# pH-Induced Conformational Transitions of Cry IA(a), Cry IA(c), and Cry IIIA δ-Endotoxins in *Bacillus thuringiensis*†

Qi Feng and Wayne J. Becktel*

*Department of Biochemistry, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210*

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ABSTRACT: Three protoxins and corresponding δ-endotoxins from *Bacillus thuringiensis* (BT) were studied by means of circular dichroism spectroscopy and size-exclusion HPLC. At neutral pH, the Cry IIIA toxin exists only as a 65-kDa monomer. The toxins of Cry IA(a) and Cry IA(c) exist both as 66-kDa monomers and as oligomers with apparent molecular masses greater than 220 kDa. At neutral pH, interconversion between monomer and oligomer is slow, and the two, separate forms exist for several days. Equilibration between the monomer and oligomer in both Cry IA(a) and Cry IA(c) toxins is facilitated by increasing the pH of the solutions to above 10. The relative amounts of monomer and oligomer depend upon temperature, pH, and buffer composition. CD spectra of the protoxins and toxins indicate a large helix content. The CD spectra of HPLC-isolated, monomeric Cry IA(a) and Cry IA(c) are quite similar, but are different from the spectrum of Cry IIIA. The Cry IA(a) and Cry IA(c) protoxins exhibit more helical CD spectra than the corresponding toxins. The CD spectra, with the pH titration of Cry IA(a), reveal that there is a significant increase in helical content as the pH is changed from neutral to alkaline values, but no decrease at low pH. A similar titration of Cry IIIA revealed no significant change in structure from pH 6 to pH 11. The CD spectrum of Cry IIIA at pH 2 indicates that the helical content of the toxin has significantly decreased. The magnitude of the 222-nm signal decreases from pH 7 to 2, with a midpoint of approximately pH 4.5. From neutral pH to weakly alkaline pH, where a great many experimental studies have been carried out, structural changes occur for all three δ-endotoxins studied. Specifically, the conformational changes take place at a pH where each toxin exhibits maximal biological activity.

The δ-endotoxins found in different strains of *Bacillus thuringiensis* (BT) cause mortality in insects by disruption of midgut membranes (Hofte & Whiteley, 1989; Huber & Luthy, 1981). They have received considerable scientific study as well as commercial application as insecticides. Crystals of the δ-endotoxins form inclusions inside cells in which they are expressed (Li et al., 1991). Protoxin crystals ingested by susceptible insects are first dissolved and then processed by proteases in the midgut to form the active toxins. These proteins bind to receptors on the surface of epithelial cells in the midgut and form an ion leakage channel. Each toxin produced is species-specific, and more than one type of protoxin may be produced by a given strain (Hofte & Whiteley, 1989).

Three variants of BT δ-endotoxins, Cry IA(a), Cry IA(c), and Cry IIIA, have been overexpressed in *Escherichia coli* and purified (Ge et al., 1990). The Cry IA protoxins are 135-kDa proteins which are processed to 66-kDa toxins. They show greatest activity against Lepidoptera. The Cry IIIA protoxins are 73-kDa proteins which are processed to 65-kDa toxins, exhibiting their greatest activity against Coleoptera.

Physical studies of the protoxins and toxins have also been carried out (Choma & Kaplan, 1990; Almond & Dean, 1993; Venugopal et al., 1992; Gringorten et al., 1992). Techniques employed by Kaplan and co-workers included UV, Raman, and circular dichroism spectroscopy (CD). Kaplan and co-workers reported that, for Cry IA(a), the toxin was approximately 30% α-helical. In addition, they report that the N-terminal and C-terminal portions of the protein could be separately unfolded with the proper choice of pH, temperature, and denaturant.

Studies of Cry IIIA pH dependence have been reported by Koller and co-workers (Koller et al., 1992). They report that the protoxin and toxin are solubilized by buffers of equivalent ionic strength with pH values lower than 4 and higher than 10. Furthermore, the acid-solubilized toxin not only crystallized better but also was equally active as the base-solubilized proteins. The significance of this observation is that, unlike the basic midguts observed in Lepidoptera, the pH for one such species of Coleoptera is between 4.5 and 5 (Koller et al., 1992).

The crystal structure of Cry IIIA toxin shows that it consists of three domains (Li et al., 1991). The N-terminal domain consists of 224 amino acids in a 7-helix bundle. The bundle consists of a central helix (helix 5) which is surrounded by the six remaining helices. The second domain consists of a series of β-sheets, and is the specific recognition binding site (Ge et al., 1991). The third domain is a β-sandwich, whose function has been hypothesized to contribute to the overall stability of the toxin (Li et al., 1991). It has been suggested that the structure of the Cry IIIA toxin is canonical for all Cry proteins (Li et al., 1991). This study directly addresses that assertion.

Carroll and Ellar determined the binding of δ-endotoxins to brush-border-membrane vesicles (BBMV) from *Manduca sexta* midgut (Carroll & Ellar, 1993). These studies measured the ability Cry IA(c) and Cry IB toxins to alter the permeability of osmotically shocked vesicles. The authors found nonselective permeability induced by Cry IA(c), but observed no effect by Cry IB. It is critical to note that these studies were carried out at pH 7.5 *in vitro*.

Gringorten et al. developed a plate assay with a lawn of suspended cells which permits analysis of δ-endotoxin activity from neutral pH to alkaline pH (Gringorten et al., 1990). The

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* Author to whom correspondence should be addressed. Telephone: 614-292-6383. Fax: 614-292-6773.

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toxins studied were of the Cry IA family. Using this assay, lowering the pH of the gels from alkaline to near neutral (10.5 to 8) significantly decreased the biological activity of the Cry IA toxins, but this decrease in activity is not caused by precipitation or aggregation at the lower values of pH (Gringorten et al., 1992). This calls into question the BBMV studies cited above as well as others carried out below pH 10.

Gringorton et al. suggest that the observed decrease in activity when the pH is lowered from above 10 to 8 is due to the protonation of tyrosine side chains. In the toxin Cry IA-(a), there are 22 tyrosines in the active toxin, and 10 of these reside in the N-terminal portion of the protein. The authors suggest that ionization of the tyrosine side chains at the alkaline pH of the lepidopteran midgut enhances solubility of the toxin as well as renders it active. This is examined in some detail below.

The current study is an extension of preliminary observations examining the physical properties of Cry IA and Cry IIIA proteins by means of CD and size-exclusion HPLC (Becktel et al., 1992). In those studies, we observed the pH dependence of structure, which is not entirely consistent with the data in the literature. In particular, we reported a significant conformational transition with increased helicity on transferring the proteins from pH 7 solutions to pH 10 solutions.

In this study, we examine δ-endotoxin monomer to oligomer interconversion as a function of pH and the type of monomers in solution. The pH dependences of the conformations of Cry IA(a), Cry IA(c), and Cry IIIA protoxins and toxins are compared. The structures of acid- or base-activated toxins are determined.

## EXPERIMENTAL PROCEDURES

*General Materials and Methods.* Water used in these studies was initially obtained from a Barnstead Nanopure II unit. Later, water from a Millipore-Q UV station was used. All salts and buffers were reagent grade or better. Stock solutions of salts and buffers were routinely filtered through a 0.22-μm filter (Gelman Supor 200). pH values of solutions were measured with a Radiometer PMH 84 pH meter and a GK2421C electrode. The size-exclusion column was calibrated with bovine serum albumin (66 kDa), ovalbumin (45 kDa), β-lactoglobulin (37 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). All were obtained from Sigma, and used without further treatment.

*Isolation of Toxins. Bacillus thuringiensis* endotoxin genes were cloned into pKK223-3 vectors. Protoxins of Cry IA(a), IA(c), and IIIA were expressed and isolated as previously described (Ge et al., 1990). Toxins were obtained by trypsin digestion of the protoxins. The protoxins were initially dissolved in carbonate buffers at pH 9.5. For the Cry IA protoxins, 10 mM dithiothreitol was also added to the solution. Trypsin, at a ratio of 1:20 to the concentration of the dissolved protoxin, was added to the solution and digestion allowed to proceed for 2 h at 37 °C. The tryptic fragments and trypsin itself were then removed by ultrafiltration through 30-kDa filters. The purity of the toxin fragments was determined by means of SDS–PAGE gel electrophoresis.

*Size-Exclusion Chromatography.* The protoxins or toxins were subjected to size-exclusion chromatography using a Waters 625 workstation 484 UV detector, and a Waters 300 SW size-exclusion column. The mobile phase consisted of 10 mM sodium phosphate at pH 6.8 and 200 mM KCl. A helium sparge of 10 mL/min and a flow rate of 0.5 mL/min were used. Detection was at 220 nm. The amount of protoxin or toxin injected onto the column varied from 1 to 100 mg. From 50 mL injections of protein solutions, different peaks were collected. This generally consisted of a 1.0–1.5 mL solution of the protein. Data collection and analysis were performed using Water's Baseline 810 software package.

*Circular Dichroism Spectroscopy.* Circular dichroism spectra (CD) were collected with a Spex CD6 spectrophotometer at room temperature (21 °C). A series of 40 scans of each spectrum were concatenated, as were the base-line spectra. Helma or Precision Cell quartz–silica cuvettes with path lengths of 1, 2, or 10 mm were employed. The wavelengths scanned were in the range of 180–350 nm. Protein solutions for studies to 180 nm contained 5 mM buffer, and those of 200 nm contained 50 mM buffer. The spectra at the two, different ionic strengths were compared after converting to molar ellipticity.

## RESULTS

*Size-Exclusion Chromatography of Protoxins and Toxins.* For Cry IA(a) at neutral pH, peaks eluted at 6.15, 10.45, and 15.13 mL. The apparent molecular masses of the fractions were determined by comparing their elution volumes to those of known molecular mass. The first peak elutes at the void volume and exhibits an apparent molecular mass in excess of 220 kDa. The second peak exhibits an apparent molecular mass of 42 kDa, and the third is low molecular mass material. A similar elution profile is seen also for Cry IA(c). Cry IIIA has one peak with a retention volume of 10.66 mL, consistent with an apparent molecular mass of 39 kDa.

The first two peaks of both of the Cry IA proteins could be collected and separately rechromatographed to yield single peaks. At 4 °C, the isolated fractions are stable for several days. With time, however, both the 42-kDa and high molecular mass peaks appear in each sample as equilibrium is established. These observations suggest that the 10.45 mL peak is likely the monomer, while the 6.15 mL high molecular mass peak is an oligomeric form of the toxin, and there is monomer: oligomer interconversion in both Cry IA(a) and Cry IA(c). If the pH of the monomer is adjusted to 11.5, and it is immediately chromatographed, oligomer is seen. The monomer and oligomer interconversion, therefore, is facilitated by high pH and inhibited at neutral pH. This observation is significant in light of the fact that Cry IA proteins are active against insects with alkaline midgut pH. It suggests that the toxins may oligomerize in solution before attaching to the membrane and forming an ion leakage channel.

The possibility of heterologous oligomer formation was explored by combining the three monomer fractions. Cry IA(a) and Cry IA(c) combine to form oligomers. Since Cry IA(a) and Cry IA(c) have almost the same apparent molecular masses, we could not determine whether the oligomers are heterologous. The same experiment was carried out with Cry IA(a) and Cry IIIA with the result that a single peak, at the elution volume of the monomer, is observed. Not only were heterologous oligomers not formed, but also Cry IA(a) did not oligomerize under these conditions.

*CD of the Protoxin and Toxin.* Given the observation of monomer:oligomer interconversion by size-exchange chromatography, it was important to determine whether or not spectral properties of the Cry IA proteins are concentration-dependent. Concentrations of Cry IA(a) ranging from 3 to 70 mg/mL yielded the same CD spectrum, indicating that association/dissociation does not occur at these concentrations over the time course of the experiment. CD spectra of the Cry IA(a) protoxin (○) and toxin (●) are shown in Figure 1. The spectra were obtained at neutral pH, at a concentration

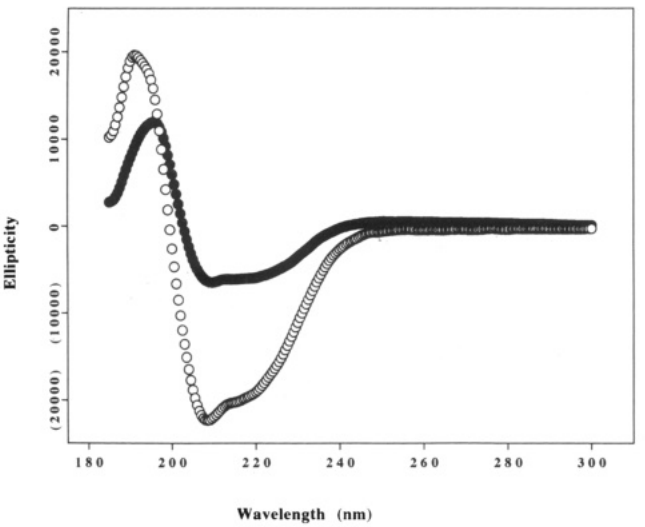



FIGURE 1: CD spectra of Cry IA(a) protoxin and toxin. Spectra were recorded using 1 cm path-length cells with solution conditions as described in the text. Unsmoothed spectra for Cry IA(a) protoxin (open circles) and toxin (closed circles) are shown. Spectra were acquired from 181 to 330 nm, and are base-line-corrected. The raw data have also been converted to molar units (molar ellipticity).

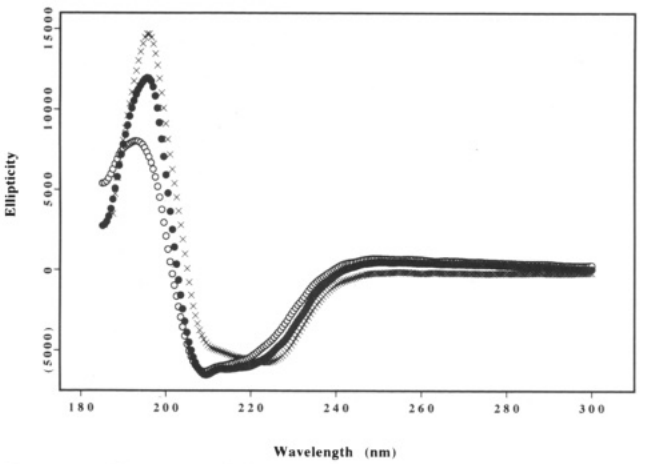



FIGURE 2: CD spectra of Cry IA(a), Cry IA(c), and Cry IIIA toxins. Spectra for Cry IA(a) (●), Cry IA(c) (○), and Cry IIIA (×) are shown. The spectra were acquired under the same conditions as for Figure 4.

of 0.07 mg/mL, at room temperature, and in a 1-mm cell. The toxin extrema are at 195–196, 209–210, and 220 mm. The protoxin peaks, however, are at 191–192, 208–209, and 220 nm.

The protoxin exhibits a significantly more helical spectrum than that of the toxin. This is apparent from both the increased magnitude of the dichroism overall and the fact that the change in sign of the $\pi$–$\pi^*$ transition is at a higher wavelength for the toxin than protoxin. These changes in helicity were observed for both Cry IA(a) and Cry IA(c), and agree with the known processing of Cry IA protoxins to toxins during which helices are lost from the C-terminal portion of the Cry IA protoxins. If we make the common assumption that 100% $\alpha$-helix corresponds to approximately –30 000 deg cm$^2$/dmol at 222 nm and 0% $\alpha$-helix approximately 0 deg cm$^2$/dmol, the amount of $\alpha$-helix in the protoxin is 67% while that of the toxin is about 20%.

*Comparison of Three Types of Cry Proteins.* CD spectra of Cry IA(c) (○), IA(a) (●), and Cry IIIA (×) are shown in Figure 2. The spectra were obtained at neutral pH and at room temperature in a 1-mm cell. Each spectrum is the result of 40 scans. Above 200 nm, spectra of the Cry IA toxins are

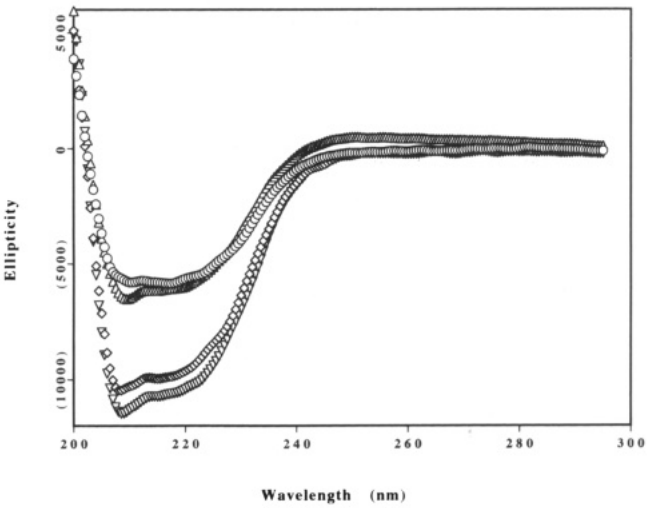



FIGURE 3: Cry IA(a) toxin pH titration curves. Spectra of Cry IA(a) toxin were obtained at pH values of 2 (○), 7.2 (△), 11 (◇), and 12 (▽). Concentrated solutions (10 mg/mL) of the protein were added to buffers at the indicated values of pH, and the resulting solution spectra were obtained. Analysis of the final pH of the resulting solution, using a microelectrode, indicated that deviation from the initial pH was insignificant in each case.

similar to one another, but differ from the Cry IIIA spectrum. Specifically, the Cry IA proteins exhibit a dichroism which becomes more positive from 210 to 225 nm, while that of Cry IIIA becomes more negative. Since Cry IA(a) and Cry IA-(c) have significant sequence homology, particularly in the N-terminal domain, this observation is consistent with a similar structure for these two proteins. Cry IIIA, which is much less homologous to the Cry IA proteins, exhibits a spectrum suggesting that its structure, at least in the first domain, which is a helix bundle (Li et al., 1991), differs somewhat from that of the Cry IA proteins.

*pH Variation of the CD and Structural Implications.* CD spectra of Cry IA(a) at four pH values are shown in Figure 3. The spectra were obtained in the same manner as those in the previous figures with one exception. Aliquots of a concentrated solution of the protein were added to buffered solutions at the different pH values and the spectra immediately determined. After the spectra were taken, the pH of the solution in the cuvette was measured with a microelectrode. In no instance did dilution into the buffer significantly modify the final pH of the solution. In addition, there was no significant difference in the CD spectrum above 220 nm at neutral pH, when compared to the lower ionic strength.

A significant increase in the helical content of Cry IA toxins is observed as the pH is changed from neutral to alkaline values when the protein is biologically active. Making the same approximations about helicity and using the 222 nm band to determine it, the helicity of the Cry IA proteins changes from approximately 15% to approximately 30%, which is comparable to the results of Covents et al. What is somewhat surprising is that there is no significant loss of helicity from neutral to acidic pH. This would seem to discount the possibility that the acidic, N-terminal domain side chain titrations are responsible for the observed change.

There are two possible explanations of the spectral changes with pH. The first is that there is a conformational change of the monomer associated with the change of pH from neutral to basic. This would most likely involve the helical portion of the protein, since that will make the greatest contribution to the CD spectrum. If there is an N-terminal helix bundle in Cry IA(a) as in Cry IIIA, this would be the most likely portion of the protein to have changed.

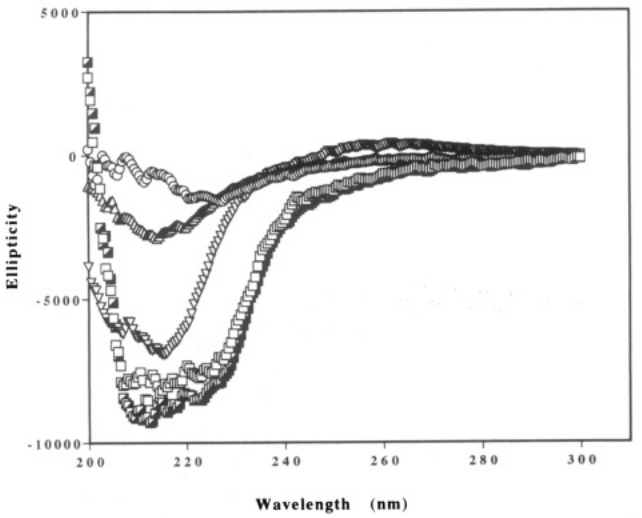



FIGURE 4: pH dependence of Cry IIIA toxin. CD spectra obtained under the conditions cited in Figure 4 at pH 2 (○), 3.4 (△), 4.8 (▽), 7.2 (◪), and 10.4 (□).

The second possibility is that the high-pH form of the toxin which yields the more helical CD spectrum is associated with the oligomeric state of the protein. To test this possibility, samples of the high-pH form of the protein which yields the more helical spectrum were chromatographed. In each instance, the samples were predominantly in the oligomeric form.

The variation of the CD spectrum of Cry IIIA with pH is shown in Figure 4. As this figure indicates, there is no change from pH 6.6 to pH 10.4. If the pH is lowered, however, a conformational transition takes place, and the toxin becomes less helical. Making the same assumptions about helicity, the toxin is approximately 15% helical at neutral and greater pH and is unfolded at acid pH. Cry IIIA toxin apparently undergoes denaturation under the conditions (low ionic strength) employed. In addition, this should be compared to the 30% value reported in the crystal structure, and to the activity profile reported by Koller et al. (1992). As most of the reported helical structure in Cry IIIA is in domain I, it is most likely that the observed conformational change arises from the titration of side chain residues in this portion of the toxin. The discrepancy between the structure, activity, and the current study must lie in the ionic strength of the solution.

## DISCUSSION

The fact that the apparent molecular masses of Cry IA(a), Cry IA(c), and Cry IIIA do not agree with those obtained by means of SDS–polyacrylamide gel electrophoresis (SDS–PAGE) could have several interpretations. One is that the proteins run anomalously on the gel and column. Another is that the proteins suffer some form of degradation either during tryptic digestion or on storage. It was observed that different preparations of each protein eluted with the same retention volumes and peak distributions: therefore, this possibility is excluded. The difference is most likely due to the fact that the proteins are denatured during SDS–PAGE and in their native form during size exclusion. It is known that the shape of the molecule can be a determining factor in the elution volume of native proteins in size-exclusion chromatography.

The conformational changes of Cry IA protoxin and toxin induced by pH have also been investigated by means of CD by Venguopal et al. (1992). *Bacillus thuringiensis* subspecies *kurstaki* endotoxins were used in these studies. The pH varied from 7 to 12. In these experiments, the ionic strength of the solutions was 50 mM, and the purification was attempted by resuspending the protoxin from the crystal form at pH 9.5. It was assumed that the toxins Cry IA(b) and Cry IA(c) did not dissolve.

The authors report Cry IA(a) protoxin exhibits a helical CD spectrum at pH 7, but that the amount of helix decreases above pH 11. The actual values range from 15 to 31%. Within the noise envelopes presented by the authors, the helicity does not actually change from pH 7 to above pH 9 and perhaps pH 10. The spectra of the toxins themselves appear somewhat helical at pH 7 but not at pH 12. In fact, there is a CD band at 235 nm for the pH 12 spectrum which is quite unusual. The toxin is 30–35% helix at neutral pH.

Covents et al. have reported the conformational transitions and domain structure of the δ-endotoxin Cry IA(b) from *Bacillus thuringiensis berliner 1715*, another member of the Cry IA family of proteins (Convents et al., 1990). Solvent denaturation studies by fluorescence and CD were used to examine the protein conformation as a function of environmental factors. The authors present evidence of a two-domain structure of this protein and a folding pathway which depends upon pH and the presence of chaotropic agents. These authors suggest that the C-terminal domain unfolds in acid and 4 M Gdn-HCl while the N-terminal domain unfolds at pH 11/4 M Gdn-HCl. In addition, the authors present CD spectra at pH 8 and 11 which indicate very little conformational change induced by the pH change. From the CD presented, the amount of helix would be estimated to be approximately 20–30%. This is comparable to the amount of helix determined by Venugopal et al. (1992).

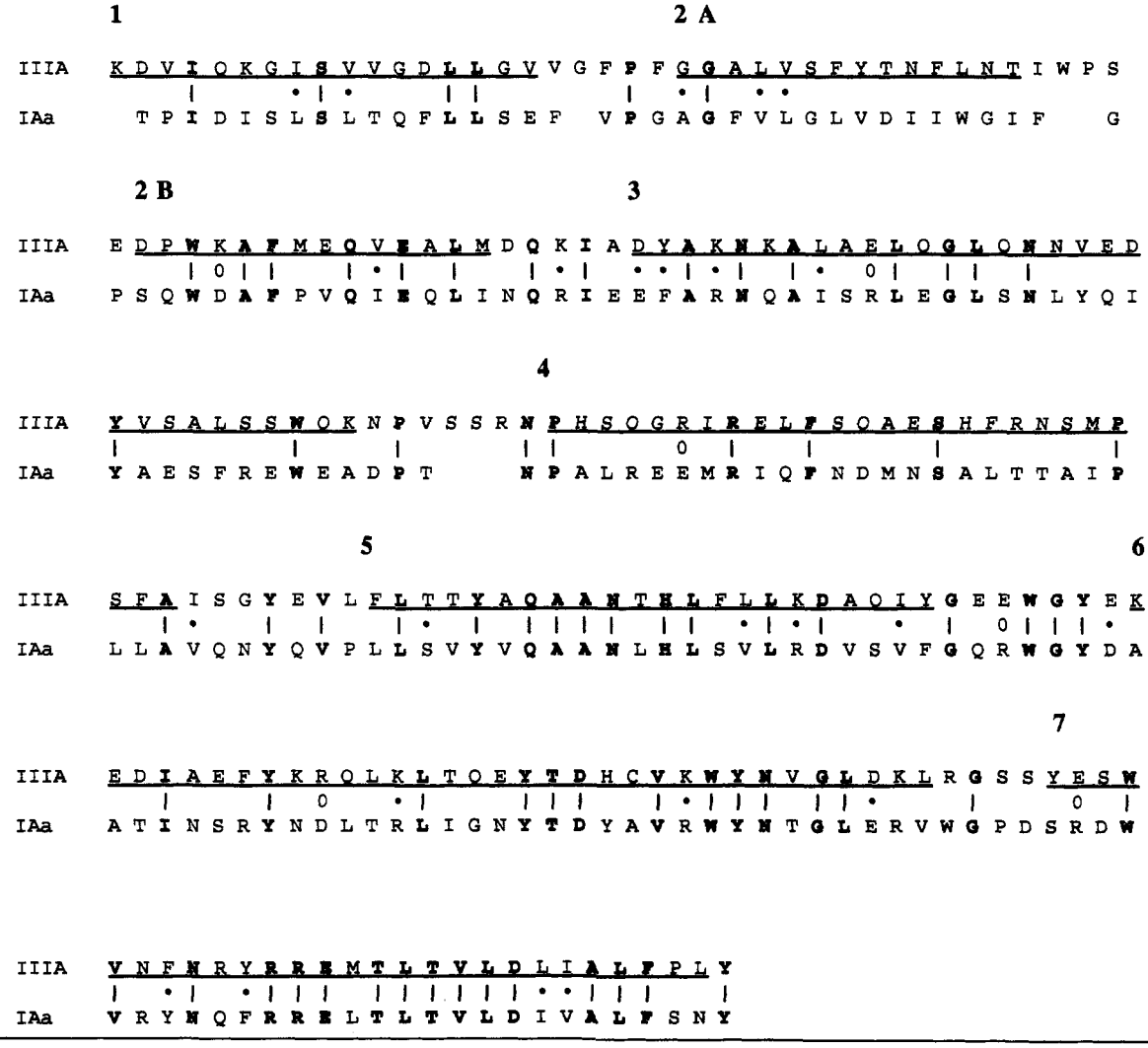
It is not clear from these results why the pH dependence presented in this study differs from that reported in previous reports (Choma & Kaplan, 1990; Venugopal et al., 1992; Convents et al., 1990). The largest differences between all of the studies, however, are found in (1) protein isolation and purification methods and (2) the solution conditions employed. The current study utilizes recombinant proteins expressed in *E. coli*. Some of the previous studies utilized proteins isolated from different strains of *B. thuringiensis*. The current study utilizes monomeric toxin purified to homogeneity by HPLC. Some of the previous studies did not indicate the purity of the samples employed or whether or not more than one Cry IA or Cry II protein was present in the solution. Differences in purification and isolation of the toxins may account for differences in their pH dependence.

Preliminary studies of the pH and ionic strength dependence of the conformation of Cry IA(a) toxin have been carried out to address the possibility that the differences in the reported pH dependence result from differences in solution composition. CD spectra were recorded at neutral and basic pH, and at 50 and 200 mM KCl for Cry IA(a) toxin. The spectra exhibit both pH and ionic strength dependence. Whether or not the changes are as great as the differences between the current study and those in the literature requires further investigation.

The CD spectra presented indicate that Cry IA and Cry IIIA toxins have somewhat different conformations. This suggests that the crystal structure of Cry IIIA is not canonical. While there are similarities, structural differences are also present.

Association of monomer to oligomer is another possible explanation of the observed CD change of the Cry IA toxins. HPLC studies reveal that basic pH facilitates formation of oligomer. From the combined CD/HPLC studies, it is apparent that the conformational change and oligomerization are linked. The increase in apparent helical dichroism could

Table 1: Domain I in Cry IIIA and Cry IAa




be attributed to helix bundle association. This is because, at the interface, portions of the helices will be removed from solvent and the more hydrophobic environment could result in a stronger signal. Another possibility is that the oligomers are of sufficient size to increase the dichroism by an artifact known as "form dichroism". This latter possibility is ruled out, however, as light scattering was not observed in these solutions.

The aligned sequences of the N-terminal domains of Cry IIIA and Cry IA(a) appear in Table 1. The alignment is patterned after that of Hodgman and Ellar (1990), with some modification. In the table, the portions of this domain which are $\alpha$-helical in Cry IIIA are underlined, and the number of each helix is placed in a line above the N-terminus. The extent of matching was scored in the following manner: (1) exact matches are given as (|); (2) similar residues are given as (•); and (3) inversion of side chain charge is given as (0). There are a total of 224 residues of Cry IIIA aligned as compared to 218 residues in Cry IAa. Of the 218 residues for the latter, 76 (or 34%) are exactly the same. If we include the "similar" residues, the number of matches increases to 100, or 45%. This degree of similarity, along with the observation that Cry IA(a) contains a significant amount of $\alpha$-helix, suggests domain I of Cry IA shares some structural motifs with Cry IIIA, but that it may exhibit some differences as noted in the discussion of the CD spectra.

There are 4 histidines and 14 glutamic acid residues in domain I of Cry IIIA. At pH 9.5, the histidine residues will be deprotonated, and the glutamic acid residues will be charged. At pH 4–5, however, the His residues will be charged, and some of the Glu residues will have become protonated. A potential 18 protons could conceivably titrate over this range of pH. Above pH 9.5, titration of 13 Tyr and 13 Lys residues in domain I of Cry IIIA must be considered. A negative change in charge of 44 protons could be expected in transferring from acid to base in this domain alone.

Cry IA(a) contains only one His residue, which is conserved from the Cry IIIA sequence in the alignment given in Table 1. The other sites in Cry IIIA in the positions of His are Ala, Ala, and Tyr in Cry IA(a). The first two putative replacements could be neutral in their effects, but the Tyr replacement likely is not. The Cry IA(a) toxin has 12 Glu residues, of which 2 are conserved from Cry IIIA, 2 are "similar", and 7 involve charge inversions to Gln, Lys, or Arg. There is less of a change in charge on going from neutral to acid pH in Cry IA(a) than in Cry IIIA. This is supported by the CD results in which there are very minor differences between the spectra at pH 7 and at pH 2.

Domain I of Cry IA(a) has no lysine residues. Instead it has 16 Arg residues, as compared to 9 for Cry IIIA. This implies that domain I of Cry IA(a) will be less negatively charged at alkaline pH than Cry IIIA. In the aligned sequence,

six Arg residues are at sites occupied by Lys in Cry IIIA. These are in helices 5 and 6 and in one of the loop regions. Since helix 5 is the amphipathic, conserved helix implicated in pore production, this may be a critical factor in pore formation, specifically to maintain a positive charge at this site, a pH below 10 would be necessary. As this toxin is active at acid pH, obviously this criterion is met. If one examines an extended alignment of this helix, the vast majority of residues at this site are Arg residues (Hodgman & Ellar, 1990). No such strong preference is seen at the other Lys replacement sites in helix 6, and one possibility is that lysine-149 in Cry IIIA toxin is directly involved in a pH-induced onset of activity.

The solubility of Cry IIIA in solutions of equal ionic strength has been determined from pH 2 through 11 (Koller et al., 1992). It was observed that the protoxin and toxin are soluble below pH 4 and above pH 10. In addition, the proteolytic activity of midgut juice from Coleoptera was higher at pH 4 and also at pH 8, although the pH 4 activity is 5 times greater than that at pH 8. Measurement of the pH of the midgut of *C. scripta* indicated that the pH varies from 6 to 4, depending upon the position in the midgut. The toxicity of the acidic and basic generated toxins was virtually identical.

What this implies is that the biologically active form of the toxin is near pH 4. Studies of the activity, stability, and structure of the Cry IIIA which are not made under acidic conditions may concern a form of the enzyme which is either not biologically active or less active. In particular, the structure of Cry IIIA which has been presented was from a solution with pH greater than 9 (Li et al., 1991). It is highly possible that this structure differs from that of the biologically active form. The His residues in the protein and some of the Glu residues are in the incorrectly charged form in the published structure.

The structure of Cry IIIA at pH 9.5 may also differ from that of the biologically active forms of toxins at pH above 10. It is known that the Cry IA toxins are inactivated by reducing the pH below 10. The CD spectra in the current study clearly indicate a conformational transition takes place with this shift in pH. It follows that the structure of the toxin at pH 9 is not the biologically active form.

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