme (51).

If the biotin binding ability of domain III turns out to be relevant to insect pathogenicity, it must mean that the Cry toxin contacts a biotin-containing protein at some point. One way to accomplish this would be to have an essential biotin-containing protein exposed on the midgut surface, in which case avidin and streptavidin themselves should be toxic to insects. However, this expectation has already been fulfilled by Morgan et al. (39). They found that avidin inhibited the growth of five coleopteran species when present in the diet at levels from 100 to 1,000 ppm (39) and that either avidin or streptavidin was lethal for European corn borer larvae when present at concentrations as low as 25 ppm. Morgan et al. (39) suggested that avidin and streptavidin caused a biotin deficiency which in turn led to stunted growth and mortality. This idea is reasonable. However, there are two lines of evidence indicating that there may be a more direct mode of action as well: (i) for the European corn borer larvae, avidin and streptavidin at 25 ppm are almost as toxic as *B. thuringiensis* itself (50% lethal concentrations, 37 μg/ml for HD-73 toxin and 3.6 μg/ml for HD-1 toxin [33]); and (ii) if the effects of avidin and streptavidin were strictly nutritional, one would not expect the five coleopteran species to require 100 to 1,000 ppm for growth retardation when, according to the data cited by Dadd (7), their nutritional requirements for biotin are 10 times lower than those of lepidopteran species. Our future research will be directed towards identifying the physiological function(s) of the biotin proteins and determining the degree to which they are targets of *B. thuringiensis* toxins during insect pathogenesis.

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REFERENCES

1. **Adang, M. J., M. J. Staver, T. A. Rocheleau, J. Leighton, R. F. Barker, and D. V. Thompson.** 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD-73 and their toxicity to *Manduca sexta*. Gene **36:**289–300.
2. **Alon, R., E. A. Bayer, and M. Wilchek.** 1990. Streptavidin contains an RYD sequence which mimics the RGD receptor domain of fibronectin. Biochem. Biophys. Res. Commun. **170:**1236–1241.
3. **Ang, B. J., and K. W. Nickerson.** 1978. Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. Appl. Environ. Microbiol. **36:**625–626.
4. **Bagci, H., F. Kohen, U. Kuscuoglu, E. A. Bayer, and M. Wilchek.** 1993. Monoclonal anti-biotin antibodies simulate avidin in the recognition of biotin. FEBS Lett. **322:**47–50.
5. **Bayer, E. A., and M. Wilchek.** 1990. Protein biotinylation. Methods Enzymol. **184:**138–160.
6. **Cummings, C. E., and D. J. Ellar.** 1994. Chemical modification of *Bacillus thuringiensis* activated δ-endotoxin and its effect on toxicity and binding to *Manduca sexta* midgut membranes. Microbiology **140:**2737–2747.
7. **Dadd, R. H.** 1985. Nutrition: organisms, p. 313–390. *In* G. A. Kerkut and L. L. Gilbert (ed.), Comprehensive insect physiology, biochemistry, and pharmacology. Pergamon Press, Oxford.
8. **Dakshinamurti, K., R. P. Bhullar, A. Scoot, E. S. Rector, G. Delespesse, and A. H. Sehon.** 1986. Production and characterization of a monoclonal antibody to biotin. Biochem. J. **237:**477–483.
9. **de Maagd, R. A., M. S. G. Kwa, H. van der Klei, T. Yamamoto, B. Schipper, J. M. Vlak, W. J. Stiekema, and D. Bosch.** 1996. Domain III substitution in *Bacillus thuringiensis* delta-endotoxin Cry1A(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Appl. Environ. Microbiol. **62:**1537–1543.
10. **Denolf, P., S. Jansens, S. Van Houdt, M. Peferoen, D. Degheele, and J. Van Rie.** 1993. Biotinylation of *Bacillus thuringiensis* insecticidal crystal proteins. Appl. Environ. Microbiol. **59:**1821–1827.
11. **Devlin, J. J., L. C. Panganiban, and P. E. Devlin.** 1990. Random peptide libraries: a source of specific protein binding molecules. Science **249:**404–406.
12. **Dow, J. A. T.** 1992. pH gradients in lepidopteran midgut. J. Exp. Biol. **172:**355–375.
13. **Du, C.** 1996. Ph.D. thesis. University of Nebraska, Lincoln.
14. **Du, C., P. A. W. Martin, and K. W. Nickerson.** 1994. Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. Appl. Environ. Microbiol. **60:**3847–3853.
15. **Feldmann, F., A. Dullemans, and C. Waalwijk.** 1995. Binding of the CryIVD toxin of *Bacillus thuringiensis* subsp. *israelensis* to larval dipteran midgut proteins. Appl. Environ. Microbiol. **61:**2601–2605.
16. **Ferre, J., M. D. Real, J. Van Rie, S. Jansens, and M. Peferoen.** 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a midgut membrane receptor. Proc. Natl. Acad. Sci. USA **88:**5119–5123.
17. **Garczynski, S. F., J. W. Crim, and M. J. Adang.** 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl. Environ. Microbiol. **57:**2816–2820.
18. **Gill, S. S., E. A. Cowles, and P. V. Pietrantonio.** 1992. The mode of action of *Bacillus thuringiensis* endotoxins. Annu. Rev. Entomol. **37:**615–636.
19. **Gould, F., A. Martinez-Ramirez, A. Anderson, J. Ferre, F. J. Silva, and W. J. Moar.** 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **89:**7986–7990.
20. **Gringorten, J. L., D. N. Crawford, and W. R. Harvey.** 1993. High pH in the ectoperitrophic space of the larval lepidopteran midgut. J. Exp. Biol. **183:**353–359.
21. **Hiller, Y., E. A. Bayer, and M. Wilchek.** 1991. Studies on the biotin-binding site of avidin. Minimized fragments that bind biotin. Biochem. J. **278:**573–585.
22. **Hofmann, C., H. Vanderbruggen, H. Höfte, J. Van Rie, S. Jansens, and H. Van Mellaert.** 1988. Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA **85:**7844–7848.
23. **Höfte, H., H. de Greve, J. Seurinck, S. Jansens, J. Mahillon, C. Ampe, J. Vandekerckhove, H. Vanderbruggen, M. Van Montagu, M. Zabeau, and M. Vaeck.** 1986. Structural and functional analysis of a cloned delta endotoxin of *Bacillus thuringiensis* berliner 1715. Eur. J. Biochem. **161:**273–280.
24. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
25. **Knight, P. J. K., B. H. Knowles, and D. J. Ellar.** 1995. Molecular cloning of an insect aminopeptidase N that serves as a receptor for *Bacillus thuringiensis* CryIA(c) toxin. J. Biol. Chem. **270:**17765–17770.
26. **Knowles, B. H.** 1994. Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxins. Adv. Insect Physiol. **24:**275–308.
27. **Koziel, M. G., N. B. Carozzi, T. C. Currier, G. W. Warren, and S. V. Evola.** 1993. The insecticidal crystal proteins of *Bacillus thuringiensis*: past, present, and future uses. Biotechnol. Genet. Eng. Rev. **11:**171–228.
28. **Lee, M. K., B. A. Young, and D. H. Dean.** 1995. Domain III exchanges of *Bacillus thuringiensis* CryIA toxins affect binding to different gypsy moth midgut receptors. Biochem. Biophys. Res. Commun. **216:**306–312.
29. **Li, J., J. Carroll, and D. J. Ellar.** 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* 2.5Å resolution. Nature (London) **353:**815–821.
30. **Livnah, O., E. A. Bayer, M. Wilchek, and J. L. Sussman.** 1993. Three-dimensional structures of avidin and the avidin-biotin complex. Proc. Natl. Acad. Sci. USA **90:**5076–5080.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
32. **MacIntosh, S. C., T. B. Stone, R. S. Jokerst, and R. L. Fuchs.** 1991. Binding of *Bacillus thuringiensis* proteins to a laboratory-selected line of *Heliothis virescens*. Proc. Natl. Acad. Sci. USA **88:**8930–8933.
33. **MacIntosh, S. C., T. B. Stone, S. R. Sims, P. L. Hunst, J. T. Greenplate, P. G. Marrone, F. J. Perlak, D. A. Fischhoff, and R. L. Fuchs.** 1990. Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. J. Invertebr. Pathol. **56:**258–266.
34. **Martin, P. A. W., and R. S. Travers.** 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. **55:**2437–2442.
35. **Martinez-Ramirez, A. C., S. Gonzalez-Nebauer, B. Escriche, and M. D. Real.** 1994. Ligand blot identification of a *Manduca sexta* midgut binding protein specific to three *Bacillus thuringiensis* CryIA-type ICPs. Biochem. Biophys. Res. Commun. **201:**782–787.
36. **Masson, L., A. Mazza, R. Brousseau, and B. Tabashnik.** 1995. Kinetics of *Bacillus thuringiensis* toxin binding with brush border membrane vesicles from susceptible and resistant larvae of *Plutella xylostella*. J. Biol. Chem. **270:**11887–11896.
37. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
38. **McGaughey, W. H.** 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science **229:**193–195.
39. **Morgan, T. D., B. Oppert, T. H. Czapla, and K. J. Kramer.** 1993. Avidin and streptavidin as insecticidal and growth inhibiting dietary proteins. Entomol. Exp. Appl. **69:**97–108.
40. **Nickerson, K. W., G. St. Julian, and L. A. Bulla.** 1974. Physiology of spore forming bacteria associated with insects. VI. A radiorespirometric survey of carbohydrate metabolism in 12 serotypes of *Bacillus thuringiensis*. Appl. Microbiol. **28:**129–132.
41. **Oddou, P., H. Hartmann, and M. Geiser.** 1991. Identification and characterization of *Heliothis virescens* midgut membrane proteins binding *Bacillus thuringiensis* δ-endotoxins. Eur. J. Biochem. **202:**673–680.
42. **Samols, D., C. G. Thornton, V. L. Murtif, G. K. Kumar, F. C. Haase, and H. G. Wood.** 1988. Evolutionary conservation among biotin enzymes. J. Biol. Chem. **263:**6461–6464.
43. **Sanchis, V., and D. J. Ellar.** 1993. Identification and partial purification of a *Bacillus thuringiensis* Cry1C δ-endotoxin binding protein from *Spodoptera littoralis* gut membranes. FEBS Lett. **316:**264–268.
44. **Sangadala, S., F. S. Walters, L. H. English, and M. J. Adang.** 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and $^{86}Rb^{+}$-K^{+} efflux in vitro. J. Biol. Chem. **269:**10088–10092.
45. **Schnepf, H. E., and H. R. Whiteley.** 1985. Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. J. Biol. Chem. **260:**6273–6280.
46. **Scrutton, M. C., M. R. Olmsted, and M. F. Utter.** 1969. Pyruvate carboxylase from chicken liver. Methods Enzymol. **13:**235–249.
47. **Tabashnik, B. E.** 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. **39:**47–79.
48. **Tabashnik, B. E., N. Finson, M. W. Johnson, and W. J. Moar.** 1993. Resistance to toxins from *Bacillus thuringiensis* subsp. *kurstaki* causes minimal cross-resistance to *B. thuringiensis* subsp. *aizawai* in the diamondback moth (Lepidoptera: Plutellidae). Appl. Environ. Microbiol. **59:**1332–1335.
49. **Toh, H., H. Kondo, and T. Tanabe.** 1993. Molecular evolution of biotin-dependent carboxylases. Eur. J. Biochem. **215:**687–696.
50. **Tu, Z., and H. H. Hagedorn.** 1992. Purification and characterization of pyruvate carboxylase from the honeybee and some properties of related biotin-containing proteins in other insects. Arch. Insect Biochem. Physiol. **19:**53–66.
51. **Tzagoloff, A.** 1982. Mitochondria. Plenum Press, New York.
52. **Vadlamudi, R. K., E. Weber, I. Ji, T. H. Ji, and L. A. Bulla, Jr.** 1995. Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J. Biol. Chem. **270:**5490–5494.
53. **Van Rie, J., S. Jansens, H. Höfte, D. Degheele, and H. Van Mellaert.** 1990. Receptors on the brush border membrane of the insect midgut as determi-

nants of the specificity of *Bacillus thuringiensis* delta-endotoxins. Appl. Environ. Microbiol. **56:**1378–1385.

54. **Van Rie, J., W. H. McGaughey, D. E. Johnson, B. D. Barnett, and H. Van Mellaert.** 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science **247:**72–74.
55. **Wolfersberger, M., P. Luethy, A. Maurer, P. Parenti, F. V. Sacchi, B. Giordana, and G. M. Hanozet.** 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol. A **86:**301–308.
56. **Wolfersberger, M. G.** 1990. The toxicity of two *Bacillus thuringiensis* δ-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. Experientia **46:**475–477.
57. **Yan, X., and W. J. McCarthy.** 1991. Chemical modification of *Bacillus thuringiensis* subsp. *thuringiensis* (HD-524) trypsin-activated endotoxin: implication of tyrosine residues in lepidopteran cell lysis. J. Invertebr. Pathol. **57:** 101–108.
58. **Ziegler, R., D. L. Engler, and N. T. Davis.** 1995. Biotin-containing proteins of the insect nervous system, a potential source of interference with immunocytochemical localization procedures. Insect Biochem. Mol. Biol. **25:**569–574.