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Structural Disulfide Bonds in the *Bacillus thuringiensis* subsp. *israelensis* Protein Crystal

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We examined disulfide bonds in mosquito larvicidal crystals produced by *Bacillus thuringiensis* subsp. *israelensis*. Intact crystals contained 2.01×10^{-8} mol of free sulfhydryls and 3.24×10^{-8} mol of disulfides per mg of protein. Reduced samples of alkali-solubilized crystals resolved into several proteins, the most prominent having apparent molecular sizes of 28, 70, 135, and 140 kilodaltons (kDa). Nonreduced samples contained two new proteins of 52 and 26 kDa. When reduced, both the 52- and 26-kDa proteins were converted to 28-kDa proteins. Furthermore, both bands reacted with antiserum prepared against reduced 28-kDa protein. Approximately 50% of the crystal proteins could be solubilized without disulfide cleavage. These proteins were 70 kDa or smaller. Solubilization of the 135- and 140-kDa proteins required disulfide cleavage. Incubation of crystals at pH 12.0 for 2 h cleaved 40% of the disulfide bonds and solubilized 83% of the crystal protein. Alkali-stable disulfides were present in both the soluble and insoluble portions. The insoluble pellet contained 12 to 14 disulfides per 100 kDa of protein and was devoid of sulfhydryl groups. Alkali-solubilized proteins contained both intrachain and interchain disulfide bonds. Despite their structural significance, it is unlikely that disulfide bonds are involved in the formation or release of the larvicidal toxin.

Bacillus thuringiensis produces protein crystals that are toxic to the larval stage of many insects. Most strains of *B. thuringiensis* form highly cross-linked bipyramidal crystals that are toxic to insects of the order *Lepidoptera*. These crystals are generally composed of one to three high-molecular-size protein chains (5) rendered insoluble by the presence of 10 to 12 disulfide bonds per 130- to 150-kilodalton (kDa) subunit (13). All crystal solubilization procedures require the cleavage of disulfide bonds either by the presence of sulfhydryl reagents or by alkaline beta-elimination (24). In contrast, the recently discovered (14) *B. thuringiensis* subsp. *israelensis* crystal is more amorphous, contains many proteins, and is toxic to *Diptera* spp. such as mosquitoes and blackflies. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Pfannenstiel et al. (28) found that both alkali-solubilized *B. thuringiensis* subsp. *israelensis* toxin and native crystals solubilized in SDS sample buffer gave protein bands, which fell into five groups: (i) a single broad band at 28 kDa; (ii) a triplet at 38, 39, and 40 kDa; (iii) a minor band at 53 kDa; (iv) a doublet at 68 and 70 kDa; and (v) a doublet at 135 and 140 kDa. Qualitative agreement with these assignments has been provided by five other groups (2, 16, 18, 32, 37).

Most investigators solubilize the *B. thuringiensis* subsp. *israelensis* crystal protein in alkaline buffers to simulate the conditions (pH 10 to 10.5) of the midguts of mosquito larvae (7). The role of alkaline disulfide cleavage in crystal solubilization has so far received little attention in research with *B. thuringiensis* subsp. *israelensis*, even though the 2.1% half-cystine composition reported for native *B. thuringiensis* subsp. *israelensis* crystals (33) is greater than that (1.1 to 1.9%) for crystals from all other *B. thuringiensis* varieties (24). In *B. thuringiensis* subsp. *thuringiensis* crystals, the half-cystines are present exclusively as disulfide bonds (13). Similar information is not yet available for *B. thuringiensis* subsp. *israelensis*. Accordingly, the present paper examines *B. thuringiensis* subsp. *israelensis* crystals with regard to the presence of both disulfide bonds and free sulfhydryl content, the function of disulfide bonds in crystal structure, disulfide bond cleavage at increasingly alkaline pH, the identity of protein subunits released by alkaline disulfide cleavage, and structural relationships among the protein bands detected by SDS-PAGE. The latter aspect complements our previous immunological comparison of the *B. thuringiensis* subsp. *israelensis* crystal proteins (27) and allows us to identify the origins of the proteins present in native and proteolytically degraded crystals.

MATERIALS AND METHODS

Toxin purification and solubilization. A single-colony isolate of *B. thuringiensis* subsp. *israelensis* taken from Bactimos powder (courtesy of Brian Federici, University of California, Riverside) was grown on GGYS medium (26). After sporulation, the protein crystals were purified on NaBr gradients as described previously (28). All analyses started with a 2-mg/ml suspension of crystals in deionized water and made use of reagent grade chemicals. The crystals were alkali solubilized for 2 h at 35°C in 50 mM NaOH (pH 12) with 10 mM EDTA. Insoluble material was removed by centrifugation at 15,000 × *g* for 10 min. The supernatants were adjusted to pH 8.0 with 0.1 M sodium phosphate-citric acid buffer (pH 3.75), and the protein content was estimated from the A_{280} [$E^{1\%}_{1\ cm\%} = 11.0$] (33).

Gel electrophoresis. Samples for electrophoresis were boiled for 5 min in SDS sample buffer (with or without mercaptoethanol) and subjected to SDS-PAGE in 7.5 to 15% or 12.5% Laemmli gels (20). Gels were stained with Coomassie blue or silver (23), depending on the desired sensitivity. Procedures for *bis*-acrylylcystamine cross-linked gels are described in Technical Bulletin no. 2045 (Bio-Rad Laboratories, Richmond, Calif.).

Production of antiserum and immunoblots. Antiserum to the 28-kDa protein of *B. thuringiensis* subsp. *israelensis* crystals was produced as described previously (27). Under

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reducing conditions, the antiserum recognized only the 28-kDa protein and the protease digestion products of the 28-kDa protein (27). In this study, the specificity of the antiserum was tested under reducing and nonreducing conditions by using Western blots (27).

Total disulfide and sulfhydryl content. Total disulfide and sulfhydryl contents were estimated by using the procedure of Thannhauser et al. (31) as modified by Damodaran (8). For all determinations, disodium 2-nitro-5-thiosulfobenzoate (NTSB) was added to samples, and the solutions were mixed by gentle inversion, incubated at room temperature in the dark for 30 min, and centrifuged at 15,000 × *g*. The A_{414} of the supernatant was recorded. For intact crystals, 500 μl of crystal suspension was added to 500 μl of deoxygenated assay buffer containing 0.4 M Tris, 6 M guanidine thiocyanate, 0.2 M Na_2SO_3, and 6 mM EDTA (pH 9.5), and the mixture was heated to 100°C for 5 min. Samples (100 μl) equivalent to 100 μg of whole crystals were diluted with 900 μl of deoxygenated NTSB assay reagent containing 0.2 M Tris, 3 M guanidine thiocyanate, 0.1 M Na_2SO_3, and 0.5 mM NTSB (pH 9.5). For the alkali-treated crystals, samples containing the equivalent of 100 μg of whole-crystal protein were diluted to 1 ml with NTSB assay reagent and processed as described above.

Sulfhydryl content. Sulfhydryl contents were estimated by using the procedure of Ellman (11). For all determinations, 5,5′-dithiobis(2-nitrobenzoic acid) was added to the samples, and the solutions were mixed by gentle inversion, incubated at room temperature in the dark for 30 min, and centrifuged at 15,000 × *g*. The A_{414} of the supernantant was recorded. Samples were prepared as described below. For intact crystals, 500 μl of crystal suspension was added to 500 μl of deoxygenated 2× SDS buffer containing 0.125 M Tris, 4% SDS, and 6 mM EDTA (pH 8.0), and the mixture was heated to 100°C for 10 min. Samples (100 μl) containing 100 μg of crystals were mixed with 900 μl of SDS buffer and 13.6 μl of 5,5′-dithiobis(2-nitrobenzoic acid) reagent (11). For the alkali-treated crystals, samples containing the equivalent of 100 μg of whole crystal protein were diluted to 1 ml with SDS buffer and processed as described above.

Determinations on pH 12-soluble and -insoluble crystal protein. Crystal suspensions (500 μl) were mixed with 500 μl of 100 mM NaOH–10 mM EDTA (final pH 12.0) and incubated for 2 h at 37°C. After centrifugation at 15,000 × *g* for 10 min, the supernatants were removed by aspiration and adjusted to pH 8.0 with dilute HCl. Protein concentrations were estimated from the A_{280}, and samples containing 100 μg of protein were processed for disulfide and sulfhydryl determinations as described for whole crystals.

The pH 12.0-insoluble pellets were washed twice with 1 ml of deionized water and suspended in 250 μl of water. Suspensions were mixed with 250 μl of the appropriate assay buffer and processed for their disulfide and sulfhydryl content.

Reactions with iodo[1-^{14}C]acetamide. In experiments for the detection of preexisting and alkali-liberated sulfhydryl groups, the samples were adjusted to pH 8.5 and incubated with 1 μCi of iodo[1-^{14}C]acetamide (59 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) for 60 min at room temperature. Reactions were terminated by addition of a 50-fold molar excess of L-cysteine. Fluorography was done as described previously (6).

RESULTS

Reduced versus nonreduced protein subunits. Our previous work (28) showing the identity of SDS-solubilized and alkali-solubilized *B. thuringiensis* subsp. *israelensis* protein subunits employed mercaptoethanol in the preparation of samples for SDS-PAGE. Thus, the gel profiles were of reduced proteins. We have now examined alkali-solubilized (pH 12) proteins prepared for SDS-PAGE in the presence and absence of 1% mercaptoethanol (Fig. 1). Differences were observed between the reduced (Fig. 1A, lane 1) and nonreduced (Fig. 1A, lane 2) proteins. The major proteins in the reduced sample were similar to those observed previously (28) except that they were present in somewhat different proportions. These differences may be caused by differences in the *B. thuringiensis* subsp. *israelensis* strains used.

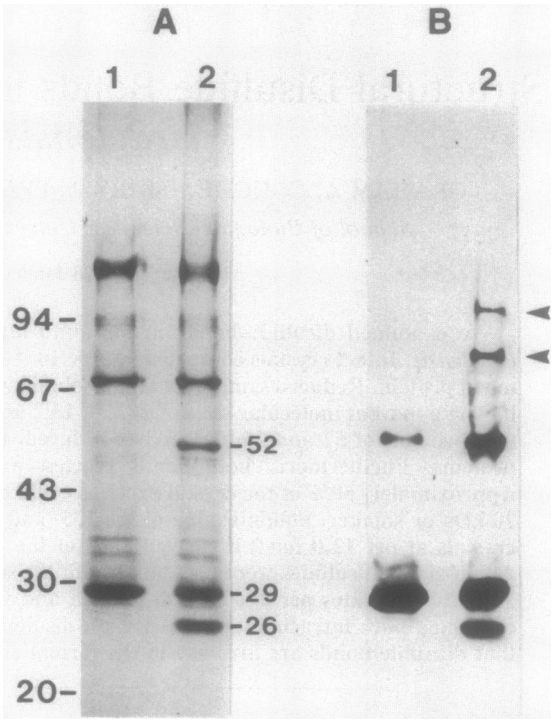

FIG. 1. Comparison of reduced and nonreduced *B. thuringiensis* subsp. *israelensis* crystal proteins. The supernatants from alkali-solubilized (pH 12) toxin were mixed with an equal volume of 2× SDS sample buffer (20), and the proteins (12.5 μg per lane) were analyzed on 7.5 to 15% gradient gels. Proteins were detected by either Coomassie blue staining (A) or immunoblot reaction with antiserum prepared against purified 28-kDa crystal protein (B). Reduced samples (lanes 1) were prepared by using a sample buffer that contained mercaptoethanol. The nonreducing sample buffer did not contain mercaptoethanol (lanes 2). The numbers at the left indicate low-molecular-size marker proteins (in kilodaltons) (Pharmacia). Arrows at the right indicate possible trimers and tetramers of the 28-kDa protein.

Gel profiles of nonreduced proteins exhibited new bands at 26 and 52 kDa. The origins of these bands were clarified by scanning densitometry of Coomassie blue-stained gels (Table 1). The 28-kDa protein accounted for 45% of the total reduced proteins but only 20% of the total nonreduced proteins. However, in nonreduced samples, the 28-kDa protein and the new 26- and 52-kDa proteins account for 42% of the total protein. We interpret this correspondence to indicate that even after solubilization at pH 12, the 26- and 52-kDa proteins contain disulfide bonds, which, if cleaved, cause the proteins to convert to a 28-kDa reduced form.

Immunological relationships. The proposed relationship

between the 26- and 52-kDa proteins and the 28-kDa protein was confirmed by immunoblots of reduced and nonreduced *B. thuringiensis* subsp. *israelensis* toxin proteins (Fig. 1B). After electrophoresis, toxin proteins were transferred to nitrocellulose and tested for reactivity with antiserum formed in response to reduced 28-kDa protein. The specificity of this antiserum was demonstrated by its reactivity with the reduced toxin proteins; only the 28-kDa protein and traces of its presumed 52-kDa dimer were detected (Fig. 1B, lane 1). The latter was not visible in Coomassie blue-stained gels.

Nonreduced proteins detected by the 28-kDa protein-specific antiserum were the 26-, 28-, and 52-kDa proteins, and minor bands at 82 and 110 kDa that were not visualized by Coomassie blue (Fig. 1B, lane 2). Thus, the 26- and 52-kDa proteins in nonreduced toxin migrate at 28 kDa in reducing gels. Most probably, the 26-kDa protein has a disulfide-containing internal loop. It is well known that reduced and nonreduced proteins have different electrophoretic migration rates, with the nonreduced proteins exhibiting lower apparent M_r values (21). This conclusion was confirmed by excising the 26-kDa band from a *bis*-acrylylcystamine cross-linked gel, solubilizing it with mercaptoethanol and noting its appearance at 28 kDa in a 12.5% polyacrylamide SDS gel. Similarly, the 52-, 82-, and 110-kDa bands represent the dimer and possible trimer and tetramer, respectively, of a 28-kDa monomer.

Disulfide bonds and free sulfhydryls. The above data contrasting reduced versus nonreduced alkali-solubilized *B. thuringiensis* subsp. *israelensis* toxin proteins strongly indicated the presence of alkali-stable disulfide bonds. Accordingly, we sought to analyze these bonds directly. By using intact crystals, the procedure of Ellman (11) with 5,5'-dithiobis(2-nitrobenzoic acid) detected 2.01×10^{-8} mol of free sulfhydryl groups per mg of protein (Table 2). The procedure of Thannhauser et al. (8, 31), making use of NTSB with excess sodium sulfite (to cleave all disulfide bonds), detected 5.25×10^{-8} mol of total sulfhydryl groups per mg of protein. This determination was done in 3 M guanidine thiocyanate to denature the protein and provide the reagents with free access to the protein interior. Since 1 mol of disulfide bonds produces 1 mol of sulfhydryl groups quantitatively in this cleavage (31), we calculate that intact crystals contain 3.24×10^{-8} mol of disulfide bonds per mg of protein (equivalent to 3.24 bonds per 100 kDa of protein).

Alkaline cleavage of disulfide bonds. Alkaline pH conditions are well known to cleave protein disulfide bonds by a beta-elimination mechanism (10, 15). However, the pH necessary for such cleavage is characteristic of the protein under study, ranging from at least 9.5 (4) to 13 (10). Disulfide bonds in the *B. thuringiensis* subsp. *israelensis* crystal also exhibited a wide range of stabilities (Table 2). Little disulfide cleavage occurred at pH values lower than 11.0, and even after pH 12.0 treatment, 1.95 disulfide bonds per 100 kDa of crystal protein were still intact. Alkali-stable disulfide bonds were located in both the pH 12-solubilized proteins and the insoluble pellet. The solubilized proteins contained approximately 0.9 disulfides and 2.8 sulfhydryls per 100 kDa of protein, whereas the insoluble pellets contained 12 to 14 disulfides per 100 kDa of protein with no detectable sulfhydryls.

TABLE 1. Major proteins in the reduced and nonreduced forms of *B. thuringiensis* subsp. *israelensis* toxin[a]

Protein band (kDa)	% Protein per band	
	Reduced	Nonreduced
Diffuse high mol wt	13.7	28.1
135 and 140	20.5	14.1
70	11.0	8.9
52	1.9	14.7
38 to 40	7.6	6.5
28	45.3	20.0
26	0	7.7

[a] Alkali-solubilized (pH 12) protein samples (20 μg) were electrophoresed in 12.5% gels. Data were obtained by integrating the peaks from scanning densitometry of Coomassie-blue stained gels by using a Gilford model 250 spectrophotometer at 595 nm (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Heterogeneous high-molecular-size material frequently appears as a smear above the 135- and 140-kDa band and may accumulate at the stacking gel-separating gel interface. This phenomenon is well known in other *B. thuringiensis* preparations (5 and references cited therein).

TABLE 2. Quantitation of alkaline disulfide cleavage[a]

pH of incubation	Disulfide bonds[b]		Free sulfhydryls[b]
	Cleaved	Intact	
7	0	3.24	2.01 ± 0.03
10	0.16	3.08	2.17 ± 0.10
11	0.28	2.96	2.29 ± 0.03
12	1.29	1.95	3.30 ± 0.00

[a] The disulfide content of intact *B. thuringiensis* subsp. *israelensis* crystals was calculated from the difference between the free sulfhydryl content ($2.01 \pm 0.03 \times 10^{-8}$ mol/mg of protein) and the total disulfide and sulfhydryl content ($5.25 \pm 0.05 \times 10^{-8}$ mol/mg of protein). After incubation (2 h at 37°C) at the indicated pH, alkaline disulfide cleavage was measured by adjusting the crystal suspension to pH 8 and detecting the number of newly formed free sulfhydryl groups. Data reported are the means ± the standard error with a sample size of three. The data for pH 10 to 12 are for total crystal suspension (i.e., both soluble and insoluble material).

[b] Values represent moles (10^{-8}) per milligram of protein.

Intrachain versus interchain disulfides. The preceding data indicate that the pH 12-solubilized *B. thuringiensis* subsp. *israelensis* toxin proteins contained intrachain disulfide bonds (responsible for the 26- to 28-kDa conversion) and interchain disulfide bonds (responsible for the 52- to 28-kDa conversion). The relative stability of these bond types was compared by determining the concentration of dithioerythritol necessary for their cleavage (Fig. 2). The 52-kDa protein was completely converted to the 28-kDa protein by 1 mM dithioerythritol (Fig. 2, lane 3), whereas the 26-kDa protein was still present after treatment with 10 mM dithioerythritol (Fig. 2, lane 5).

Location of the free sulfhydryls. To determine which protein subunits contained preexisting sulfhydryl groups, intact crystals were boiled in SDS and iodo[1-^{14}C]acetamide without added reducing agents. Fluorography of SDS gels (Fig. 3) indicated that the 28-, 52-, and 68- to 70-kDa bands were radiolabeled (Fig. 3A, lane 3) as was a band at 80 to 82 kDa not visualized by Coomassie blue staining. The 135- and 140-kDa bands were not solubilized under these conditions.

Mosquito guts are intensely proteolytic (19), and the ultimate toxin(s) responsible for larvicidal activity probably resides in protease-resistant domains (27). Digestion of crystal proteins with chymotrypsin (Fig. 4A, lane 2) and thermolysin (Fig. 4A, lane 3) identified domains at 24, 27, and 65 kDa. These correspond closely with those previously detected (22, 31, and 65 kDa) after digestion with mosquito larval gut enzymes (27). The protease-resistant domains were readily detected in Coomassie blue-stained gels (Fig. 4A, lanes 2 and 3), but not detected by fluorography of protease digests of iodo[1-^{14}C]acetamide-radiolabeled toxin proteins (Fig. 4B, lanes 2 and 3).

Location of the alkali-cleaved disulfide bonds. Approxi-

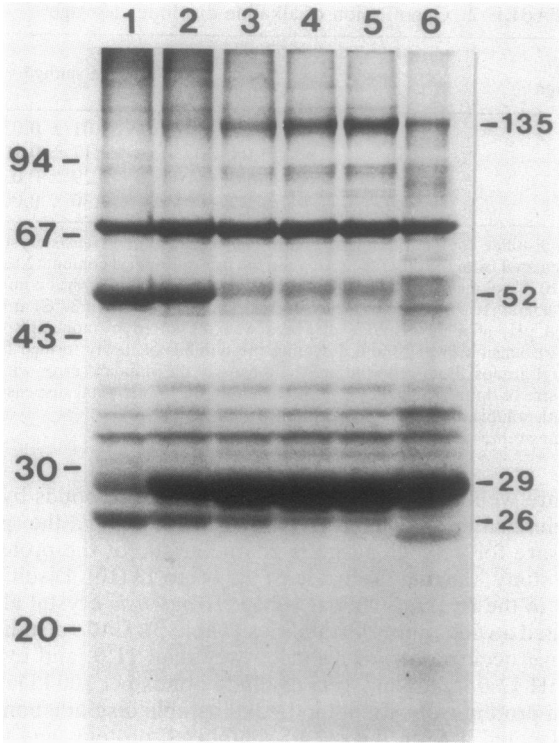

FIG. 2. Stability of *B. thuringiensis* subsp. *israelensis* toxin disulfide bonds to reduction by dithioerythritol. All samples were solubilized at pH 12, precipitated by adjusting the pH to 5.0 with 0.1 M sodium phosphate-citric acid buffer (pH 3.75) and suspended in 50 mM Tris hydrochloride (pH 8.25). To each 100-μl sample of solubilized protein (1.33 mg/ml), 10 μl of an 11× dithioerythritol stock solution in 50 mM Tris hydrochloride (pH 8.25) was added to give the indicated final dithioerythritol concentration. After 30 min at room temperature, the reduction reaction was quenched by 10-μl additions of iodoacetamide stock solutions sufficient to give molar ratios of iodoacetamide to dithioerythritol greater than 2.2. The proteins (30 μg) were mixed with nonreducing SDS sample buffer, separated on a 12.5% polyacrylamide gel, and stained with Coomassie blue. The reductions were carried out in the presence of 0 (lane 1), 0.1 (lane 2), 1.0 (lane 3), 5.0 (lane 4), 10.0 (lane 5), or 25.0 (lane 6) mM dithioerythritol. The numbers at the left are standard molecular-size markers (in kilodaltons), and those at the right indicate the toxin proteins (in kilodaltons) of greatest interest.

mately 40% of the disulfide bonds of the crystal were cleaved by a 2-h incubation at pH 12 (Table 2). To determine which protein subunits contained alkali-labile disulfide bonds, intact crystals were boiled in SDS containing nonradioactive iodoacetamide at pH 8.5 so that all preexisting free sulfhydryls were blocked. Unreacted iodoacetamide was removed by washing the pellet (centrifuged at 15,000 × *g*) three times with SDS buffer. The derivatized toxin proteins were then incubated at pH 12 and returned to pH 8.5 for reaction with iodo[1-^{14}C]acetamide. Labeled proteins were detected by fluorography of SDS gels (Fig. 3). Sulfhydryls newly generated by exposure to pH 12 were located primarily in the 28-, 135-, and 140-kDa subunits (Fig. 3B, lane 2).

Solubility. The above data concern the presence and stability of crystal disulfide bonds, not their structural significance. Accordingly, the idea that disulfide bonds are responsible for crystal insolubility was investigated. A total of 50% of the crystal protein was solubilized by conditions which do not cause significant disulfide cleavage, e.g., 2% SDS at pH 6.8 or 50 mM NaOH (pH 11.0) (Table 3). However, further crystal solubilization requires disulfide cleavage, either in the form of a reducing agent such as mercaptoethanol or pH values of 11.75 or greater. The SDS-soluble crystal material included the 28- and 70-kDa proteins, the 38- to 40-kDa proteins, the 52-kDa dimer, and minor amounts of the higher multimers, but no 135- or 140-kDa proteins (Fig. 3A, lane 2). The SDS-insoluble pellet contained all of the 135- and 140-kDa proteins and higher-molecular-size proteins combined with various levels of the other major proteins.

Identity of the alkali-solubilized protein subunits. Crystals were incubated at pH 10 to 12, and soluble proteins were analyzed by SDS-PAGE to determine the protein subunits present (Fig. 5). With equivalent amounts of protein applied to each lane, it was apparent that increasing pH had no effect on the degree to which proteins with a molecular mass of 70 kDa or smaller were solubilized. Significantly, these are the subunits solubilized by boiling in 2% SDS at pH 6.8 (Fig. 3A, lane 2). However, the 135- and 140-kDa subunits were solubilized only at pH values higher than 11.0 (Fig. 5, lanes 6–8). This indication that the high-molecular-size protein requires disulfide cleavage before solubilization is in agreement with the demonstration (Fig. 3B, lane 2) that sulfhydryl groups resulting from alkaline cleavage are located in the 135- and 140-kDa subunits.

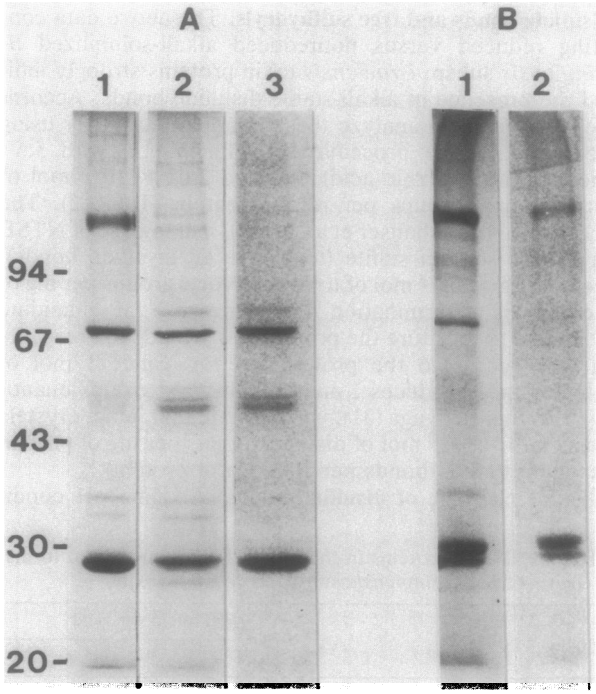

FIG. 3. Location of preexisting and alkali-generated sulfhydryls in *B. thuringiensis* subsp. *israelensis* crystal protein. (A) Preexisting sulfhydryls were identified by solubilizing the crystal protein at neutral pH. Lanes: 1, crystal proteins solubilized in the presence of mercaptoethanol; 2, crystal proteins solubilized in SDS (pH 6.8 at 100°C) after reaction with iodo[1-^{14}C]acetamide. Lanes 1 and 2 are from Coomassie blue-stained gels. Lane 3 is a fluorogram of lane 2. (B) Identification of sulfhydryls generated by alkali solubilization. Crystal proteins were solubilized with SDS and incubated with nonradioactive iodoacetamide before pH 12 disulfide cleavage and reaction with iodo[1-^{14}C]acetamide. Lane 1 is from a Coomassie blue-stained gel, and lane 2 is a fluorogram of lane 1. The numbers at the left indicate low-molecular-size marker proteins (in kilodaltons) (Pharamcia). Each lane contains 15 μg of protein.

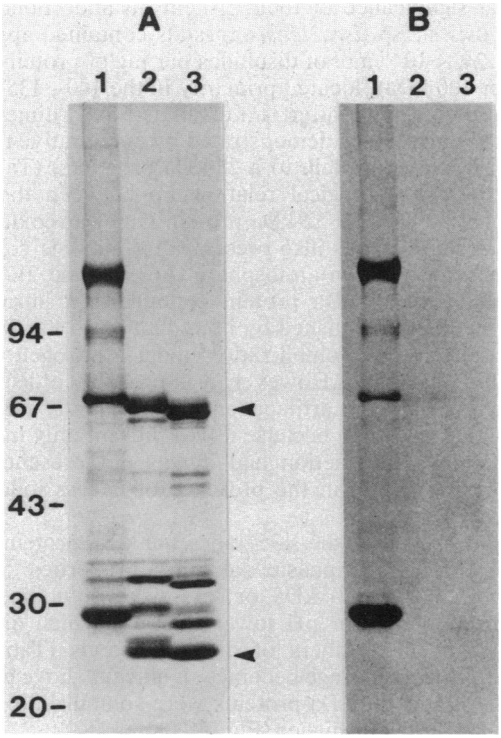

FIG. 4. Protease-resistant domains and sulfhydryl localization. Alkali-solubilized (pH 11.75) *B. thuringiensis* subsp. *israelensis* toxin proteins in 40% methanol were radiolabeled with iodo[1-^{14}C]acetamide as described previously (6) and incubated at pH 8.0 (with 50 mM Tris hydrochloride) with either chymotrypsin (25 μg/ml) at 30°C for 2 h or thermolysin (25 μg/ml) at 38°C for 2 h. The reactions were stopped by the addition of phenylmethylsulfonyl fluoride (to 2.5 mM) and EDTA (to 5 mM), respectively. Samples (20 μg per lane) were analyzed on 7.5 to 15% gradient gels and detected by either Coomassie blue staining (A) or fluorography (B) with En^{3}Hance (New England Nuclear Corp., Boston, Mass.)-impregnated gels. Lanes: 1, ^{14}C-radiolabeled proteins without proteolysis; 2, chymotrypsin-digested toxin proteins; 3, thermolysin-digested toxin proteins. The numbers at the left indicate low-molecular-size marker proteins (in kilodaltons). Arrows indicate the 25- and 65-kDa protease-resistant domains.

A further pH-induced modification evident in Fig. 5 is the conversion of the 28-kDa protein to a form which migrates as a 29-kDa protein. When the toxin was solubilized at pH 10.0, the protein migrated at 28 kDa, whereas the protein from the toxin solubilized at pH 12.0 migrated entirely at 29 kDa. The protein migrated at 28 and 29 kDa in equal quantities when crystals were solubilized at pH 11.0 to 11.25 (Fig. 5). The 28-kDa form is clearly the one present in native crystals because no 29-kDa protein was detected after extraction with 2% SDS at pH 6.8. The existence of the 28- to 29-kDa derivatization explains why a broad 28-kDa band was observed after alkaline (pH 11.0 to 11.75) solubilization (28).

Native crystals. Analysis of protein subunits by SDS-PAGE requires prior solubilization of the crystal proteins. However, the presence of substantial free sulfhydryl groups (2.01 per 100 kDa of protein), combined with the prevalence of disulfide-sulfhydryl exchange reactions (12), raises the question of whether the disulfide-containing proteins detected in Fig. 1 and 2 are intrinsic proteins of native crystals or artifacts produced during crystal solubilization. Three variations of our standard alkaline-solubilization conditions (pH 12) were used to identify such artifacts (Fig. 6). First,

TABLE 3. Solubility and toxicity of the *B. thuringiensis* subsp. *israelensis* crystal proteins[a]

Solubilization conditions	% Protein solubilized	LC_{50} (ng/ml)[b]	95% confidence interval (ng/ml)
2% SDS (pH 6.8)	50 ± 0.5 (2)	nontoxic	
pH 10.00 to 10.25	32 ± 5.0 (4)	124 (3)	96 to 152
pH 10.75 to 11.00	50 ± 4.0 (4)	95 (3)	71 to 119
pH 11.75	80[c]	108 (3)	100 to 116
pH 12.0	83 ± 6.0 (4)	141 (3)	119 to 163
pH 12.00, 2% mercaptoethanol	100		

[a] A 24-h bioassay was used to determine protein toxicity to third-instar *Aedes aegypti* larvae. Because these larvae are filter-feeders, the solubilized proteins were first attached to 0.8-μm latex beads (30). Data reported are the means ± standard error followed by the sample size in parentheses. SDS both inactivated the toxin (26) and prevented its attachment to latex beads (6).

[b] LC_{50}, Concentration at which 50% of the larvae are dead.

[c] Data taken from a previous publication (28).

crystal solubilization and sample preparation were done under an atmosphere of ntirogen to prevent the formation of disulfide bonds by aerobic reoxidation. The protein profile of crystals solubilized under nitrogen (Fig. 6, lane 2) was identical to that obtained under standard aerobic conditions (Fig. 6, lane 1). Second, 25 mM iodoacetamide was added to the solubilizing buffer to trap free sulfhydryls and prevent disulfide-sulfhydryl exchange reactions (Fig. 6. lane 3). Under these conditions, more of the 52-kDa protein was detected and the 26-kDa protein was not present. Addition-

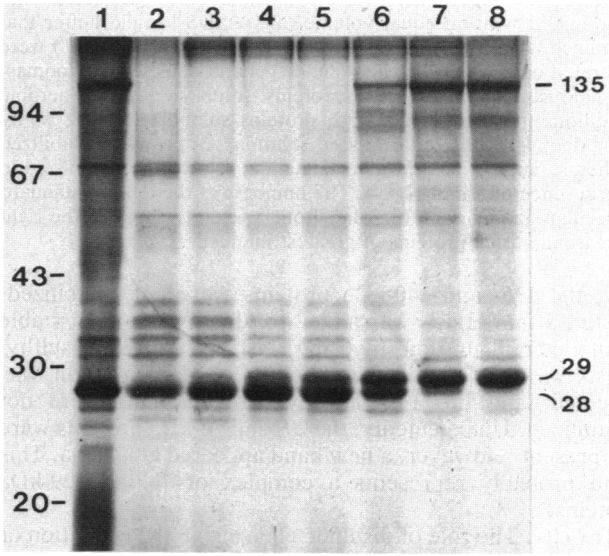

FIG. 5. *B. thuringiensis* subsp. *israelensis* crystal proteins solubilized at increasingly alkaline pH values. The supernatants from alkali-solubilized toxin were adjusted to pH 5.0 with 0.1 M sodium phosphate-citric acid buffer (pH 3.75) containing 10 mM EDTA and incubated at room temperature for 1 h. After centrifugation (15,000 × *g*), the protein pellets were dissolved in 50 mM Tris hydrochloride–10 mM EDTA (pH 8.3) and centrifuged again, and the protein concentration of the supernatants was determined from the A_{280}. Protein samples (5 μg per lane) were mixed with SDS sample buffer containing mercaptoethanol, separated on a 12.5% polyacrylamide gel, and detected by silver staining. Lanes: 1, reduced protein from intact crystals; 2–8, proteins solubilized at pH 10.0, 10.25, 10.5, 10.75, 11.25, 11.75, and 12.0, respectively. The numbers at the left are standard molecular-size markers (in kilodaltons), whereas those at the right indicate the toxin proteins of greater interest.

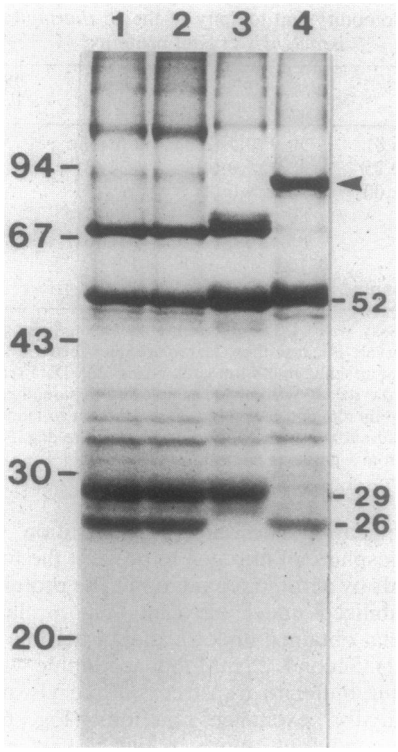

FIG. 6. Sulfhydryl-related and nonoxidizing variations on the alkaline solubilization of *B. thuringiensis* subsp. *israelensis* crystal proteins. The supernatants from alkali-solubilized (pH 12.0) toxin were mixed with an equal volume of 2× SDS sample buffer that contained no mercaptoethanol. The proteins (30 μg per lane) were separated on a 12.5% polyacrylamide gel and detected by Coomassie blue staining. Lanes: 1, proteins solubilized under aerobic conditions (no modifications); 2, proteins solubilized under N_2 by using deoxygenated and degassed solutions; 3, proteins solubilized in the presence of 25 mM iodoacetamide; 4, proteins solubilized in the presence of 5 mM $HgCl_2$. The numbers at the left are standard molecular-size markers (in kilodaltons), whereas those at the right indicate the toxin proteins of greatest interest.

ally, the 135- and 140-kDa proteins were not solubilized. Lastly, 5 mm $HgCl_2$ was added to identify the most stable disulfide configuration, by using Hg(II) as a bivalent sulfhydryl cross-link. Again, more of the 52-kDa protein was detected, and the 135- and 140-kDa proteins were not solubilized. Unexpectedly, the 29- and 70-kDa proteins were not present. However, a new band appeared at 90 kDa. This band probably represents a complex of 70- and 29-kDa proteins.

Toxicity. The role of disulfide cleavage in the formation or release of active toxin was examined. As the pH increased from 10.0 to 12.0, greater amounts of crystal protein were solubilized (Table 3) and more disulfide bonds were cleaved (Table 2). However, the larval toxicity per milligram of solubilized protein remained approximately constant. This result is in agreement with our previous observation (26) that the *B. thuringiensis* subsp. *israelensis* toxin does not require free sulfhydryl groups for biological activity. In contrast, when the disulfide bonds of *B. thuringiensis* subsp. *kurstaki* are reduced and blocked, there is a 160-fold reduction in toxicity (29).

DISCUSSION

We have analyzed mosquito larvicidal *B. thuringiensis* subsp. *israelensis* crystals with regard to the structural and functional significance of their protein disulfide bonds. *B. thuringiensis* subsp. *israelensis* crystals contained approximately 3.24×10^{-8} mol of disulfides per mg of protein (3.24 bonds per 100 kDa), located primarily in the 140-, 135-, and 52-kDa proteins. The latter is a disulfide-linked dimer of a 28-kDa monomer(s), as demonstrated by quantitative reduction of the 52-kDa protein to a 28-kDa protein(s) (Table 1) and by the immunological relationship between the two proteins (Fig. 1B). The 52-kDa protein is not a reoxidation artifact, because it was also present after alkaline solubilization under a nitrogen atmosphere (Fig. 6, lane 2). Similarly, solubilized 26-kDa protein contained an intrachain disulfide bond as established by its reduction to the 28-kDa protein after alkaline solubilization under a nitrogen atmosphere (Fig. 6, lane 2). However, it is probably a disulfide-sulfhydryl interchange artifact (and consequently not present in native crystals), because it was present only in trace levels after SDS extraction and it was not present after alkaline solubilization in the presence of excess iodoacetamide (Fig. 6, lane 3).

Disulfide bonds in the 135- and 140-kDa proteins are responsible for the biphasic solubility properties of the crystal. Proteins of 70 kDa or less were solubilized by conditions (2% SDS or pH lower than 11) which did not cleave significant numbers of disulfide bonds (Table 2). Alkaline disulfide cleavage becomes significant above pH 11, and the 135- and 140-kDa proteins were solubilized only at pH values of 11.25 or higher (Fig. 5).

Alkaline solubilization of the 135- and 140-kDa proteins was prevented by the presence of excess iodoacetamide. This indication that a disulfide-sulfhydryl interchange is operative during solubilization is consistent with the presence of sulfhydryls in the pH 12-solubilized 140- and 135-kDa proteins (Fig. 3) and their absence in the highly cross-linked insoluble pellet.

Even after incubation at pH 12, 60% of the disulfide bonds were still intact (Table 2). The pH 12-solubilized proteins contained both intrachain and interchain disulfide bonds, responsible for the 26- and 52-kDa entities, respectively. The stabilities of these two bond types were compared by their sensitivity to reduction by dithioerythritol (Fig. 2). The greater stability of intrachain disulfide bonds had been predicted on thermodynamic grounds (24), and these expectations were confirmed by the lower levels (1 mM) of dithioerythritol necessary for reduction of the 52-kDa dimer. Confirmation of the stability of the 26-kDa protein intrachain disulfide bond comes from its formation by disulfide-sulfhydryl interchange and its appearance as a Hg(II) cross-linked product (Fig. 6, lane 4).

We have previously shown (26) that the disulfide bonds in *B. thuringiensis* subsp. *israelensis* crystals can be cleaved and blocked with greater than 90% retention of toxicity. Thus, despite their structural significance, it is unlikely that crystal disulfide bonds participate in larvicidal activity. This conclusion is supported by two aspects of our present data. Increasingly alkaline pH levels lead to greater disulfide cleavage (Table 2) and greater protein solubilization (Table 3). However, they do not lead to increased toxicity (Table 3). Furthermore, because of the intensely proteolytic nature of the mosquito larval gut (19), the ultimate toxin(s) most likely resides in the protease-resistant domains, as is the case for *B. thuringiensis* subsp. *kurstaki* (1) and *Bacillus sphaericus* (3) toxins. However, the protease-resistant domains of iodo[1-^{14}C]acetamide-labeled *Bacillus thuringiensis* subsp. *israelensis* toxin could not be detected by fluorography (Fig. 4B). The apparent absence of sulfhydryls in these domains is

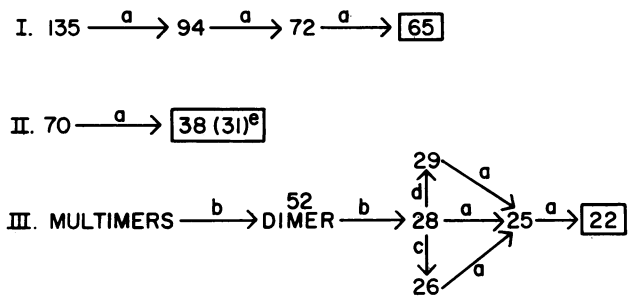

FIG. 7. Structural relationships among the *B. thuringiensis* subsp. *israelensis* crystal proteins. The roman numerals indicate three lines of immunologically distinct proteins (27). Numbers represent apparent size in kilodaltons. Symbols: a, proteolytic degradation (data from reference 27); b, disulfide cleavage; c, disulfide formation; d, high pH conversion; e, proteins migrating at 38, 39, and 40 kDa in 12.5% polyacrylamide gels (5) but at 31, 34, and 36 kDa in 7.5 to 15% gradient gels (27). Numbers in boxes are protease-resistant domains.

in agreement with amino acid analysis data reported by Armstrong et al. (2) in which no cysteine was detected in a 25-kDa protein obtained after digestion with trypsin and proteinase K. Preferential localization of cysteine and cystine in protease-sensitive regions can lead to underestimates of their presence if the analyses are done on protease-contaminated crystals, an ever-present danger for products released during bacterial sporulation (25). Recently, the amino acid sequence of the 28-kDA protein from another strain of *B. thuringiensis* subsp. *israelensis* has been deduced from the nucleotide sequence of its gene (35). The sequence indicated a protein of 27,340 Da containing two cysteine residues, one in the protease-sensitive region and the other, surprisingly, in the protease-resistant region.

The actual identity of the *B. thuringiensis* subsp. *israelensis* toxin(s) is still unresolved. The 28-kDa protein (2, 9, 18, 32), the 65- to 70-kDa proteins (16), and the proteins of 130 kDa and larger (34) have all been identified as being responsible for larval mortality. More recently, Wu and Chang (36) concluded that larvicidal activity was caused by a synergistic combination of the 28-kDa protein and proteins 65 kDa and larger. Ibarra and Federici (17) noted that the initially low toxicity of purified 65-kDa protein increased with increasing contamination by the 28-kDa protein. Definitive resolution of this controversy requires protein separation under nondenaturing conditions. Unfortunately, the *B. thuringiensis* subsp. *israelensis* toxin proteins have a high affinity for one another (27), and the existence of a stable dimer of the 28-kDa protein provides added complexity. Larvicidal column fractions from size exclusion chromatography can easily be enriched in the 65- to 70-kDa proteins and still contain the 52-kDa dimer.

When combined with our previous immunological and proteolytic data (27), the present data allow us to generate a unifying model for the structural relationships among *B. thuringiensis* subsp. *israelensis* crystal proteins (Fig. 7). The structural complexity depicted in Fig. 7 can also be related to the observed morphological complexity of *B. thuringiensis* subsp. *israelensis* crystals. Ultrastructural analyses have shown the crystals to be composed of two (18), three (17), or four (22) inclusion types. Significantly, all three research groups (17, 18, 22) found an inclusion type composed primarily of the 65- to 70-kDa and 38-kDa proteins, corresponding to the second of our immunologically distinct protein categories (Fig. 7). Furthermore, Lee et al. (22) distinguished two sizes of refractile bodies of similar protein composition except that the larger refractile bodies contained all the 52-kDa protein. We hope that the data presented in Fig. 7 will resolve controversies resulting from observed differences in electrophoretic mobility of toxic crystal proteins which may be caused in part by proteolysis, disulfide interchange, or altered environmental conditions, such as pH.

The structural diagram for *B. thuringiensis* subsp. *israelensis* is far more complex than similar diagrams for the insecticidal crystals produced by *B. sphaericus* (3) and the *Lepidoptera*-active strains of *B. thuringiensis* (1), primarily because of the presence of at least three immunologically distinct lines of proteins (27) in the *B. thuringiensis* subsp. *israelensis* crystals (Fig. 7). We have studied the structural relationships among the crystal components in the belief that such knowledge will lead to structure-function correlations for the toxin(s).

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