Folding and Unfolding of the Protoxin from *Bacillus thuringiensis*: Evidence That the Toxic Moiety Is Present in an Active Conformation†

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Received March 16, 1990; Revised Manuscript Received August 8, 1990

ABSTRACT: The action of trypsin or papain on the 130-kDa crystal protein (protoxin) from *Bacillus thuringiensis* subsp. *kurstaki* HD-73 yields a 67-kDa proteinase-resistant toxic fragment (toxin) which is derived from the N-terminal half of the molecule. Sensitivity to proteolysis and fluorescence emission spectroscopy showed that the toxin unfolded to a much greater extent in 6 M guanidinium chloride (GuHCl) than in 8 M urea. Protoxin also unfolded extensively in 6 M GuHCl, whereas in 8 M urea only the C-terminal half of the molecule had unfolded extensively. Both unfolded protoxin and unfolded toxin refolded to their native and biologically active conformations. The biphasic unfolding observed for protoxin suggests that the C-terminal half of the molecule unfolded rapidly, whereas the N-terminal toxic moiety unfolded at a much slower rate, similar to that of the free 67-kDa toxin. A 67-kDa fragment, derived from the N-terminal half of the molecule, could be generated from the protoxin in the presence of either urea or GuHCl by treatment with proteinases. Compared to toxin in denaturants, this fragment was found to be more sensitive to proteolysis. However, on removal of the denaturants the fragment had the same proteinase resistance and cytolytic activity as native toxin. The increased proteinase sensitivity of the fragment generated in the presence of denaturants appears to be due to a perturbation in the conformation of the N-terminal toxic moiety. This perturbation is attributed to the unfolding of the C-terminal region of the protoxin prior to its proteolysis to yield the 67-kDa fragment. It is concluded that unfolding and folding of the N-terminal half of the protoxin molecule are essentially independent of the C-terminal half and that the proteolytic processing of protoxin to toxin is not accompanied by a major conformational change in the toxic moiety.

Bacillus thuringiensis subsp. *kurstaki* HD-73 produces a 130-kDa protein, protoxin, in the form of a parasporal crystal. On ingestion by susceptible lepidopteran larvae, the protein is rendered toxic by the action of larval gut proteinases which convert the protoxin into a 58–70-kDa toxin (Fast, 1981; Aronson et al., 1986; Brousseau & Masson, 1988). The protoxin contains 16 cysteine residues: 14 in the C-terminal half of the molecule and 2 near the amino terminus (Adang et al., 1985). Bovine trypsin removes the first 28 amino acids and the C-terminal half of the protoxin, leaving a proteinase-resistant 67-kDa toxin devoid of cysteine residues (Bietlot et al., 1989).

The protoxin molecule appears to be composed of two distinct structural regions. In addition to the difference in the proteinase sensitivity and cysteine content between the N- and C-terminal halves of the molecule, only 3 of the 34 lysine residues remain in the trypsin-generated toxin. Furthermore, the N-terminal half of protoxin is predominantly hydrophobic in character whereas the C-terminal half is much more hydrophilic (Andrews et al., 1987). The C-terminal half of protoxin is not required for toxicity; nevertheless, its primary structure is highly conserved among the lepidopteran-specific crystal proteins (Höfte & Whiteley, 1989). It is unclear whether this sequence conservation reflects an essential structural requirement for crystal formation and/or a requirement for correct folding of the N-terminal toxic moiety. In addition, it has not been established whether a conformational change occurs in the N-terminal toxic moiety during the proteolytic processing of the protoxin.

Experimental Procedures

Reagents. Trypsin, elastase, and papain were obtained from Sigma Chemical Corp. (St. Louis, MO). Sequenal-grade urea and guanidinium chloride were obtained from Pierce (Rockford, IL). All other chemicals were reagent grade.

Protein Preparation. B. thuringiensis subsp. *kurstaki* HD-73 was grown in half-strength trypticase broth, the cells were lysed in double-distilled water, and the crystals were purified by Renograffin (Squibb, Montreal, Canada) gradients, as described previously (Carey et al., 1986). Toxin was prepared from purified crystals and characterized as described by Bietlot et al. (1989).

Protein Quantification. Amino acid analysis of several protoxin and toxin preparations of known absorbance at 280 nm showed that toxin at a concentration of 1 mg/mL gives an absorbance of 1.61 for a 1-cm path length; the corresponding absorbance of protoxin is 1.37 (Bietlot et al., 1989).

Designations for Denatured and Renatured Protein. The protocol used to investigate the effect of urea and GuHCl[1] on protoxin and their effect on toxin generated by trypsin (T_t) from protoxin in both the presence and absence of denaturants is summarized in Figure 1. An identical protocol was used for generating toxin by papain digestion of protoxin (T_p). Bold-face type designates proteins which were assayed for biological activity.

Bioassays. The in vitro biological activities of native and renatured toxins were assessed by use of CF-1 insect cells in

† This research was supported by the Natural Sciences and Engineering Research Council of Canada.

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[1] Abbreviations: CAPS, 3-cyclohexyl-1-propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; GuHCl, guanidinium hydrochloride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; E64, 1-[N^{α}-(*trans*-epoxysuccinyl)-L-leucylamido]-4-guanidinobutane.

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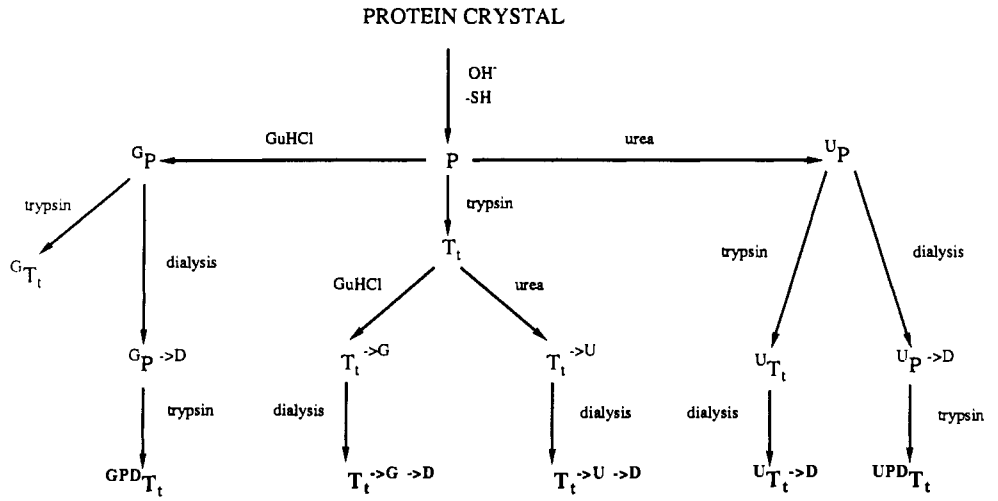

FIGURE 1: Generation of toxins from the protein crystal by trypsin: Protocol for unfolding and refolding of the protoxin and/or toxin from urea and GuHCl. P, protoxin; ^{G}P, protoxin incubated in GuHCl; $^{G}T_t$, toxin generated from protoxin in GuHCl by trypsin; $^{GP\rightarrow D}$, dialyzed ^{G}P; $^{GPD}T_t$, toxin generated by trypsin from $^{GP\rightarrow D}$. T_t, native trypsin-generated toxin; $T_t^{\rightarrow G}$, T_t incubated in GuHCl; $T_t^{\rightarrow G\rightarrow D}$, dialyzed $T_t^{\rightarrow G}$; $T_t^{\rightarrow U}$, T_t incubated in urea; $T_t^{\rightarrow U\rightarrow D}$, dialyzed $T_t^{\rightarrow U}$. ^{U}P, protoxin incubated in urea; $^{U}T_t$, toxin generated by trypsin from protoxin in urea; $^{U}T_t^{\rightarrow D}$, dialyzed $^{U}T_t$; $^{U P\rightarrow D}$, dialyzed ^{U}P; $^{UPD}T_t$, toxin generated by trypsin from $^{U P\rightarrow D}$. Note: the same protocol was used to prepare papain-generated toxins; these samples are identified in the text as T_p.

a lawn assay (Gringorten et al., 1990). Samples were adjusted to a concentration of 20 ng/μL in 0.1 M CAPS buffer, pH 10.5, and 2-fold serial dilutions were applied to the assay plates. Threshold levels were estimated to lie between the last visible spot colored by trypan blue, indicating injured cells, and the following unaffected sample spot.

Proteolysis of Protoxin and Toxin in Denaturants. Protoxin (8 mg/mL) or toxin (4 mg/mL) was incubated in 8 M urea at either pH 8.0 (0.1 M Tris buffer), pH 9.0 (0.1 M carbonate/bicarbonate buffer), or pH 10.0 (0.1 M CAPS buffer) at 20 °C for periods ranging from 15 min to 20 h. After a given incubation time, 20 μL of reaction mixture was removed, and 5 μL of either activated papain (15 mg/mL), trypsin (25 mg/mL), or elastase (25 mg/mL) was added. After 5 min, the inhibitor E64 was added to the papain-containing samples, and the samples were boiled for 2 min in SDS sample buffer (2.5% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 10 mM Tris-HCl, pH 8.3). Addition of inhibitor was necessary as papain retains residual activity even under strongly denaturing conditions (Sluterman, 1967). In order to inhibit further proteolysis, boiling SDS sample buffer was added directly to samples containing trypsin or elastase. After boiling, samples were cooled and analyzed by SDS–PAGE.

Proteolysis of protoxin and toxin in GuHCl was conducted in a similar manner, except that after the 5-min incubation with proteinase the proteins were precipitated from GuHCl by addition of 200 μL of 20‰ (w/v) TCA. The precipitate was washed three times with water and then solubilized by boiling in SDS sample buffer. This procedure, which was shown to recover proteins quantitatively from GuHCl, eliminated salt-induced streaking of proteins on the gels.

Limited Proteolysis by Papain in SDS. Protoxin or toxin (0.5–1.0 mg) was dissolved in 200 μL of either 0.1 M CAPS buffer, pH 10.5, or 8 M urea, pH 8.0. Activated papain (0.05 mg) was added, followed by boiling SDS sample buffer (2.5% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 10 mM Tris-HCl, pH 8.3). No papain inhibitor was added prior to boiling of the samples; thus, residual active papain proteolyzed the protoxin or toxin as the protein unfolded under these strongly denaturing conditions.

Gel Electrophoresis. Polyacrylamide–SDS gels (10–15% gradient) were run on a Pharmacia Phast electrophoresis system with preformed gels and other materials supplied by Pharmacia. Gels were stained with Coomassie blue, and densitometric measurements were made on an LKB-2222-010 Ultro Scan XL laser densitometer.

N-Terminal Sequencing. The N-terminal sequence of toxin generated by the action of papain on protoxin was determined with a Model 470A Applied Biosystems gas-phase sequencer (Watson et al., 1988).

Fluorescence. Emission spectra of toxin and protoxin under native and denaturing conditions were collected with a SLM-800C spectrofluorometer. Samples, contained in 3-mL quartz cuvettes with 1-cm path lengths, were maintained at 22 °C. The samples were excited at 295 nm, and emission was monitored at 340 nm. Emitted light was passed through an emission polarizer oriented at 35.3° to the vertical in order to minimize distortions due to Brownian motion (Badeau & Brand, 1979). Corrections were made for the signal from the appropriate blank and the wavelength dependence of the instrument response (Ghiggino et al., 1983). For all fluorescence measurements, the absorbance of the sample at the excitation wavelength was less than 0.1. Samples were maintained at pH 9.0 with 0.1 M carbonate/bicarbonate buffer. To ensure that the protoxin molecules were in a reduced state, the samples contained 0.01% β-mercaptoethanol. The fluorescence spectrum of the completely unfolded state of both protoxin and toxin was estimated from the emission spectra of peptic digests (pepsin:protein 1:100) of each of these two proteins (Bietlot et al., 1990).

The effect of rapid dilution on the fluorescence spectra of denatured toxin and protoxin was observed by adding 15 μL of 10 mg/mL protein, incubated in either 6 M GuHCl containing 0.1% β-mercaptoethanol or 8 M urea containing 0.1% β-mercaptoethanol, to 1.5 mL of 0.1 M carbonate/bicarbonate buffer, pH 9.0. The buffer was rapidly stirred during sample addition. Fluorescence spectra of the diluted samples were collected immediately and on an hourly basis thereafter.

Results and Discussion

The striking asymmetry between the two halves of the protoxin molecule in terms of amino acid composition and physical properties raises the question as to whether these two regions behave independently or interact in a cooperative

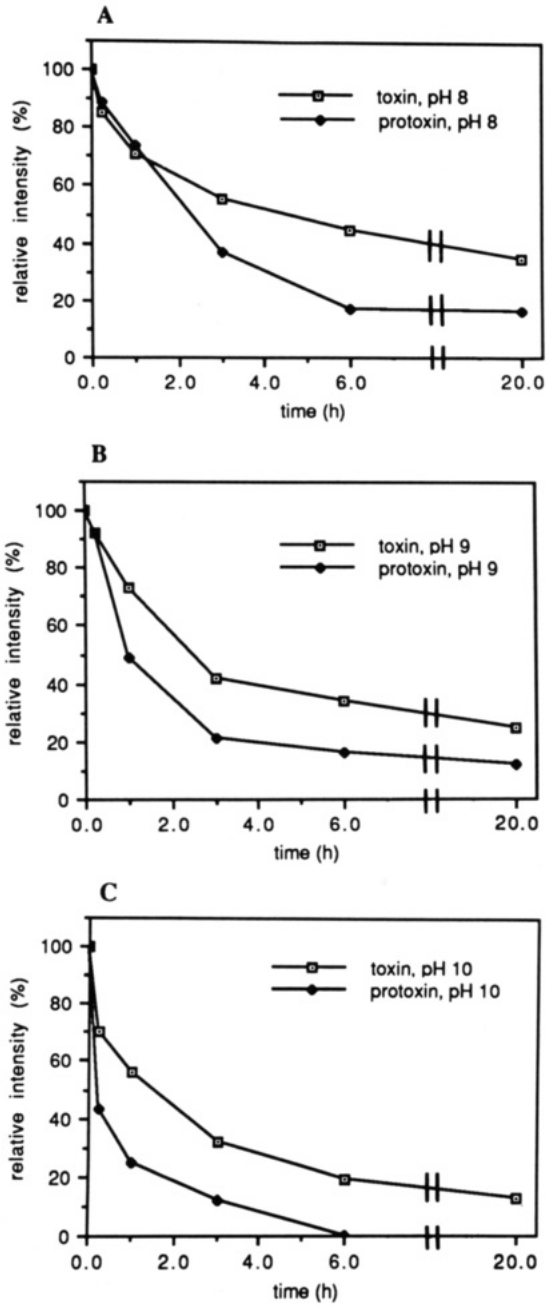

FIGURE 2: Sensitivity to digestion by papain of toxin incubated in 8 M urea ($T_p^{\rightarrow U}$) (⊡) and toxin generated in 8 M urea from protoxin ($^{U}T_p$) (◆). The amount of toxin at each time point was determined by quantifying the Coomassie blue staining intensity of the 67-kDa band. The zero-time sample point for protoxin was determined by quantifying the amount of toxin generated in the absence of denaturant. (A) Proteolysis conducted at pH 8.0; (B) proteolysis at pH 9.0; (C) proteolysis at pH 10.0.

manner. Of particular interest is whether the C-terminal half interacts with the N-terminal region and affects the conformation of the toxic moiety. If the two halves of the protoxin molecule unfold and fold independently of each other, this would suggest that the two regions do not interact in a cooperative manner. Unfolding/folding studies therefore provide a direct approach for addressing this problem. In the present study, the unfolding/refolding processes in protoxin and toxin were monitored by the proteins' susceptibility to proteinase digestion and by fluorescence emission spectroscopy. Cytolytic assays were used to confirm whether the toxin, and toxic moiety of the protoxin, had refolded into a biologically active conformation.

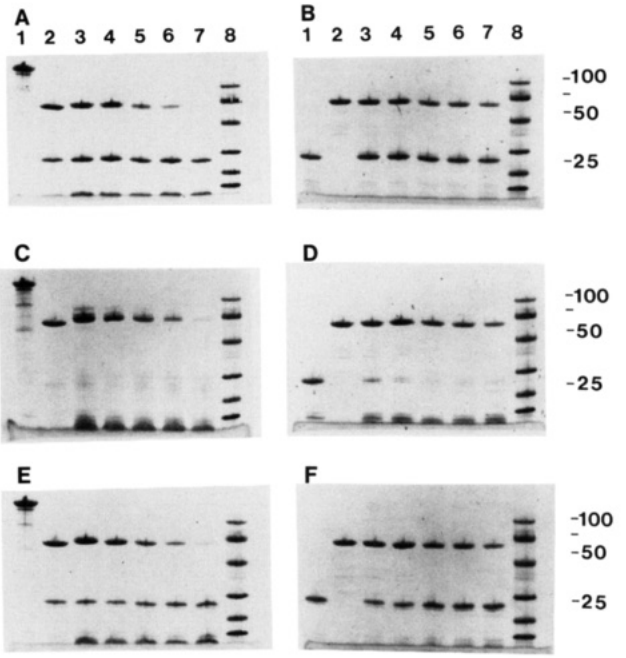

FIGURE 3: Proteinase sensitivity at pH 9.0 of toxin incubated in 8 M urea ($T_t^{\rightarrow U}$) and of toxin generated from protoxin in 8 M urea ($^{U}T_t$ and $^{U}T_p$). Samples of protoxin or toxin were incubated in 8 M urea at pH 9.0 for various time intervals (15 min to 20 h) before addition of proteinase. The samples were then subjected to SDS–gel electrophoresis followed by staining with Coomassie blue. Proteolysis of protoxin by papain (gel A), trypsin (gel C), and elastase (gel E) is shown. Lane 1 shows the protoxin protein; lane 8 shows the molecular mass markers, with molecular masses indicated in kilodaltons. Lane 2 indicates the amount of toxin generated by the proteinase from the protoxin sample in the absence of urea. Lanes 3–7 are 15-min 1-h, 3-h, 6-h, and 20-h incubations of protoxin in 8 M urea prior to addition of proteinase. Gels B (papain), D (trypsin), and F (elastase) show the results of the corresponding incubations of toxin in 8 M urea with the respective proteinase. Lane 1 shows the proteinase alone, and lane 2 is the zero-time toxin sample (no proteinase added). Lanes 3–7 are 15-min, 1-h, 3-h, 6-h, and 20-h incubations of toxin in 8 M urea prior to addition of proteinase. Lane 8 shows the molecular mass markers. Note that trypsin and, to a lesser extent, elastase autodigest under the denaturing conditions used.

Unfolding and Refolding of Protoxin and Toxin. The generation of toxin from protoxin has been shown to occur by an unusual proteolytic process in which fragments of approximately 10 kDa are removed in sequence from the C-terminus until the proteinase-resistant toxin is released (Choma et al., 1990). Protoxin incubated in 8 M urea for 6 h or in 6 M GuHCl for 1 h, followed by dialysis ($^{U}P^{\rightarrow D}$ and $^{G}P^{\rightarrow D}$; Figure 1), exhibited the same sequential proteolysis (data not shown) and generated 67-kDa fragments ($^{UPD}T_t$ and $^{GPD}T_t$) which were proteinase resistant. These results suggest that both the N-terminal toxic moiety and the C-terminal half of the unfolded protoxin refolded to their native conformations. When the protoxin was incubated in 6 M GuHCl for 6 h, the sequential proteolysis was not observed after dialysis, although a proteinase-resistant fragment was still obtained. These results indicate that after prolonged incubation in 6 M GuHCl the N-terminal toxic moiety could still refold to the native conformation but that the C-terminal region could not.

Accessibility of peptide bonds to proteolytic enzymes is a useful approach for monitoring unfolding/folding processes in proteins (Ghélis & Yon, 1982a). Since toxin is unusually resistant to further proteolysis by most proteinases, including papain (Choma et al., 1990), susceptibility to proteolysis should provide a convenient method for following the unfolding of the toxin. The experimental protocol adopted in the present study was to incubate protoxin or toxin in denaturant for a

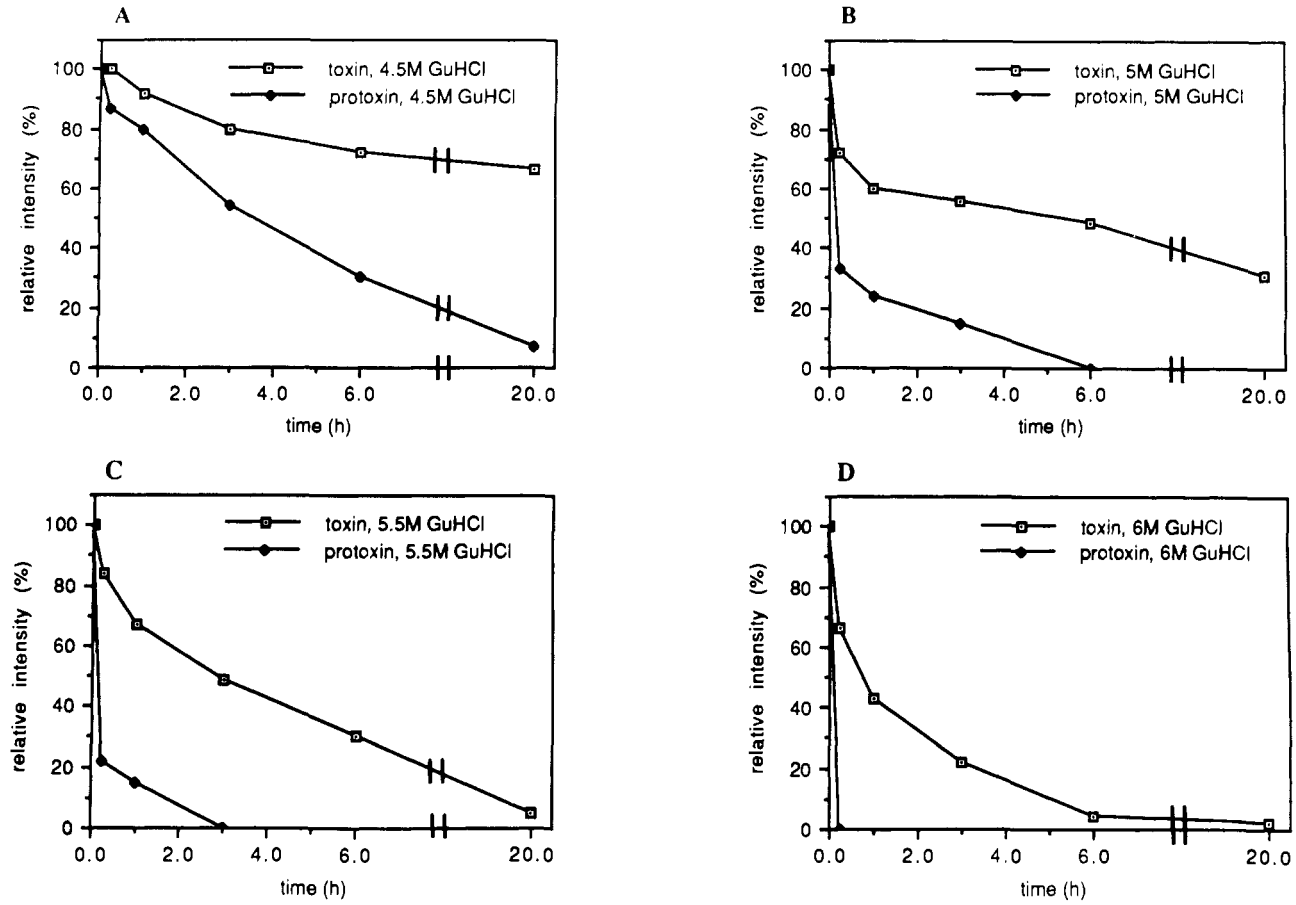

FIGURE 4: Sensitivity to papain digestion at pH 9.0 of toxin incubated in GuHCl ($T_p^{\rightarrow G}$) (□) and toxin generated in GuHCl (^GT_p) (◆). The amount of toxin at each time point was determined by quantifying the Coomassie blue staining intensity of the 67-kDa band. The zero-time sample point for protoxin was determined by quantifying the amount of toxin generated from the protoxin sample in the absence of denaturant.

specified period of time and then to add the proteinase for 5 min followed by the addition of boiling SDS sample buffer. In the case of papain, boiling SDS did not rapidly inactivate the enzyme, and it was necessary to add the inhibitor E64 to the digest. The amount of toxin remaining was quantified by SDS gel electrophoresis and laser densitometry. Most proteinases are rapidly inactivated in the presence of denaturants, but if relatively large quantities (ca. 1:1 mol/mol) are added, activity remains for a sufficient length of time to proteolyze any susceptible peptide bonds. Papain has unusual stability in denaturants, and therefore, it was the most suitable enzyme to use for quantifying the proteolytic susceptibility of protoxin and toxin under various denaturing conditions.

Papain can be used to generate active toxin (T_p) from protoxin. Amino acid sequencing of T_p gave the N-terminal sequence Gly-Glu-Arg-Ile. This sequence shows that the activation of protoxin by papain removes the first 25 amino acids, as compared to the first 28 removed during bovine trypsin activation. The C-terminal cleavage site was not determined, but as T_p has the same apparent M_r value as T_t, the terminal cleavage sites by the two proteinases must lie close together on the polypeptide chain.

Toxin in 8 M urea ($T_p^{\rightarrow U}$) was found to be surprisingly resistant to proteolysis by papain. The extent of proteolysis increased at higher pH values (Figure 2) and with incubation time (Figures 2 and 3B). After 1 h incubation most of the toxin remained resistant to proteolysis, and even after 20 h a significant amount of toxin was still proteinase resistant (Figure 3B). Trypsin-generated toxin in urea ($T_t^{\rightarrow U}$) showed similar resistance toward proteolysis by trypsin (Figure 3D) and elastase (Figure 3F). These results demonstrate that toxin unfolds very slowly in 8 M urea and, for such a large protein, is unusually resistant to urea denaturation.

When papain is added to protoxin in 8 M urea (^UP), a fragment (^UT_p) with the same apparent M_r as T_p is rapidly generated. The remainder of the protoxin molecule is digested by the proteinase into small peptides. The amount of ^UT_p generated decreases with increasing pH (Figure 2) and with incubation time in 8 M urea (Figures 2 and 3A). However, the rate of proteolysis of this fragment by papain is significantly faster than that observed for toxin ($T_p^{\rightarrow U}$) incubated for the same length of time in 8 M urea (Figure 2 and panel A vs panel B of Figure 3). A similar increased sensitivity to proteolysis is observed with ^UT_t relative to $T_t^{\rightarrow U}$ (panel C vs panel D of Figure 3). The fragment generated by elastase from protoxin in urea also shows greater proteinase sensitivity (panel E vs panel F of Figure 3).

The difference in proteinase sensitivity between toxin generated in GuHCl (^GT_p and ^GP_t) and native toxin incubated in GuHCl ($T_p^{\rightarrow G}$ and $T_t^{\rightarrow G}$) is much greater than the corresponding difference observed in urea (Figure 4). Native toxin appears to be relatively stable to proteolysis in up to 5 M GuHCl and is completely proteolyzed after 6 h in 6 M GuHCl. In contrast, toxin generated in GuHCl (^GT_p) is rapidly proteolyzed even in 4.5 M GuHCl and is so rapidly digested in 6 M GuHCl that none could be visualized on SDS gels (Figure 4D).

The increased susceptibility to proteolysis of the toxic fragments generated in denaturants (^UT_t, ^UT_p, ^GT_t, and ^GT_p) indicates that they have a different conformation in the presence of denaturant than do native toxins (T_t and T_p) incubated in urea or GuHCl ($T_t^{\rightarrow U}$, $T_t^{\rightarrow G}$, $T_p^{\rightarrow U}$, and $T_p^{\rightarrow G}$).

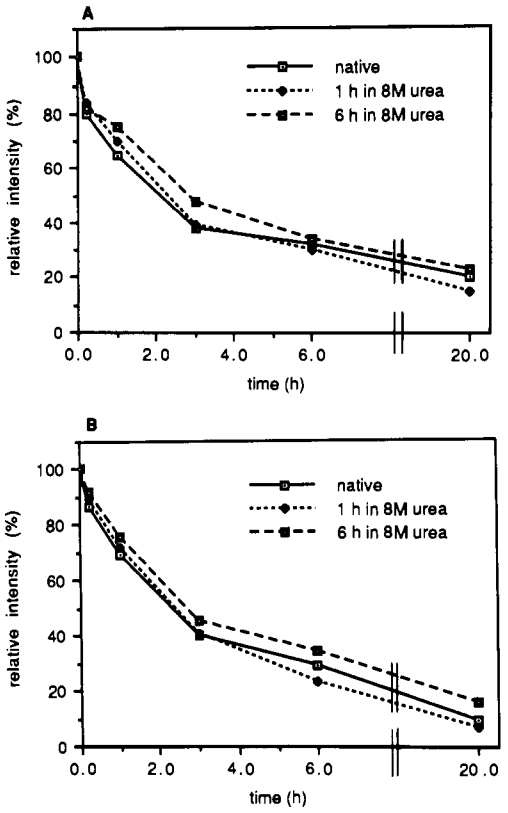

FIGURE 5: Sensitivity to proteolysis at pH 9.0 of native toxin and of toxin generated from protoxin during incubation in 8 M urea. (A) Sensitivity to trypsin: (i) native trypsin-generated toxin ($T_t^{\rightarrow U}$) (□), (ii) toxin generated by trypsin digestion of protoxin after 1-h incubation in 8 M urea, pH 9.0, and then dialyzed ($^{U}T_t^{\rightarrow D}$) and reincubated in 8 M urea (◆), and (iii) toxin generated by trypsin digestion of protoxin after 6 h in 8 M urea, pH 9.0, and then dialyzed ($^{U}T_t^{\rightarrow D}$) and reincubated in 8 M urea (□). (B) Sensitivity to papain: procedure as above, except that papain was used to generate the native toxin (T_P) and the urea-generated toxin ($^{U}T_p^{\rightarrow D}$). The stability of these proteins toward papain upon readdition of 8 M urea is shown. Note that the inhibitor E64 was added to samples following the 5-min incubation period with papain.

If the proteinase-sensitive toxins generated in denaturant are dialyzed against 0.1 M carbonate/bicarbonate buffer, pH 9.0, they become as proteinase resistant as the native toxins (data not shown). Their proteinase resistance is also indistinguishable from that of native toxins on readdition of denaturant (Figure 5). Similarly, if native toxins are incubated in denaturant and then dialyzed ($T_p^{\rightarrow G\rightarrow D}$, $T_t^{\rightarrow G\rightarrow D}$, $T_p^{\rightarrow U\rightarrow D}$, and $T_t^{\rightarrow U\rightarrow D}$), their original proteinase resistance is restored.

The results of toxicity assays quantifying the threshold levels of protein required to injure CF-1 insect cells are presented in Table I. Native toxins exposed to either 8 M urea or 6 M GuHCl for up to 6 h, followed by dialysis ($T_t^{\rightarrow U\rightarrow D}$, $T_p^{\rightarrow U\rightarrow D}$, $T_t^{\rightarrow G\rightarrow D}$, and $T_p^{\rightarrow G\rightarrow D}$), retain full toxicity. If the corresponding toxins are first generated in the presence of urea ($^{U}T_t$ and $^{U}T_p$) and then dialyzed ($^{U}T_t^{\rightarrow D}$ and $^{U}T_p^{\rightarrow D}$), they also have the same toxicity as native toxin. Similarly, fully toxic 67-kDa proteins ($^{UPD}T_t$, $^{UPD}T_p$, $^{GPD}T_t$, and $^{GPD}T_p$) could be generated from protoxin incubated in 8 M urea or 6 M GuHCl for up to 6 h, followed by dialysis and addition of papain or trypsin. Within the limits of the sensitivity of this assay (±1 dilution unit; Gringorten et al., 1990), these results demonstrate that biological activity is regenerated in the refolding of the toxic moiety in the protoxin molecule and in the refolding of free toxin.

Fluorescence Emission of Toxin. Figure 6A shows the effect of 8 M urea of the fluorescence emission spectrum of

Table I: Toxicity of *B. thuringiensis* subsp. *kurstaki* HD-73 Toxins[a] to CF-1 Cells: Effect of Denaturants on the Threshold Levels of Toxicity

toxin	threshold level (ng/μL)[b]	toxin	threshold level (ng/μL)[b]
T_t	0.32–0.16	T_p	0.64–0.32
$T_t^{\rightarrow U\rightarrow D}$	0.32–0.16	$T_p^{\rightarrow U\rightarrow D}$	0.64–0.32
$^{U}T_t^{\rightarrow D}$	0.32–0.16	$^{U}T_p^{\rightarrow D}$	0.64–0.32
$^{UPD}T_t$	0.32–0.16	$^{UPD}T_p$	0.64–0.32
$T_t^{\rightarrow G\rightarrow D}$	0.64–0.32	$T_p^{\rightarrow G\rightarrow D}$	0.64–0.32
$^{GPD}T_t$	0.64–0.32	$^{GPD}T_p$	0.64–0.32

[a] Preparation protocol for each toxin is given in Figure 1. [b] Threshold level lies between the given values.

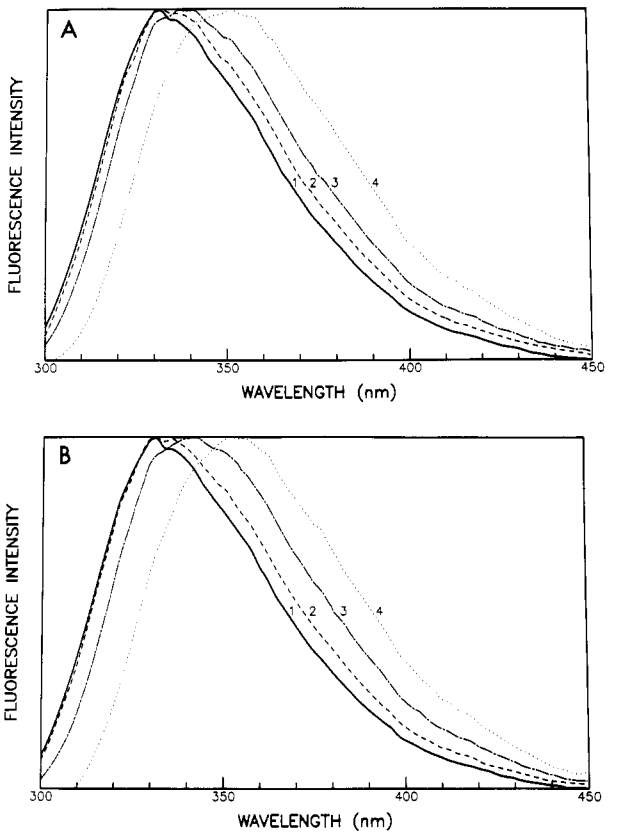

FIGURE 6: Fluorescence emission spectra of trypsin-generated toxin. (A) (1) Native toxin (T_t); (2) toxin incubated in 8 M urea for 1 h ($T_t^{\rightarrow U}$); (3) toxin incubated in 8 M urea for 6 h ($T_t^{\rightarrow U}$); (4) peptic digest of toxin in 8 M urea. (B) (1) native toxin (T_t); (2) toxin incubated in 6 M GuHCl for 1 h ($T_t^{\rightarrow G}$); (3) toxin incubated in 6 M GuHCl for 6 h ($T_t^{\rightarrow G}$); (4) peptic digest of toxin in 6 M GuHCl. All samples were at pH 9.0. For both panels A and B, rapid dilution of the samples which gave spectra 2 and 3 resulted in the generation of spectra which were superimposable onto spectrum 1.

toxin. On addition of urea, the emission spectrum shifts to longer wavelengths. If toxin incubated for 6 h in 8 M urea ($T_t^{\rightarrow U}$) is diluted rapidly in buffer, its fluorescence spectrum is superimposable on that of the native toxin (spectrum 1, Figure 6A). A larger red shift in the emission spectrum is observed when toxin is exposed to 6 M GuHCl (Figure 6B). As observed with urea, the emission spectrum of native toxin could be regenerated upon rapid dilution of toxin which had been incubated for 6 h in 6 M GuHCl ($T_t^{\rightarrow G}$; spectrum 1, Figure 6B). These fluorescence results suggest that toxin unfolds to a much greater extent in GuHCl than in urea and that it refolds into its native conformation upon removal of denaturant. If it is assumed that the emission spectrum of a peptic digest of toxin reflects the completely unfolded state,

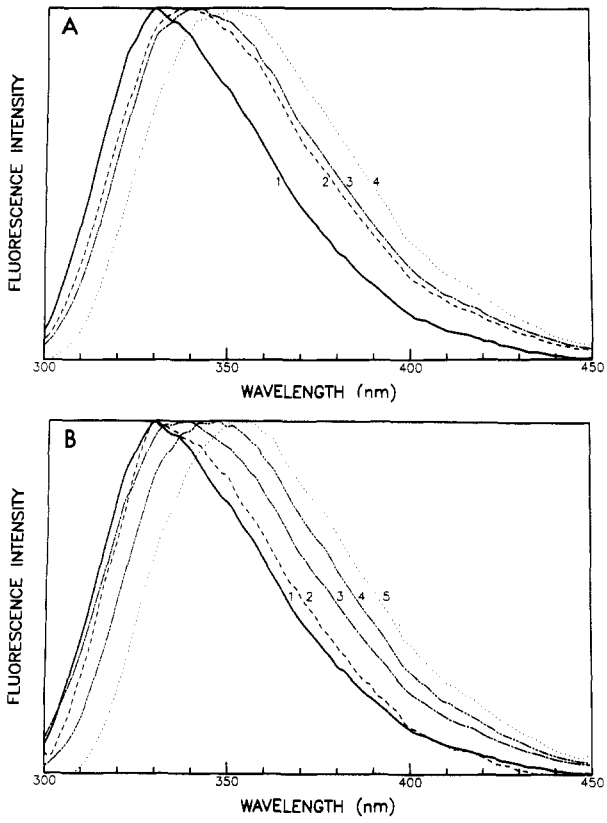

FIGURE 7: Fluorescence emission spectra of protoxin. (A) (1) Native protoxin (P); (2) protoxin incubated in 8 M urea for 1 h (^{U}P); (3) protoxin incubated in 8 M urea for 6 h (^{U}P); (4) peptic digest of protoxin in 8 M urea. Rapid dilution of the samples which gave spectra 2 and 3 resulted in the generation of spectra which were superimposable onto spectrum 1. (B) (1) Native protoxin (P); (2) dilution of the sample which gave spectrum 4; (3) protoxin incubated in 6 M GuHCl for 1 h (^{G}P); (4) protoxin incubated in 6 M GuHCl for 6 h (^{G}P); (5) peptic digest of protoxin in 6 M GuHCl. Rapid dilution of the sample which gave spectrum 3 resulted in the regeneration of spectrum 1. All samples were at pH 9.0.

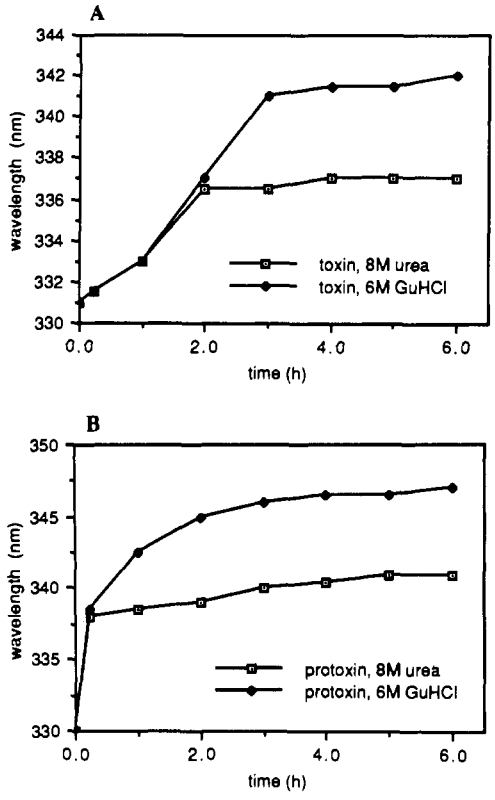

FIGURE 8: Fluorescence emission maxima of toxin and protoxin as a function of incubation time in denaturants. (A) Trypsin-generated toxin in 8 M urea ($T_t^{\rightarrow U}$) (⊡) and trypsin-generated toxin in 6 M GuHCl ($T_t^{\rightarrow G}$) (◆). (B) Protoxin in 8 M urea (^{U}P) (⊡) and protoxin in 6 M GuHCl (^{G}P) (◆). All samples were at pH 9.0.

it appears that toxin does not completely unfold even in 6 M GuHCl (Figure 6B).

Fluorescence Emission of Protoxin. Native protoxin and toxin have very similar fluorescence emission spectra (Figures 6 and 7). In the presence of either urea or GuHCl, the emission spectrum of protoxin is shifted toward longer wavelengths than that observed for toxin. As in the case of the toxin, there is a much larger red shift with GuHCl. Upon dilution of protoxin incubated in 8 M urea for 6 h, the emission spectrum of native protoxin was rapidly regenerated and was superimposable on that of the native protoxin (spectrum 1, Figure 7A). The emission spectrum of native protoxin could be regenerated from protoxin incubated for 1 h in 6 M GuHCl but not after 6-h incubation (Figure 7B). In the latter case, the emission spectrum remained slightly shifted to longer wavelengths (spectrum 2, Figure 7B). Protoxin therefore appears to readily unfold in GuHCl, and even after prolonged incubation, much of the native structure can apparently be restored upon dilution of the denaturant. As the bioassays (Table I) indicate that the toxic moiety refolds to its native conformation, some random oxidation of the cysteine residues in the C-terminal region probably accounts for the failure to regenerate completely the native spectrum of the protoxin. Therefore, the fluorescence data, susceptibility to proteolysis, and cytolytic activity all indicate that the unfolded 130-kDa protoxin and, in particular, its constituent 67-kDa toxic moiety refold to the native conformation.

Fluorescence Maxima of Protoxin and Toxin in Denaturants. Convents et al. (1990) found that toxin from *Bacillus thuringiensis* subsp. *berliner* unfolded to a greater extent in GuHCl than in urea. It is clear from the red shifts in the fluorescence maxima that protoxin and toxin from *B. thuringiensis* subsp. *kurstaki* HD-73 also unfold to a much greater extent in GuHCl than in urea (Figure 8). An interesting feature of the unfolding process in protoxin is that it appears to consist of two distinct events: a rapid step which is complete within 15 min followed by a slow unfolding over the next 2–3 h (Figure 8B). In both urea and GuHCl, the fast step results in a similar red shift in the fluorescence maximum. Furthermore, the rate of the slower process in protoxin is very similar to the overall rate of toxin unfolding (Figure 8A). These results suggest that the toxic moiety within the N-terminal half of protoxin unfolds at the same rate as free toxin. The initial rapid shift in the fluorescence spectrum of protoxin is therefore primarily due to the unfolding of the C-terminal half of the molecule.

Structural Implications. Most proteins contain relatively few cysteine residues, and these are usually present in the disulfide form. The denatured and reduced states of cysteine-containing proteins often form incorrect disulfide bridges and, in the absence of reducing agent, do not refold to the native conformation (Ghélis & Yon, 1982b). Since the protoxin contains 16 cysteine residues, 14 of which are in the C-terminal half, the observed rapid refolding of this region is unusual. The present results provide additional support for the conclusion of Bietlot et al. (1990) that all the cysteine residues of the native protoxin are on the surface of the molecule and do not form intrachain disulfide bonds.

The primary structure of the C-terminal portion of the protoxin molecule is highly conserved even though it is not

required for toxic activity. Höfte and Whiteley (1989) have suggested that the sequence is conserved because the C-terminal region serves an essential structural role in crystal formation. The finding of Bietlot et al. (1990) that all the cysteine residues in the C-terminal half of protoxin form parallel interchain disulfide bridges suggests that the sequence conservation arises from constraints imposed by the formation of multiple interchain disulfide bridges arranged in a crystalline array. The results obtained in this study show that the N-terminal toxic moiety and C-terminal half of the protoxin molecule fold and unfold independently. Indeed, the two halves of the molecule behave as if two autonomous proteins were fused together. This observation is consistent with the view that the two halves of the molecule have independent functions and that the primary role of the C-terminal region is for crystal formation.

It has not been established whether the toxic moiety in the protoxin molecule undergoes a conformational change upon activation. If, as it appears, the C-terminal region serves only to stabilize the crystal and therefore functions quite independently of the N-terminal half, then it is quite possible that the toxic moiety is present in an active conformation. Indeed, protoxin displays toxic activity toward CF-1 insect cells, although the threshold level is approximately 2 orders of magnitude higher than it is for toxin (Milne, 1989). The low toxicity of the protoxin molecule may be due to steric hindrance caused by the C-terminal half of the molecule between the protein's binding domain and the insect cell binding site.

Toxin generated in denaturants is more unfolded than native toxin, but the amount of unfolding is clearly small. It is possible that this observation reflects the fact that the activation process is accompanied by a minor conformational change which is prevented under denaturing conditions. However, in the presence of denaturants, the C-terminal region of the protoxin is unfolded, and it is still linked to the C-terminal end of the toxic moiety. It is likely that under these conditions a partial unfolding of the proximal portion of the N-terminal toxic moiety will take place and decrease the region's structural stability. While it does not necessarily follow that this would destabilize the entire toxic moiety, there is evidence that toxins truncated at the C-terminal end are much less stable molecules (Schnepf & Whiteley, 1985; Adang et al., 1985; Aronson et al., 1986). The results obtained in this study show that no major conformational change occurs on activation of protoxin to toxin. A similar conclusion was reached by Huber-Lukac et al. (1983), who found that monoclonal antibodies raised to toxin also bound to protoxin. Nevertheless, the possibility that some minor conformational change occurs upon activation cannot be ruled out.

Under controlled denaturing conditions, limited proteolysis of toxin with papain cleaves peptide bonds in the interdomain regions, yielding two fragments (Convents et al., 1990) or three fragments (Choma et al., 1990). The primary cleavage occurs in the interdomain area between the constant and hypervariable regions deduced from analysis of gene nucleotide sequences (Höfte & Whiteley, 1989). The second cleavage site reported by Choma et al. (1990) lies within the hypervariable region. When protoxin is incubated under identical denaturing conditions, the same fragments are observed on SDS gels (data not shown). Furthermore, it was found that limited proteolysis with papain of toxin or protoxin in 8 M urea resulted in the generation of the same three-fragment pattern (data not shown). It therefore appears that the partial unfolding in 8 M urea observed by fluorescence emission spectroscopy does not affect the integral domain structure of either the toxin or toxic moiety in the protoxin molecule. This observation further indicates that the conformation of the toxic moiety in the protoxin is very similar, if not identical, to that of the free toxin.

ACKNOWLEDGMENTS

We are especially grateful to Luc Pêloquin of the Biotechnology Research Institute in Montreal for his help in performing the bioassays, to Art Szabo of the National Research Council of Canada for the use of his fluorescence facility, and to Tim Lessard of the National Research Council of Canada for his help in protoxin crystal preparation. We thank Ross Milne and Witold Surewicz for their advice and valuable discussions during the course of this work. C.T.C. is a recipient of an Ontario Graduate Research Scholarship.

Registry No. GuHCl, 50-01-1; urea, 57-13-6.

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