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Membrane interactions and surface hydrophobicity of *Bacillus thuringiensis* δ-endotoxin CryIC**

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Abstract

The interaction between *Bacillus thuringiensis* insecticidal δ-endotoxin CryIC and phospholipid vesicles was studied by fluorescence spectroscopy. The toxin dissipates the diffusion potential across vesicular membranes, presumably by creating ion permeable channels or pores. This effect is pH-dependent and strongly increases under acidic conditions. The enhanced membrane-perturbing activity of CryIC at low pH correlates with the increased surface hydrophobicity of the toxin molecule. The membrane permeabilizing effect of the toxin is further increased by the presence of acidic phospholipids. These findings are discussed in relation to the mode of insecticidal action of the toxin.

Key words: δ-Endotoxin; Fluorescence probes; Membrane permeabilization; Surface hydrophobicity; *Bacillus thuringiensis*

1. Introduction

The δ-endotoxins are a large family of proteins occurring in crystalline form in spores of *Bacillus thuringiensis* [1]. These toxins are of considerable economic importance as environmentally safe insecticides. The mode of action of δ-endotoxins appears to be a complex, multicomponent process. Upon ingestion by the insect, the proteins are proteolytically activated and bind to high-affinity receptors on the midgut epithelium [2–4]. The membrane-binding event is followed by swelling and lysis of epithelial cells, resulting in eventual larval death. While the details of this lytic mechanism are not yet established, it has been postulated that the toxin acts by generating membrane pores [2,3,5]. The notion of pore-forming ability of δ-endotoxins has been recently supported by the observations that at least some proteins belonging to the *B. thuringiensis* family interact with membrane phospholipids [6,7] and can form ion-selective channels in receptor-free planar lipid bilayers [8,9]. However, neither the details of this interaction, nor the actual mechanism of the postulated toxin insertion into the membrane and assembly into functional channels are understood. It remains also unclear whether the channel-forming ability constitutes a general property of the whole family of *B. thuringiensis* endotoxins.

In this communication we describe the interaction of Lepidoptera-specific toxin CryIC with the membranes of unilamellar phospholipid vesicles. We demonstrate that the protein dissipates transmembrane potential by inducing a flux of Cl^- and/or Na^+ ions. This membrane-perturbing activity of the toxin is pH-dependent and correlates with the surface hydrophobicity of the protein molecule.

2. Materials and methods

2.1. Materials

L-α-phosphatidylcholine (PC), type V-E, dicetylphosphate, valinomycin and the buffer salts were obtained from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG) was from Avanti Polar Lipids (Alabaster, AL). The fluorescent probes 3–3′-diethylthiadicarbocyanine iodide ($diSC_2(5)$) and 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) were from Molecular Probes Inc. (Eugene, OR). The toxin CryIC was obtained from the parasporal inclusions from the HD-133 strain of *Bacillus thuringiensis* var. *aizawai*, kindly provided by Dr. L. Masson (Biotechnology Research Institute, National Research Council, Montreal, Quebec). The toxin was proteolytically activated and purified essentially as described previously [9].

2.2. Vesicle preparation

Lipids were mixed in a desired molar ratio in chloroform and thoroughly dried under nitrogen to create a thin film on the test tube wall. Vortexing of the lipid with an appropriate buffer (100 mM KCl and either 10 mM HEPES, 50 mM CAPS or 50 mM citrate buffer for pH

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Abbreviations: bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; $diSC_2(5)$, 3,3′-diethylthiadicarbocyanine iodide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); LUV, large unilamellar vesicles; PC, L-α-phosphatidylcholine; PG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)].

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7.4, 10.0 or 4.0, respectively) resulted in a suspension of multilamellar liposomes. Large unilamellar vesicles (LUV) were obtained by six cycles of extrusion of multilamellar liposomes through two stacked 100 nm pore size polycarbonate filters (Nucleopore Corp.) in a pressure extruder (Lipex Biomembranes Inc., Vancouver, Canada), followed two cycles of freezing-thawing and additional six cycles of extrusion. This procedure yields relatively uniform preparation of LUV with the mean diameter of about 80 nm [10]. The stock suspension contained 10 mg/ml lipid.

2.3. Potential-dissipation assay

Electric potential difference across the membranes of LUV was monitored fluorometrically using a potential-sensitive dye $diSC_2(5)$ [11,12]. Initially, the cuvette contained 2 ml of K^+-free buffer (100 mM NaCl and either 10 mM HEPES, 50 mM CAPS or 50 mM citrate buffer for pH 7.4, 10.0 or 4.0, respectively) and 250 nM dye, into which 2 μl of the LUV stock was added. Subsequently, diffusion potential of the K^+ ions across the LUV membranes was created by addition of 25 nM valinomycin. Finally, the toxin was injected and the time course of fluorescence changes was recorded with an SLM 8000C spectrofluorometer at 670 nm using the excitation at 645 nm. A long pass filter with the cut-off at 660 nm was used on the emission side. The cuvette was thermostatted at 20°C.

2.4. Binding of bis-ANS

Fluorescence of the amphiphilic fluorophore bis-ANS was excited at 395 nm and observed at 495 nm through a 460 nm cut-off filter. Sample contained 15 μM bis-ANS and 90 nM cryIC in 100 mM NaCl, 10 mM HEPES. pH was changed by injecting small aliquots of concentrated HCl or NaOH.

3. Results

3.1. Dissipation of membrane potential

The fluorescence of carbocyanine dye $diSC_2(5)$ responds to changes in electric potential across the vesicular membrane [11,12]. In the presence of transmembrane potential the dye accumulates in the membrane and/or interior of the vesicle, resulting in self-quenching of fluorescence. When the potential dissipates, the dye is released into the aqueous phase where it is no longer self-quenched and the fluorescence increases. In our experiments, K^+ ions are in thermodynamic equilibrium and dissipation of the membrane potential reflects the transmembrane movement of other ions present, namely efflux of Cl^- and/or influx of Na^+. Fig. 1 shows the effect of CryIC on the rate of potential dissipation in vesicles consisting of the mixture of PC and PG (9:1 molar ratio). In the absence of the protein there is no fluorescence recovery during the time course of the experiment (trace A), indicating high stability of the vesicles. Addition of CryIC (1.5 μM) results in a discharge of the membrane potential. The time-course of this effect is very similar at pH 7.4 and 10. In both cases the discharge of ions is relatively slow and is preceded by a lag period of approximately 5 min (trace B). This may be contrasted with the response under acidic conditions (pH below 5), in which case the rate of potential dissipation is considerably increased (trace C).

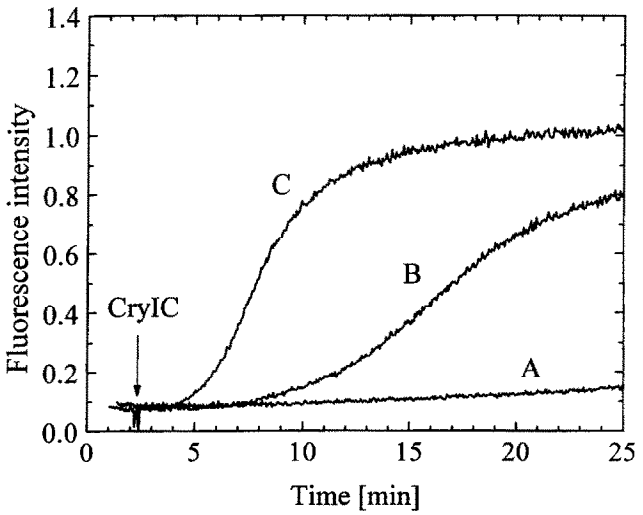

Fig. 1. Effect of pH on the CryIC-induced potential dissipation in PC/PG (9:1) LUV. The sample contained 13 μM lipid, 250 nM $diSC_2(5)$ and 25 nM valinomycin in 100 mM NaCl, buffered with 10 mM HEPES or 50 mM citrate for pH 7.4 or 4, respectively. Trace A, no CryIC added, pH 7.4; trace B, 1.5 μM CryIC, pH 7.4; trace C, 1.5 μM CryIC, pH 4. The arrow indicates the time of addition of the toxin.

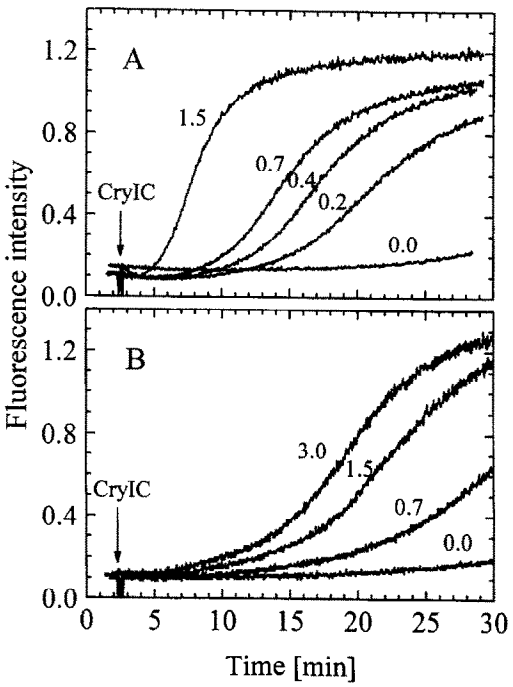

Fig. 2. CryIC-induced potential dissipation at pH 4 in LUV prepared from the mixture of PC and PG at a molar ratio of 9:1 (A) and PC alone (B). Concentration of the toxin in μM is indicated at each trace. The arrow indicates the time of addition of the toxin.

Fig. 2A shows the time course of potential dissipation in PC/PG vesicles at pH 4 recorded at various concentrations of CryIC. Under these conditions, a significant ion flux can be detected at a protein concentration as low as 0.1 μM. Comparison of the data of Fig. 1 and Fig. 2A allows us to estimate that approximately 5 times less protein is required at pH 4 than at pH 7.4 or 10 to produce a similar response.

In order to asses the effect of membrane lipid composition on the toxin-membrane interaction, experiments similar to those described above were performed with vesicles composed solely of phosphatidylcholine (Fig. 2B). The ability of CryIC to induce ion leakage in these vesicles (at pH 4) was significantly lower compared to the vesicles containing phosphatidylglycerol. A similar enhancement of the rate of toxin-induced potential dissipation was observed if PG was replaced by a synthetic negatively charged amphiphile dicetylphosphate (data not shown).

3.2. pH-dependent binding of bis-ANS to CryIC

In order to gain some insight into the mechanism of the pH-dependent interaction of CryIC with phospholipid vesicles, we have probed the effect of acidic pH on the toxin conformation. The fluorescence quantum yield of a dye 8-anilino-1-naphthalene-sulfonic acid (ANS), or its dimeric conjugate bis-ANS, depends strongly on the polarity of the environment, increasing upon binding to hydrophobic sites of proteins [13]. This class of compounds has been widely used as a probe to monitor conformational changes in proteins and to asses surface exposure of hydrophobic sites [14,15].

Fig. 3 shows pH dependence of the fluorescence intensity of bis-ANS in the presence of CryIC. While the quantum yield of the dye remains essentially unchanged in the pH range between approximately 10 and 5, further decrease in pH results in a dramatic enhancement of fluorescence intensity. Since the fluorescence of bis-ANS in the absence of the protein is pH-independent, we conclude that the enhancement observed under acidic conditions in the presence of CryIC reflects increased binding of the probe as a result of the exposure of hydrophobic sites on the surface of the protein molecule. The apparent pK of this conformational transition in CryIC is 4.2.

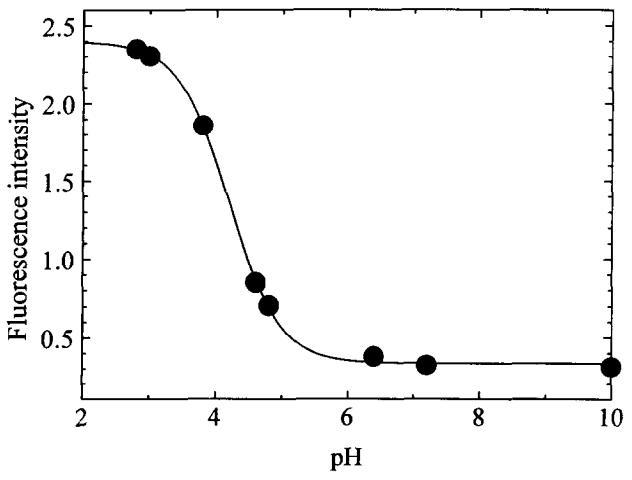

Fig. 3. pH dependence of the fluorescence intensity of bis-ANS in the presence of cryIC. The concentration of the dye was 15 μM and that of the toxin 90 nM.

4. Discussion

A crucial step in the cascade of events during the insecticidal action of *Bacillus thuringiensis* toxins is the interaction of the toxin molecules with the membranes of midgut epithelial cells. It has been postulated that upon binding to specific receptors, the toxin may act by generating channels or pores in the membranes of susceptible cells [2,3,5]. In this communication we show that the δ-endotoxin CryIC has the ability to dissipate membrane potential across the membranes of phospholipid vesicles. This confirms earlier indications that insecticidal δ-endotoxins from *B. thuringiensis* can interact with membrane lipids and induce permeabilization of the receptor-free lipid membranes [5–9].

Although pH in the midgut of lepidopteran larvae is generally alkaline [16], it may vary considerably within and among larvae [17] and pH is considered an important determinant of larval susceptibility to δ-endotoxins [2]. In this context, it is of particular interest to explore the effect of pH on the structural and functional properties of *B. thuringiensis* endotoxins. Previous circular dichroism study has demonstrated small pH-dependent changes in the secondary structure of one of the proteins belonging to the Cry family [18]. However, very little information is available regarding the effect of pH on the membrane interaction of the toxin. The data obtained in this study show that the membrane permeabilizing activity of CryIC at neutral and basic pH is rather weak. The toxin-induced leakage of ions is slow and can be observed only at relatively high concentrations of the protein (in a micromolar range). Such a weak membrane-perturbing activity of the toxin at basic/neutral pH is generally consistent with the results of recent experiments with planar lipid bilayers [9]. These experiments have shown that, in a similar pH range, CryIC can form ion-selective channels. However, spontaneous association of the toxin with the membrane was weak and successful observation of channel activity often required the use of a special membrane incorporation procedure.

The most notable finding of the present study is the observation that the membrane permeabilizing activity of CryIC is rapidly increased when pH of the medium is below 5. No previous study has explored the behavior of *B. thuringiensis* endotoxins under conditions of low pH. The increased ability of CryIC to induce leakage of ions from lipid vesicles at acidic pH suggests that the protein may undergo pH-dependent conformational changes that are conducive to membrane binding and/or penetration of the toxin. Indeed, the presence of a conformational transition, resulting in an increased exposure of hydrophobic sites on the surface of CryIC, is clearly indicated by the bis-ANS binding experiments. The behavior of CryIC under conditions of acidic pH shares certain characteristics common to a number of other bacterial toxins, such as diphtheria toxin [19], *Pseudo-*

monas aeruginosa exotoxin A [20,21], tetanus toxin [22,23] and colicins [24,25]. All these proteins show a dramatic increase in the affinity for phospholipid membranes at acidic pH. Furthermore, at least for some of them, the increased membrane interaction has been correlated with a conformational transition to a folding intermediate known as a 'molten globule' state [19,20,24–27]. In this state the protein retains a large proportion of its native secondary structure, while the tertiary structure is considerably loosened [27–29]. One of the characteristic features of the molten globule state is an increased exposure of hydrophobic residues that are buried in the native state [28,29]. While this characteristic is clearly shared by CryIC, further spectroscopic studies are required to characterize in more detail conformational properties of the protein under different conditions and, especially, to determine whether the particular conformation adopted by CryIC at low pH can be described as a molten globule state.

Another similarity between δ-endotoxin and some other membrane-active bacterial toxins is the preferential membrane interaction in the presence of acidic phospholipids [19,21,22,25]. In the case of CryIC, the effect of acidic lipids is clearly nonspecific and indicates that electrostatic interactions play a role in binding of the toxin to the membrane.

The physiological significance of the effect of acidic pH on the membrane interaction of *B. thuringiensis* δ-endotoxin is at present not clear since the pH of the target cells is rather alkaline than acidic [16]. Nevertheless, the present data demonstrate that under certain conditions in vitro the protein can undergo transition to a conformational state that is conducive to membrane interaction and promotes flux of ions across the lipid bilayer. It is conceivable that in vivo a transition to a similar conformational state can be triggered by means other than low pH (e.g. by binding to the receptor or proteolytic processing of the receptor-bound species). The above possibility is fully consistent with recent crystallographic data [30] which revealed structural similarities between the putative pore-forming domain of one of *B. thuringiensis* δ-endotoxins and the respective domain of colicin A, the protein believed to undergo physiologically-relevant transition to a membrane insertion-competent conformation [27].

5. References

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