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Unfolding affects insect cell permeabilization by *Bacillus thuringiensis* Cry1C toxin

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Abstract

Bacillus thuringiensis Cry toxins are efficient, environment-friendly biological insecticides. Their molecular mode of action on target insect cells remains largely unknown. The aim of this study was to investigate the relation between the conformational state of the Cry1C toxin and its ionophoric activity on live Sf9 cells of *Spodoptera frugiperda*, a target insect for this protein. Potassium ion movement induced by Cry1C across the cell membrane was measured with a fluorescent assay developed previously and the conformation of the toxin was studied using tryptophan spectroscopy. Following treatment with 4 M guanidinium hydrochloride, which resulted in the unfolding of its N-terminal half, the toxin retained its full capacity to permeabilize the cells while the fully unfolded toxin did not induce potassium leakage. Therefore, permeabilization of Sf9 cells by Cry1C requires the integrity of the C-terminal half of the toxin and may depend on an initial unfolding step provided by the acidic environment of the cells.

Keywords: Bacterial toxin; K^+ efflux; Protein spectroscopy; Ion fluorescent probe; Protein folding; Sf9 cultured cell

1. Introduction

Bacillus thuringiensis (*Bt*), a Gram-positive soil bacterium, produces large parasporal proteic inclusions containing one or more protoxins in crystalline form (the Cry proteins). *Bt* products are of major economic importance as environment-friendly control agents against a variety of insect pests. The proposed killing mechanism involves the following steps: solubilization of the protoxin in the midgut environment which, in the case of lepidopteran insect larvae, is highly alkaline, proteolytic activation by gut proteases, toxin binding to receptors located at the apical membrane of midgut epithelial columnar cells and formation of pores responsible for intracellular ionic balance disruption, collapse of the cell membrane potential and osmotic cell lysis (reviewed in [1]).

The three-dimensional atomic structures of two members of the Cry toxin family (i.e. Cry3A and Cry1Aa) show that in solution they are organized in three structural domains [2,3]. The N-terminal domain is an eight α-helix bundle that is thought to be responsible for pore activity [4–6] while the central and C-terminal domains are involved in toxin–recep-

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tor interaction and structural stability [7–9]. It has been proposed that interaction between the different domains modulate pore activity [10,11].

Aminopeptidases N (120 kDa) and cadherin-like proteins (210 kDa) from target insect midgut epithelial cells are recognized as specific receptors by Cry1A and Cry1C toxins ([1] for a review), [12–18]. In Sf9 cells (*Spodoptera frugiperda*, Lepidoptera), which are susceptible to *Bt* toxins but are of ovarian origin, a 40 kDa protein was reported to bind specifically Cry1C [19]. Cry toxins spontaneously insert into phospholipid membranes [20–23] and form ion-selective channels in artificial lipid bilayers [24–28]. Channel formation is strongly promoted by the presence of Cry toxin receptors in the membrane [29–32]. Ion channels are likely to be responsible for the increase in membrane permeability and for cell membrane depolarization that are observed in insect cell lines and midgut preparations (reviewed in [6]) [33].

Despite the wealth of data accumulated on Cry toxin structure and biological activity [1], the molecular mechanism responsible for toxin insertion into the plasma membrane of insect cells is not well understood. It has been shown that full length Cry toxins undergo conformational changes upon insertion into planar lipid bilayers [34–36] or phospholipid vesicles [22,23,37]. Furthermore, several studies with α-helical peptides mimicking parts of Cry toxin domain 1, alone or in combination, support the concept of a major tertiary structure reorganization upon insertion into phospholipid membranes [38–43]. Such changes, however, have not been directly demonstrated in living cells. In the present study, the relation between Cry1C unfolding and cell permeabilization was explored. We took advantage of the results of several studies in which the structural effects of chaotropic agents on Cry1Ab, Cry1Ac, Cry1C and Cry3A toxins in solution have been investigated [37,44–47]. In particular, it has been demonstrated that Cry1C treated with increasing concentrations of guanidinium hydrochloride (GuHCl) underwent a two-step unfolding process from the N- to the C-terminus [46]. Using a potassium ion efflux fluorescent assay [33,48], we show that the ionophoric activity of Cry1C on Sf9 cells is unaffected by N-terminal unfolding of the toxin by 4 M GuHCl, indicating that unfolding of the N-terminus of the molecule does not affect cell permeabilization while unfolding of its C-terminal half does alter the permeabilization process. Furthermore, our data suggest that acidification of the toxin environment leads to structural changes that may facilitate its insertion of the toxin in the cell membrane of target insects.

2. Materials and methods

2.1. Chemicals

Grace's insect cell culture medium and Pluronic F-68 were obtained from Gibco BRL (Burlington, ON, Canada) and fetal bovine serum from Hyclone (Logan, UT, USA). CD-222 (coumarin diacid cryptand [2′.2′.2′]) was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were of analytical grade.

2.2. Cell cultures

Sf9 cells (ATCC CRL 1711) were grown as described previously [48]. They were harvested during mid-logarithmic phase by centrifugation ($180\times g$, 20 min at room temperature). Pellets were resuspended in G*Ko, a simplified Grace's medium [49] from which KCl was omitted. It contained (in mM): NaCl 21, $MgCl_2$ 14, $MgSO_4$ 11, $CaCl_2$ 6.8, PIPES/Tris 10, pH 6.3. The osmolarity of the medium was adjusted to 380 mosm kg^{-1} H_2O with sucrose. Under these conditions, cell viability was 96% [48]. Cells were used within 3 h after harvesting.

2.3. Cry1C purification and treatment

Cry1C (*Bt* subsp. *aizawai*) was produced, trypsin-activated and purified as described previously [50] except that the purified toxin was dialyzed overnight at 4°C against 50 mM Na_2CO_3, pH 11 before freezing in liquid nitrogen and storage at −80°C (for a month or less). For unfolding experiments, 77 nM Cry1C was incubated overnight at 4°C in 50 mM Na_2CO_3, pH 11 with various concentrations of GuHCl. Sham-treated toxin was prepared the same way but without GuHCl. For refolding experiments, 7.7–15.4 μM Cry1C was pretreated overnight at 4°C with 4 M GuHCl in 50 mM Na_2CO_3, pH 11. It was then diluted to a final toxin concentration of 77 nM

either in 50 mM Na_2CO_3, pH 11 or in G*Ko, pH 6.3. Under these conditions, the maximal residual concentration of GuHCl was 40 mM.

2.4. Fluorescence measurements

GuHCl-induced Cry1C toxin unfolding and GuHCl-pretreated toxin refolding were monitored by tryptophan emission fluorescence as described by Choma and Kaplan [44] and Convents et al. [45,46]. The activated Cry1C protein possesses 10 tryptophan residues which are well distributed between the two halves of the toxin: four are located in domain 1, four in domain 2 and two in domain 3. Spectra were recorded using a Spex-Fluorolog CM-3 spectrofluorimeter (Jobin Yvon Horiba, Edison, NJ, USA) equipped with a 3 ml, 1 cm optical path quartz cuvette. The temperature was regulated at 27°C. A 295 nm excitation wavelength was selected [44] for recording emission spectra (scanning rate = 1 nm s^{-1}) between 320 and 400 nm. Slits were adjusted to achieve 5 nm bandwidth.

Extracellular potassium concentration changes ($[K^+]_{out}$) were measured as described previously [33,48]. Briefly, 2 ml of Sf9 cells were incubated at 27°C with 5 μM CD-222 in G*Ko medium under continuous stirring. Sham-treated or GuHCl-pretreated Cry1C toxin was directly added to the cuvette. $[K^+]_{out}$ (expressed as μmol mg^{-1} protein) was deduced from calibrated CD-222 fluorescence changes ($\lambda_{exc} = 360$ nm, $\lambda_{em} = 470$ nm, 5 nm bandwidth, 1 Hz acquisition frequency), considering that 10^6 cells corresponded to 0.16 mg protein [48].

2.5. Data analysis

Data analysis was performed on a personal computer using SigmaPlot 5.0 software (Jandel Scientific, San Rafael, CA, USA). The rate of K^+ efflux (VK_I^+) and the delay to its onset (t_{10},), defined as the time between toxin addition and 10% of maximal K^+ efflux, were determined from 5th order polynomial fitted potassium efflux curves. The kinetic parameters of the efflux were deduced from VK_I^+ vs. toxin concentration plots fitted with a Hill-like equation [33,48]. Statistical significance was determined using two-way ANOVA, followed by a Bonferroni test ($P < 0.05$).

3. Results

3.1. Effect of Cry1C unfolding on its ionophoric activity in Sf9 cells

To determine the experimental conditions to be used for testing the effect of unfolded Cry1C toxin on Sf9 cells, it was verified that the protein exposed to the chaotropic agent GuHCl followed the same unfolding pattern in solution as that reported by Convents et al. [46]. Increasing doses of GuHCl at pH 11 induced a two-step unfolding of Cry1C, with a stable intermediate between 3 M and 4 M GuHCl and a fully unfolded protein above 6 M of GuHCl (data not shown).

The onset of the K^+ efflux induced by 25 nM sham-treated Cry1C, i.e. preincubated under the same conditions but without GuHCl, was preceded by a delay (t_{10}) of about 105 s and the maximum rate of change of the efflux (VK_I^+) was 1.1 μmol min^{-1} mg^{-1} protein (Fig. 1, trace 1), in accordance to previously published data [33]. When Sf9 cells were exposed to 25 nM of toxin that had been pretreated with 7 M GuHCl, no K^+ efflux was observed (Fig. 1,

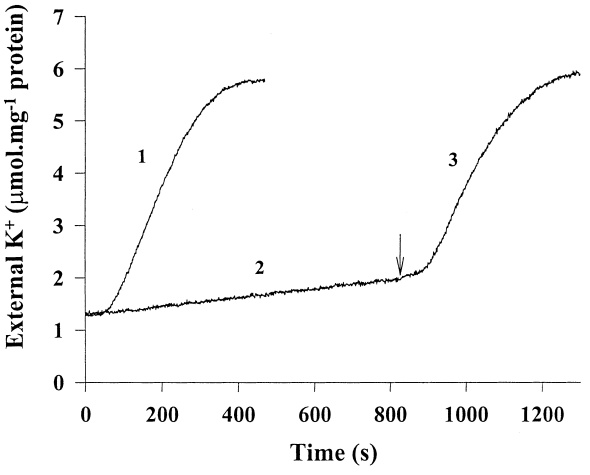

Fig. 1. Effect of Cry1C unfolding on toxin-induced K^+ efflux from Sf9 cells. Sf9 cells were incubated in G*Ko with 5 μM CD-222 and external K^+ concentration changes were deduced from CD-222 fluorescence changes as described in Section 2. At $t = 0$, the cells were incubated with 25 nM Cry1C toxin, sham-treated (trace 1) or pretreated with 7 M GuHCl (trace 2). Sham-treated toxin (25 nM) was then added to the cells in the spectrometer cuvette at the time indicated by the arrow (trace 3). Each trace is representative of three independent experiments.

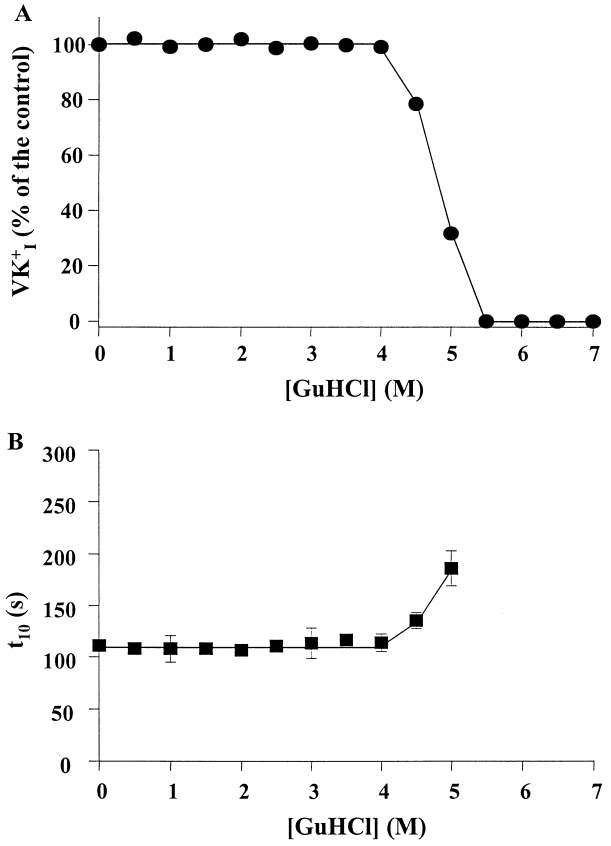

Fig. 2. Effect of GuHCl pretreatment dose on the kinetic parameters of Cry1C-induced K^+ efflux. Sf9 cells were incubated as indicated in Fig. 1 and treated with 25 nM Cry1C toxin preincubated overnight in Na_2CO_3, pH 11 with various GuHCl concentrations. The relative change of VK^+_I compared to that induced by sham-treated Cry1C toxin (A) and the delay t_{10} to the onset of the K^+ efflux (B) were determined as described in Section 2. Data points are means ± S.E.M. from three independent experiments. Solid lines represent visual fits to the data points.

trace 2). Subsequent addition of 25 nM of sham-treated Cry1C induced a K^+ efflux that was similar to that observed with sham-treated Cry1C alone (Fig. 1, trace 3). It was verified that the absence of Cry1C ionophoric activity observed with 7 M GuHCl-pretreated toxin was not due to an inhibitory effect of the chaotropic agent itself on Sf9 K^+ efflux, since addition to the cells of 45.5 mM GuHCl (the residual concentration of GuHCl after dilution of toxin pretreated at the highest GuHCl concentration used in this study) did not affect the K^+ efflux induced by subsequent exposure of the cells to 25 nM sham-treated Cry1C (data not shown).

3.2. Effect of GuHCl on the kinetic properties of Cry1C-induced K^+ efflux

VK^+_I and t_{10} were determined for Sf9 cells exposed to 25 nM Cry1C pretreated at pH 11 with various concentrations of GuHCl (Fig. 2). Up to 4 M GuHCl, neither VK^+_I (Fig. 2A) nor t_{10} (Fig. 2B) were affected, compared to sham-treated Cry1C. However, at concentrations of GuHCl between 4 and 5 M, a sharp reduction of VK^+_I and a rapid increase of t_{10} were observed. Above 5 M GuHCl

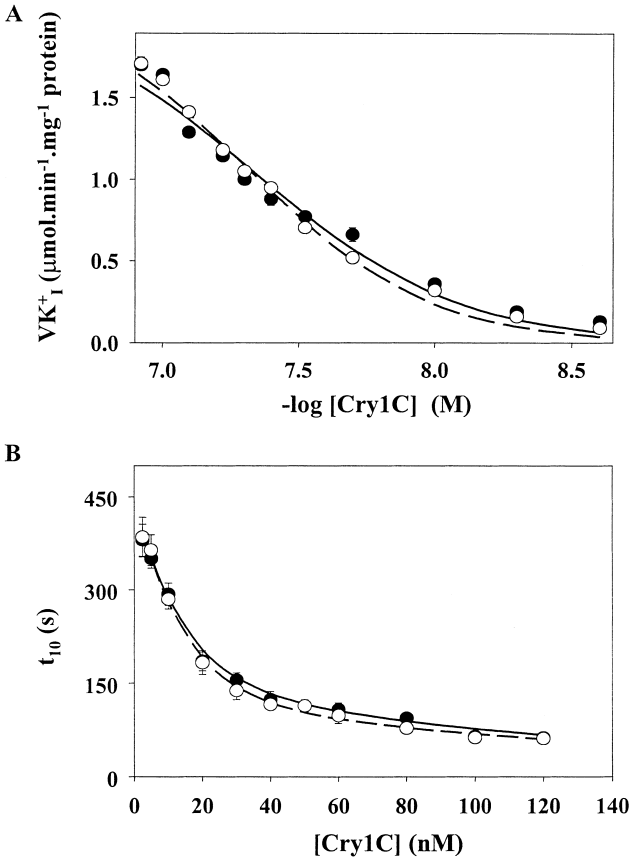

Fig. 3. Dose–effect of 4 M GuHCl-pretreated Cry1C toxin on K^+ efflux from Sf9 cells. Sf9 cells were incubated as described in Fig. 1 and treated with various concentrations of Cry1C preincubated in Na_2CO_3, pH 11 with 4 M GuHCl (○) or sham-treated (●). VK^+_I (A) and t_{10}, the delay to the onset of the K^+ efflux (B), were determined for each concentration of GuHCl-pretreated or sham-treated Cry1C. Data points are means ± S.E.M. of three independent experiments. The Hill-like equation [48] fits of VK^+_I (A) and the double exponential fits of t_{10} (B) are represented by the solid and dashed lines corresponding to the effect on K^+ efflux of sham-treated and GuHCl-pretreated Cry1C, respectively.

concentrations, no K^+ efflux could be recorded anymore.

The toxin dose dependence of the K^+ efflux kinetic properties was determined for 4 M GuHCl-pretreated Cry1C (Fig. 3). Both VK_I^+ (Fig. 3A) and t_{10} (Fig. 3B) plots were similar to those obtained with sham-treated toxin. None of the parameters derived from a Hill-like analysis of VK_I^+ vs. Cry1C concentration relations [33], i.e. VK_{max}^+, EC_{50} or the Hill number, were significantly affected by Cry1C pretreatment with the denaturing agent (Table 1). Similarly, χ_1 and χ_2, the two constants derived from a double exponential fit of the t_{10} plots, did not significantly differ, whether the toxin had been pretreated with 4 M GuHCl or not (Table 1).

3.3. Cry1C unfolding and refolding

The results of the above experiments conducted on Sf9 cells showed that at pH 6.3 both sham-treated and 4 M GuHCl-pretreated Cry1C toxins had the same permeabilizing effect on the cells. This suggested that the toxin may have been in the same conformational state under these conditions, and particularly that the sham-treated toxin may have been unfolded. To check this hypothesis, the following refolding experiments were performed.

The kinetics of Cry1C refolding following GuHCl treatment was determined from the measurement, at different times, of the emission spectra of 4 M GuHCl-pretreated toxin following toxin dilution in either 50 mM Na_2CO_3, pH 11 or G*Ko, pH 6.3 to a final concentration of 77 nM (Fig. 4). The fluorescence ratio F^{335}/F^{348} of GuHCl-pretreated Cry1C increased with time after dilution in 50 mM Na_2CO_3, pH 11, a clear indication of toxin refolding (Fig. 4, open circles) and reached a steady-state value close to that of the sham-treated toxin (Fig. 4, filled circles). A time constant of 95 s for the renaturation process was derived assuming single exponential refolding kinetics. These data are consistent with the results of a previous study showing that rapid dilution of unfolded Cry1Ac toxin in denaturant-free buffer at pH 9.0 resulted in the full return of the protein to its native conformation [44].

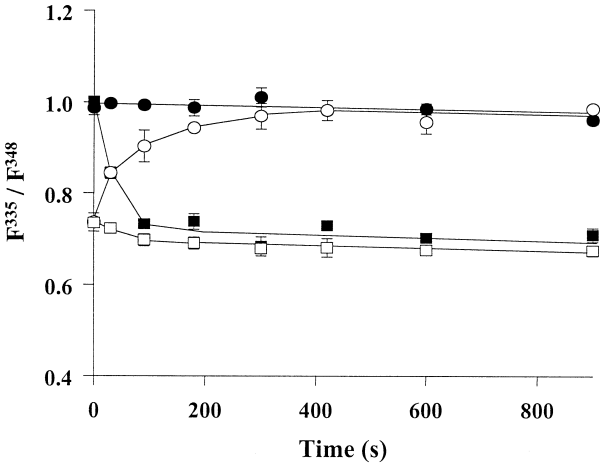

Fig. 4. Time course of Cry1C refolding. Cry1C toxin was incubated overnight in 50 mM Na_2CO_3, pH 11 with 4 M GuHCl (○,□) or without the denaturating agent (sham treatment) (●,■). The toxin solution was diluted to a final concentration of 77 nM in 50 mM Na_2CO_3, pH 11 (○,●) or G*Ko, pH 6.3 (□,■). Emission spectra were recorded at various times following toxin dilution. Corresponding F^{335}/F^{348} ratios were plotted as a function of time. Data points are means ± S.E.M. from three independent experiments. Solid lines represent visual fits to the data points.

No variation of fluorescence could be detected in the 15 min following sham-treated toxin dilution in either 50 mM Na_2CO_3, pH 11 (Fig. 4, filled circles) or G*Ko, pH 11 (data not shown). In contrast, the behavior of both 4 M GuHCl-pretreated and sham-treated Cry1C was affected by dilution into G*Ko, pH 6.3. A small decrease of F^{335}/F^{348} was observed with the toxin pretreated with the chaotropic agent

Table 1
Kinetic parameters of the K^+ efflux from Sf9 cells exposed to 4 M GuHCl-pretreated or sham-treated Cry1C toxin

	GuHCl-pretreated Cry1C	Sham-treated Cry1C
VK_{max}^+ (μmol $min^{-1} \times (mg\ protein)^{-1}$)	2.08	2.13
EC_{50} (nM)	34.3	33.8
Hill number	1.082	1.078
χ_1^a (nM)	3.74	3.05
χ_2^a (nM)	97	98

[a] χ_1 and χ_2 are the constants derived from the analysis of t_{10} plots fitted by a double exponential function (Fig. 3).

(Fig. 4, open squares). Unexpectedly, a large reduction of the fluorescence ratio took place with the sham-treated toxin (Fig. 4, filled squares), reaching a steady-state value close to that obtained for the 4 M GuHCl-pretreated toxin. Therefore, lowering the pH of the environment from a highly alkaline level to a slightly acidic level promoted a significant unfolding of sham-treated Cry1C and induced further unfolding of the toxin that had been denatured overnight.

4. Discussion

The purpose of this study was to explore the relation between the conformational state of a *Bt* toxin and its capacity to permeabilize live cells of a target insect. While the mechanism responsible for pore formation has been extensively studied in model membranes (reviewed in [6]), showing that major conformational changes are required, including interdomain movements [34] and unfolding of the pore-forming domain [34–36,42], very few data are available on the permeabilizing effect of Cry toxins on live cells [6,51] and most of the information was obtained with insect cell lines [11,28,33,49,52–54]. Cry1C is a *Bt* toxin which specifically kills a variety of lepidopteran insects, particularly from the *Spodoptera* family (from: K. van Frankenhuyzen and C. Nystrom (1999) The *Bacillus thuringiensis* toxin specificity database, http://www.glfc.forestry.ca/Bacillus/Bt_HomePage/netintro99.htm), and is also specifically cytolytic to Sf9 cells, an ovarian cell line derived from *S. frugiperda* larvae [25]. In the present work, experiments were conducted with a K^+ efflux fluorescent assay developed previously [33,48] for investigating the permeabilization of live insect cells by pore-forming toxins. Moreover, considering the peculiar environment in which Sf9 cells are grown compared to that of the larval midgut, the effect of pH on the conformational state of the protein in solution was also investigated.

Our data clearly demonstrate that 4 M GuHCl-pretreated Cry1C activated toxin remained as active against Sf9 cells as the sham-treated protein. The behavior of Cry1C and other Cry toxins in solution and in the presence of chaotropic agents has been investigated previously [37,44–47]. Unfolding of Cry1C toxins by GuHCl displayed two phases: at low to middle concentration of the agent (1–4 M), a stable unfolded intermediate was observed [45,46] in which the N-terminal half of the activated toxin was unfolded to the extent where its tertiary structure was lost. Further unfolding of the protein took place when it was exposed to higher concentrations of GuHCl, eventually leading to a random coil state. Our data show that partial unfolding of Cry1C pretreated with moderate concentration of GuHCl (up to 4 M) did not affect the structural machinery needed for pore formation in Sf9 cells. Therefore, under our conditions, the critical structural components required for binding the toxin to the receptors of the cells, inserting it into their membranes and assembling it into functional transmembrane pores were conserved.

The three-dimensional atomic structure of Cry1C is unknown. In its activated form, Cry1C is a 618 residue protein which shares 48% identity with Cry1Aa, another lepidopteran toxin, and 36% identity with Cry3A, a coleopteran toxin, whose 3-D structures have been elucidated by X-ray crystallography [2,3]. Therefore the Cry1C tertiary structure should be very similar to that of Cry1Aa, which itself does not differ much from that of Cry3A [3]. In particular, amino acid identity between domains 1, domains 2 and domains 3 of activated Cry1C and Cry1Aa is 55%, 49% and 46%, respectively, and the N-terminal halves of the two toxins are 56% identical (R. Brousseau, personal communication). The N-terminal half of the toxin encompasses domain 1, which is mainly α-helical and therefore provides the crucial pore-forming structural components, and a small stretch of domain 2, including loop 1 which has been shown to be one of the three loops of domain 2 involved in receptor binding [1]. In view of the high level of homology between Cry1C and Cry1Aa in this region, it is expected that, similar to what is supposed to take place in lipid membranes, (i) pore formation requires a significant conformational change involving the displacement of domain 1 vs. domains 2 and 3 around the hinge loop between domains 1 and 2 [34], (ii) pore formation depends on the insertion of the hairpin formed by α-helix 4, α-helix 5 and the loop that links them [34,36], and (iii) the pore may be lined by a set of α_4-helices possibly arranged in a tetrameric fashion [35,36,55].

If this holds, our data imply that the partial unfolding of Cry1C by 4 M GuHCl provided the proper domain 1–domain 2 conformational change, the correct positioning of the α_4–α_5 hairpin in the vicinity of the membrane and the appropriate oligomerization capacity of the protein to form a pore functionally similar to that of the sham-treated toxin. Obviously, because the kinetic components of the K^+ efflux from Sf9 cells remained identical to those measured with the sham-treated toxin, the level of unfolding achieved by 4 M GuHCl treatment did not result in better membrane permeabilization, i.e. it did not improve toxin binding or pore formation. On the other hand, integrity of the tertiary structure of the C-terminal half of the toxin appeared to be required for cell permeabilization, as Cry1C pretreated with higher doses of GuHCl was unable to permeabilize the cells, suggesting that the binding regions located in the C-terminal half of the protein, like loops 2 and 3 of domain 2 and other regions of domain 3 that may be critical to binding [1], had to remain properly folded. Similarly, permeabilization of the cells required the appropriate folding of the domain 2 and 3 regions which may be involved in pore formation and/or function [10,11].

When the toxin was pretreated with 7 M GuHCl, it lost its ability to permeabilize Sf9 cells. Considering that dilution of 4 M GuHCl-pretreated toxin in G*Ko at pH 6.3 resulted in further unfolding of the protein (Fig. 4, empty squares), it was assumed that the 7 M GuHCl-pretreated toxin did not refold in G*Ko, pH 6.3 and therefore that the cells were exposed to a fully unfolded toxin. Under these conditions, lack of permeabilization could have resulted from the inability of the protein to insert and assemble into a functional pore, which could be related to deficient domain 1 helical components and/or the ability to assemble them into a functional pore. Alternatively, the unfolding of domain 2 loops could have resulted in the lack of binding of the toxin to its receptor. Our data support the latter because normal Sf9 permeabilization by sham-treated toxin was achieved following exposure of the cells to 7 M GuHCl-pretreated toxin, suggesting that the fully unfolded toxin did not bind to, or was easily dislodged from the receptors at the cell surface.

Spectroscopic examination of the Cry1C toxin in solution, under different pH and GuHCl treatment conditions, showed that acidification of the medium from pH 11 to pH 6.3 resulted in the same fluorescence change for the sham-treated toxin as that achieved by 4 M GuHCl. This suggests that in Sf9 experiments which were conducted at pH 6.3, the optimum pH for culturing these cells [57], the sham-treated toxin underwent partial unfolding. It explains why both 4 M GuHCl-pretreated toxin and sham-treated toxin induced the same K^+ efflux from Sf9 cells (Fig. 3). Therefore the results of previous work conducted on cell lines under these slightly acidic conditions may better reflect the effects of a partially unfolded, though fully active, Cry1C protein than those of the native toxin [11,33,49,52–54]. Interestingly, the acidic environment of the cells may in fact promote their permeabilization by Cry1C, as indicated by the fact that the delay preceding the onset of the Cry1C-induced K^+ efflux from Sf9 cells depended on extracellular pH with a 14 s/pH unit increase when the pH was raised above 7 [33]. A similar pH dependence on Cry1C pore formation ability was observed in brush border membrane vesicles made from *Manduca sexta* midgut epithelial cells [56]. It is also consistent with the results of a study showing that negatively charged liposomes were better permeabilized by Cry1C at low pH [22]. In contrast, pore formation by Cry1C was not pH-dependent in neutral lipid bilayers [25].

It is tempting to propose that partial unfolding of the activated Cry1C toxin, equivalent to that achieved by 4 M GuHCl, took place at the immediate vicinity of Sf9 cells. This would represent the initial step of toxin partitioning and pore formation into the plasma membrane of the cells. While this appears to be caused by acidification under the particular experimental conditions required for Sf9 cells, the exact role of receptors, membrane composition and other environmental factors in promoting pore formation by *Bt* Cry toxins remains to be elucidated, especially in midgut epithelial cells.

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