# Restriction of intramolecular movements within the Cry1Aa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering

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Received 22 February 1997; revised version received 23 April 1997

**Abstract Disulfide bridges were introduced into Cry1Aa, a *Bacillus thuringiensis* lepidopteran toxin, to stabilize different protein domains including domain I α-helical regions thought to be involved in membrane integration and permeation. Bridged mutants could not form functional ion channels in lipid bilayers in the oxidized state, but upon reduction with β-mercaptoethanol, regained parental toxin channel activity. Our results show that unfolding of the protein around a hinge region linking domain I and II is a necessary step for pore formation. They also suggest that membrane insertion of the hydrophobic hairpin made of α-helices 4 and 5 in domain I plays a critical role in the formation of a functional pore.**

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*Key words:* Cry1Aa; Planar lipid bilayer; Ion channel; Disulfide bridge; *Bacillus thuringiensis*

## 1. Introduction

*Bacillus thuringiensis* (*Bt*) synthesizes a variety of insecticidal crystal proteins called ICPs or Cry proteins which, after solubilization in the gut and modification by gut juice proteases, are toxic to a number of lepidopteran, dipteran and coleopteran larvae [1]. The description of the first lepidopteran toxin atomic structure, Cry1Aa [2], revealed it to be a globular molecule comprised of three distinct domains with a high degree of overall structural similarity to that determined for the coleopteran specific toxin Cry3A [3]. In both structures domain I is composed entirely of eight α-helices, domain II is composed of three antiparallel β-sheets and two short α-helices, and domain III is a β-sandwich of two antiparallel β-sheets. Interestingly the α-helical bundles of both Cry toxins share a structural similarity to the pore-forming domains of two other well characterized bacterial toxins, diphtheria toxin and colicin A [4], although they do not share any sequence similarity. Among all four toxins, one or more hydrophobic helices, large enough to span a membrane, are found embedded between the other α-helices. This suggests a common mechanism of membrane permeation whereby these water soluble toxins undergo a conformational change to permit two or more helices to partition into a lipid membrane and form a functional pore. Indeed, in the case of colicin A and diphtheria toxin, spontaneous insertion into the membrane follows an unfolding of the helical bundles [5,6]. Although Cry toxins can also partition into planar lipid bilayers, in the absence of a receptor, to make functional ion channels [2,7–11], little is known about the *Bt* toxin-pore formation process, the biophysical properties of the ion channels, or their regulation and molecular architecture. Recent reviews [12,13] have speculated that Cry toxin insertion into membranes may be the result of a penknife- or umbrella-like conformational change in the toxin triggered by binding of the toxin to a specific membrane protein followed by insertion of a domain I helical hairpin composed of either helices α5 and α6 (hairpin α5α6) or α4 and α5 (hairpin α4α5).

Disulfide bridge engineering has been used to stabilize protein conformation and to provide information on protein structure and conformational changes [5,14,15]. Since trypsin-activated Cry1Aa does not contain cysteine residues, site-directed mutagenesis was used to introduce cysteines and create intramolecular disulfide bonds within the putative pore-forming region or interdomain regions. To investigate the mechanism of toxin insertion and channel formation in planar lipid bilayers, these disulfide bridges were strategically located to restrict the flexibility of either helix movement within domain I or movement of domain I and domain II relative to each other. Our results demonstrate that channel formation requires domain I to swing away from the rest of the protein. They suggest that among the helical hairpins of domain I, α4α5 is the most likely to partition into the bilayer and line the ion channel pore.

## 2. Materials and methods

### 2.1. Disulfide bridge mutants

The potential sites for amino acid conversion to cysteines were determined by examination of the Cry1Aa toxin atomic structure [2]. In order for disulfide bridging to occur, it was important to select amino acids within the two regions targeted for bridging which had a β-carbon distance within an appropriate range (3.6–5.4 Å) thus bringing the potential sulfur atoms to within 2 Å. All the Cry1Aa amino acids selected for cysteine mutation were created within the double-stranded DNA plasmid, pMP39 [16], by oligonucleotide-directed in vitro mutagenesis using the double oligonucleotide method of Deng and Nickolof [17] (Clontech Transformer kit, Clontech Laboratories, Palo Alto, CA, USA). All mutant and native Cry1Aa protoxins were expressed in the form of insoluble inclusions in *Escherichia coli*. A total of five bridged mutants was created (Fig. 1). Four mutants were designed to restrict flexibility within domain I: MP159 (arg99cys and ala144cys) links the middle of α3 to α4, MP169 (ile88cys and tyr153cys) links the interhelical loop of α2α3 with that of α4α5, MP178 (val162cys and ala207cys) links the middle of α5 to α6 and MP206 (ser176cys and ser252cys) links the C-terminal ends of α5 and α7. The interdomain linking bridge clone MP186 (arg224cys and ser279cys) was designed to link domain I (N-terminus of α7) to domain II (between the small helices α8a and α8). All Cry1Aa mutants were sequenced using the automated fluorescent sequencer from Applied Biosystems model 370A (Foster City, CA, USA).

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*PII* S0014-5793(97)00626-1

*2.2. Toxin expression and purification*

The various protoxin inclusion bodies were grown in *E. coli* at 30°C as described elsewhere [16]. All bridged clones except MP169 appeared to retain similar biochemical properties as the parental Cry1Aa protoxin with respect to expression, solubility at alkaline pH and trypsin sensitivity. In the case of MP169, although it retained wild-type solubility and trypsin sensitivity, the level of total protoxin expression was approximately 20% that of the wild-type protein. All toxins were activated at 37°C by resuspending the purified inclusion bodies in 0.1 M $Na_2CO_3$ (pH 11.0), containing 1% (w/v) trypsin for 1–2 h. Since there are numerous trypsin cleavage sites within the trypsin-resistant activated toxins, any misfolding of mutant proteins would have resulted in complete digestion of the protoxins to small peptides. The toxin was then purified by FPLC-Mono Q (Pharmacia Biotech, Montreal, Quebec, Canada) ion-exchange chromatography and then dialyzed extensively against water with continuous stirring to precipitate the toxins in their oxidized form. All protein concentrations were determined by the method of Bradford [18] using bovine serum albumin as a standard.

*2.3. Planar lipid bilayers*

Reconstitution of *Bt* toxin in planar lipid bilayers has been described in detail elsewhere [8]. Briefly, phospholipid membranes were formed from a 7:2:1 lipid mixture of phosphatidylethanolamine, phosphatidylcholine and cholesterol painted on a 250 μm circular aperture in a Delrin wall separating two low volume chambers (4 ml *trans*, 3.5 ml *cis*). Channel activity, following injection of 1–20 μg/ml (15–300 nM) of activated protein near the membrane in the *cis* chamber, was monitored by step changes in the current recorded during holding test voltages across the planar lipid bilayer. Under the experimental conditions used in this study, membranes had a capacitance of approximately 150–200 pF and remained stable for hours. In control experiments, it was verified that the addition of 0.5–20 mM β-mercaptoethanol alone to the *cis* chamber did not affect membrane electrical properties or stability and did not induce channel activity. The channels formed by the parental toxin Cry1Aa [2] were unaffected by the presence of β-mercaptoethanol at doses ranging from 0.5 to 20 mM. Reduction of bridge mutants in situ was accomplished by the addition of 1–20 mM β-mercaptoethanol to the *cis* chamber. All experiments were performed at room temperature (20–22°C) in buffer solutions containing either 150 or 450 mM KCl, 1 mM $CaCl_2$ and 10 mM Tris base, pH 9.0. Single channel currents were recorded with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Analysis was performed on a personal computer using pClamp and Axotape software (Axon Instruments, Foster City, CA, USA).

## 3. Results and discussion

*3.1. Interdomain bridging*

Through the creation of strategically placed disulfide bridges, control over the flexibility of protein target areas can be secured [5,14]. Structurally, the trypsin-resistant, activated Cry1Aa toxin consists of three tightly associated domains. Domain III of Cry1Aa is stacked upon domain II with both domains being aligned against domain I along helix α7. In our initial attempt to map the topography of toxin rearrangements on the surface of the lipid bilayer, we hypothesized that a certain degree of flexibility between domain I and the domain II/domain III stack may be needed to permit the eventual insertion of helices from domain I into the bilayer membrane. Such a partially unfolded structure, or 'molten globule' state, has been shown to occur with both colicin A and diphtheria toxins prior to the insertion of their α-helical rich insertion/translocation domains into membranes [4]. To this end, a double cysteine mutant (MP186) was created which locked α7 of domain I to domain II thus preventing any potential increase in interdomain distance. No ion channel activity was observed after the addition of MP186, in the oxidized or 'locked' state, to the *cis* chamber of the planar lipid bilayer apparatus. To ensure that the absence of channel activity was not due to a longer lag period caused by slower spontaneous integration of the oxidized form of the toxin, experiments with 5 μg/ml of MP186 in the *cis* chamber were monitored for periods up to 1 h. Addition of the reducing agent β-mercaptoethanol to the *cis* side of the bilayer resulted in the appearance of channel activity. The extent of channel activity was dependent on the concentration of reducing agent, with full activity (i.e., parental Cry1Aa level activity, Fig. 3A) being reached within 10–20 min in the presence of 15 mM β-mercaptoethanol (Fig. 2). Since native Cry1Aa toxin channel activity usually appears within 1 min after addition to the bath [2], it was possible that the longer lag period was due to a slow rate of toxin reduction rather than a decrease in the rate of toxin integration. This hypothesis was confirmed by preincubating MP186 in 10 mM β-mercaptoethanol between 5 and 12 h. Following addition of preincubated toxin to the *cis* chamber, rectangular current jumps occurred rapidly, usually within 10–20 s (Fig. 2, second trace from the top). The conductance, selectivity and kinetic properties of the channels produced by the reduced form of MP186 resembled those of the native Cry1Aa toxin [2] (data not shown). In particular, several subconducting states were observed (Fig. 2, third trace from the top), suggesting a multimeric organization in the bilayer, similar to those reported for other native Cry toxins [2,8–10]. The current-voltage relation-

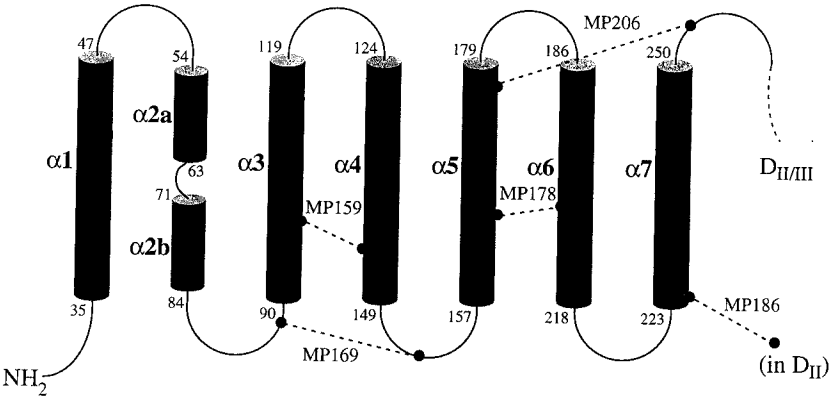


Fig. 1. Schematic representation of the mutation sites created in domain I of Cry1Aa. Helices are represented by narrow cylinders. Numbers next to helix extremities are the actual residue positions [2]. Mutant names are shown next to the dashed lines indicating engineered disulphide bonds. $D_{II/III}$ represents the stack formed by domains II and III.

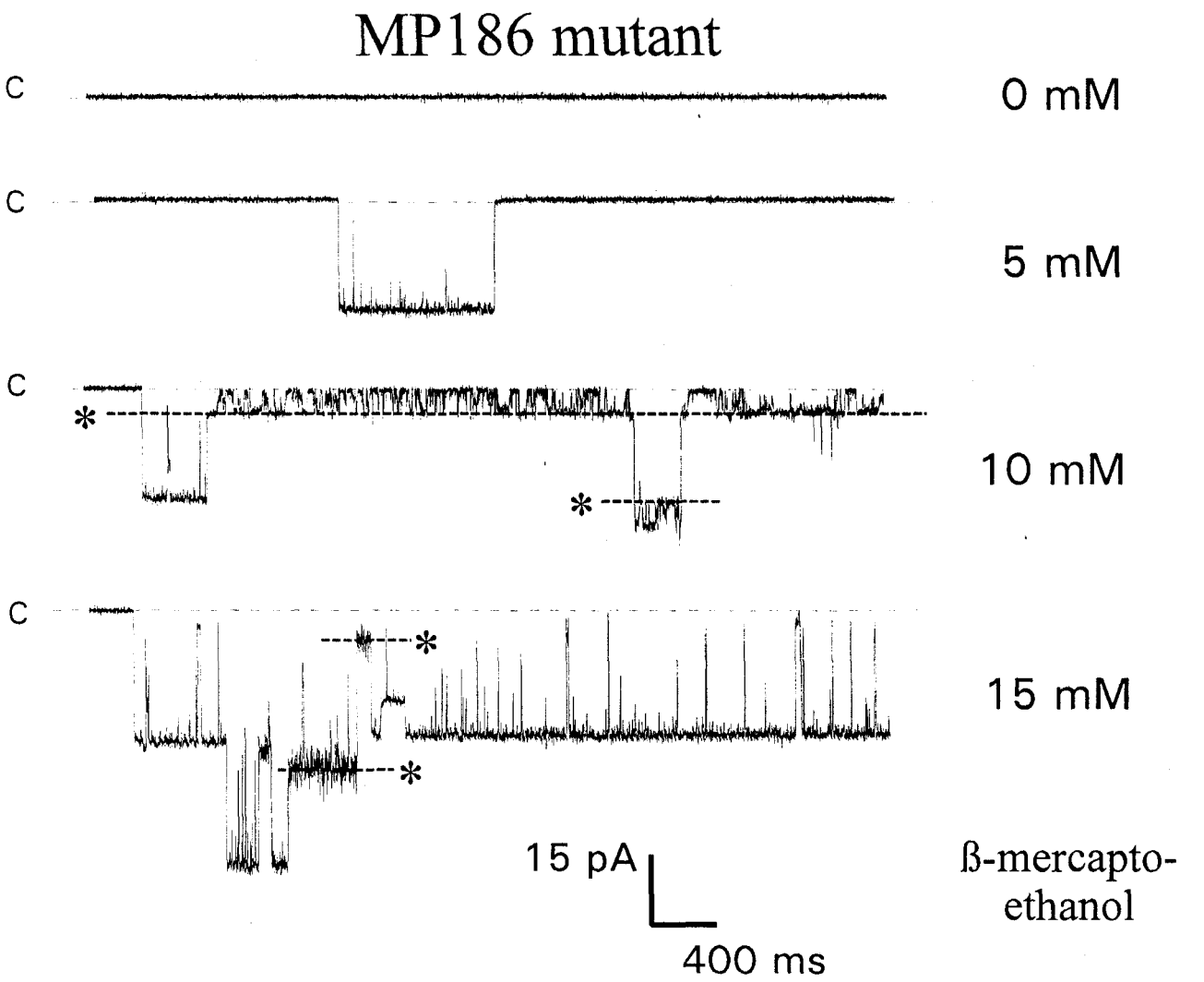


Fig. 2. Effect of increasing the concentration of β-mercaptoethanol on channel activity. Cry1Aa MP186 mutant (5 μg/ml) was present on the *cis* side of the bilayer separating symmetrical 150 mM KCl solutions. β-Mercaptoethanol was added to the *cis* side of the membrane. Holding potential: −60 mV. Letter C indicates the closed state. Asterisks (*) near the dashed lines indicate subconducting states.

ship of the reduced MP186 mutant was rectilinear and the channel conductance, i.e., the largest measurable conductance determined in the principal conducting state, was 485 ± 25 pS (mean ± SEM, $n = 6$) under symmetrical KCl conditions. At −40 mV, for both the MP186 mutant and the parental toxin, the probability of N channels to be open was approximately 60% and the mean open and closed times were approximately 30 and 120 ms, respectively. Under non-symmetrical KCl conditions, the zero-current voltage shifted in a direction consistent with cation selectivity.

Thus, our results show that, in the absence of a specific membrane binding protein, the ability of domain I to swing away from the rest of the toxin molecule, presumably through the interdomain hinge region (arg254-thr264), appears to be a necessary step preceding membrane partition into planar lipid bilayers.

### 3.2. Intradomain bridging

Bacterial toxins like diphtheria and colicin A, possessing distinct α-helical bundles, are thought to partition or translocate into lipid bilayers through the insertion of one or more helical hairpins [4]. In the case of Cry1Aa, three of the eight α-helices in domain I (α1, α2a and α2b) are of insufficient length to span a lipid bilayer, being less than 14 amino acids in size. However, the remaining five consecutive helices, ranging from 22 to 36 amino acids in length, have the potential to create four membrane spanning helical hairpins [2]. Through the creation of disulfide bridges, we have attempted to restrict interhelical movement within domain I in order to determine which hairpin(s) insert into the membrane. Four mutants were designed which linked α3 and α4 (MP159), interhelical loops between α2α3 and α4α5 (MP169), α5 and α6 (MP178) and finally α5 to the interdomain hinge region (MP206) (Fig. 1). The rationale behind selecting the target sites for cysteine mutagenesis was based on the high probability that α5 is involved in membrane integration [19,20] and the low probability that α1, α2a and α2b could cross the membrane due to their small size [2]. In planar lipid bilayers, all four mutants in the oxidized state exhibited the same lack of channel activity as was observed with MP186. Similarly, Cry1Aa-type activity was regained after addition of β-mercaptoethanol to the *cis* chamber. The intradomain mutants, once reduced, had the

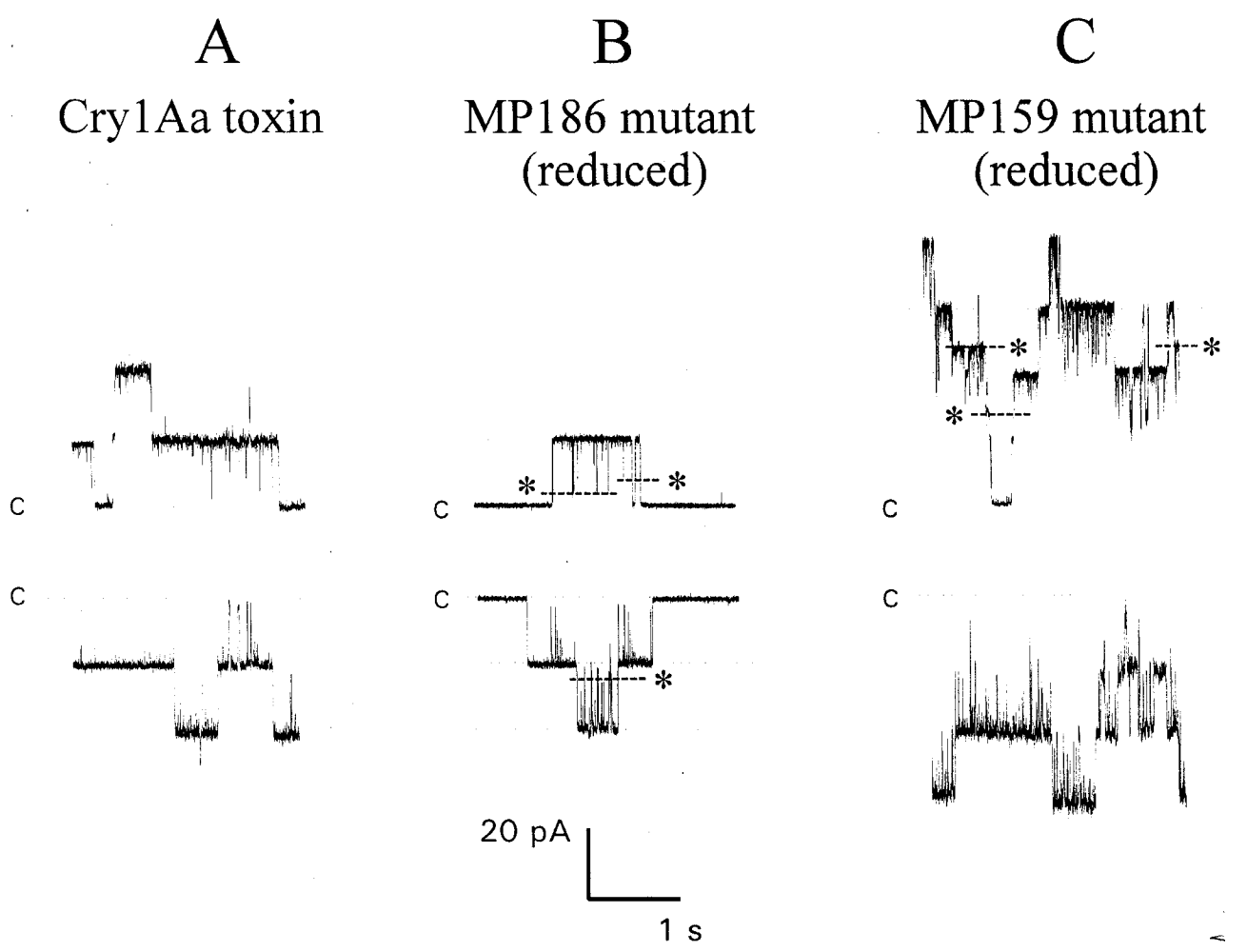


Fig. 3. Single channel currents recorded in the presence of β-mercaptoethanol under symmetrical KCl concentration conditions: (A) Cry1Aa, (B) MP186, and (C) MP159. Holding potential: +40 mV (upper traces); −40 mV (lower traces). Letter C indicates the closed state. Asterisks (*) near the dashed lines indicate subconducting states.

same biophysical properties as the parental toxin (not shown) and displayed multiple subconducting levels, as seen with MP186, which again suggests oligomeric association of the individual proteins (Fig. 3C). Fig. 3C shows typical ion channel currents produced by MP159, one of the reduced intradomain bridge mutant. Three of the mutants, MP169, MP178 and MP206, have disulfide bridges designed to restrict interhelical movement of α5 by linking it to other regions within domain I. The observed inactivity of these mutants in their oxidized form is consistent with observations by Gazit and Shai [19,20] who demonstrated that a synthetic α5 peptide could self-assemble in a membrane-bound state and cause a leakage of vesicular contents. Moreover, this helix could co-assemble with a separate helix (α7) which lacked the ability to self-assemble. These data strongly suggests that α5 represents a likely candidate, within the domain I helical bundle, to act as a driving force as part of a hairpin that eventually crosses the lipid bilayer. The inactivity of oxidized MP159 which links α3 to α4 strongly suggests that α1α2, α5α6 and α6α7 are not involved in early membrane insertion. It further implies that α2α3, α3α4 or α4α5 may have the potential to integrate into lipid bilayers. However, as indicated earlier, hairpins α1α2 or α2α3 can be eliminated strictly due to size constraints thus reducing the potential candidates to α3α4 and α4α5. Moreover, since mutants MP178, MP206 and MP169 do not impose restrictions on α3α4 movements and yet prevent channel formation while in the oxidized state, the α3α4 hairpin can be eliminated as the candidate for insertion. Therefore, from the indirect evidence provided above, our data suggest that the building of the transmembrane pore is initiated by insertion of the α4α5 hairpin. This observation is consistent with both the length (>30 Å) and the biochemical nature of α4α5. All charged residues are found on the N-terminal end of α4 and the C-terminal end of α5, i.e., furthest away from the loop which connects helices α4 and α5 in the hairpin. The helical segments nearest the loop, which would be the section initially exposed to the membrane surface, forms the most hydrophobic segment of domain I [2]. However, it cannot be excluded that added flexibility in other regions of domain I is necessary to attain the oligomeric arrangement required to produce a stable, functional pore, which could provide an alternative explanation as to why no channel activity was observed for the mutants while in the oxidized state.

### *3.3. Pore model*

The data presented in this study allow us to speculate on the sequence of events which occur during toxin integration into lipid membranes. Cry1Aa toxin, upon binding to a GPI-

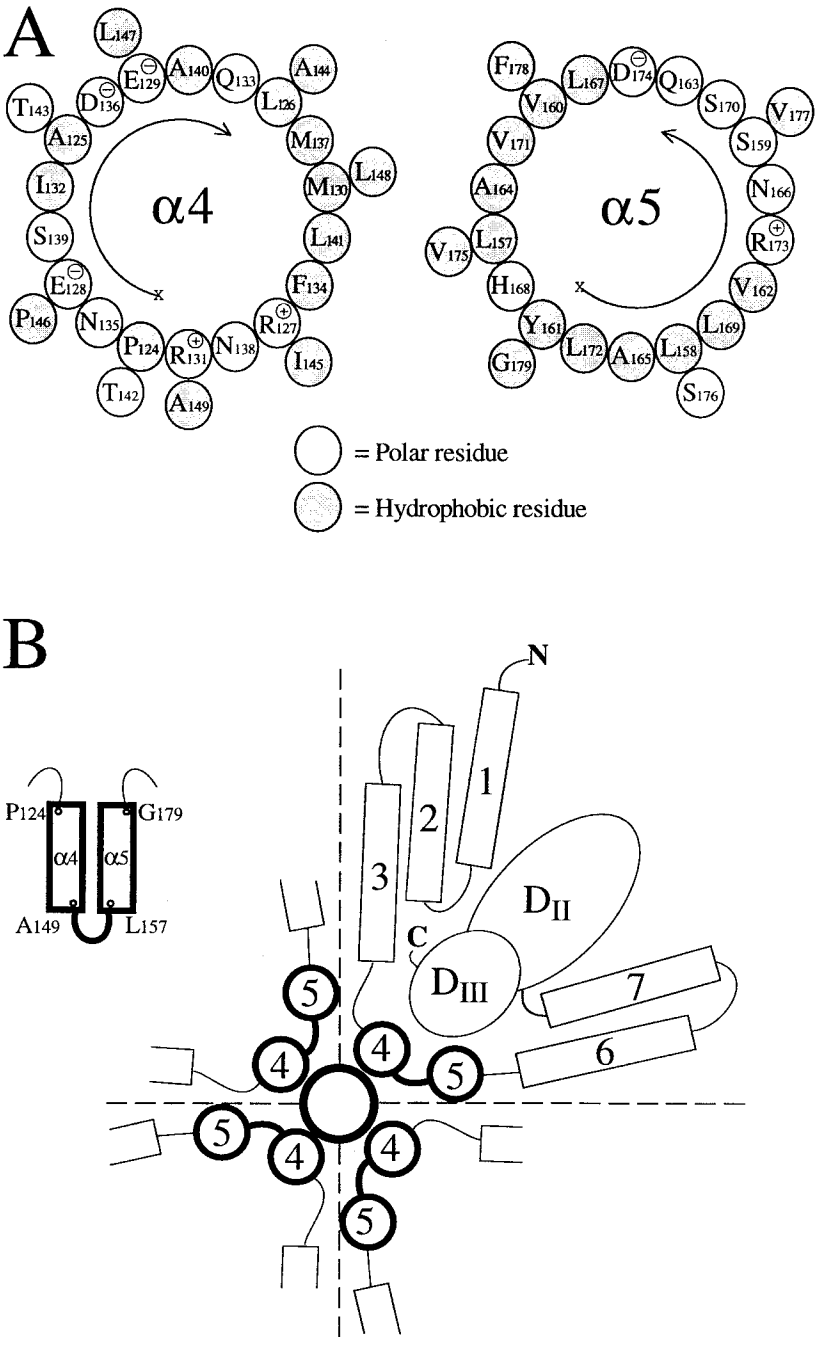


Fig. 4. Hydropathic profile of the α4α5 hairpin and a working model for pore formation. (A) Helical wheel projections [27] of both helices within the hairpin. The axis of the helices have been straightened from their 26° angled conformation found in the native crystal. The X represents the starting amino acid and the arrow indicates the direction of rotation of the α-carbon backbone. Charged amino acids at pH 9.0 are indicated by circled + and − signs. (B) Schematic top view representation of a tetrameric model of the pore formed by Cry1Aa toxin in a phospholipid membrane. The small insert indicates the identity of the N- and C-terminal amino acids of α4 and α5. Domain I helices are numbered in rectangular boxes and small circles. The larger thick-walled central circle represents the transmembrane toxin pore. $D_{II}$ and $D_{III}$ represent domain II and domain III.

linked aminopeptidase N receptor at the surface of the membrane [21], or alternatively to membrane lipids (S.F. Garczynski and M.J. Adang, personal communication), or spontaneously to artificial phospholipid membranes at nanomolar concentrations (this study), undergoes a conformational change analogous to a 'molten globule' state described for other bacterial toxins [4]. In this transition from the compact, trypsin-resistant, Cry toxin structure, domain I separates from the other two domains through the hinge region connecting domain I to domain II, as demonstrated by MP186. Subsequent to the movement of domain I away from the rest of the molecule, the α4α5 hairpin partitions into and crosses the membrane bilayer while the other helices of domain I presumably flatten out on the membrane surface in an umbrella-like arrangement. The question now remains as to how these two helices arrange themselves within the membrane environment

to form one of the transmembrane subunits of an oligomeric ion channel that satisfies established Cry toxin pore characteristics. It is clear that any such arrangement must take into account at least four important factors; namely the hydropathic features of the two helices, ion channel specificity, pore size, and helix-helix interactions. An examination of the hydropathic nature and charge distribution of α4 and α5 revealed a number of interesting features. Fig. 4A shows the two helices aligned with respect to each other in a manner similar to that found in the crystal structure [2]. The orientation of the neighboring hydrophobic faces presumably remains unchanged after membrane integration [22]. Fig. 4A shows that α5 is amphipathic in nature with most of the helical surface being hydrophobic and with one side essentially hydrophilic. Helix 4, which also displays amphipathic properties, appears to be the opposite of α5 in that most of the helical surface is hydrophilic in nature with one side being completely hydrophobic. The large hydrophilic surface of α4 suggests that it is this region which may line the aqueous pore surface and that the role of α5, which is more hydrophobic, may be to provide an anchor within the lipid bilayer for the α4α5 hairpin. Consistent with this type of arrangement is the fact that α5 is located within a highly conserved region in Cry toxins [1]. Since the conductance of ion channels formed by different toxins under the same conditions appear to be subclass-specific, at least in the case of Cry1Aa, Cry1Ac and Cry1C (approximately 450 pS for the first two toxins vs. 25–135 pS for the latter [2,8,9]), it is reasonable to assume that only α4, having a non-conserved hydrophilic side, lines the aqueous surface of the pore thus accounting for the observed toxin subclass-specific variations in ion transport. It is important to mention that any influence exerted by extramembranous elements (i.e., domains II or III) on channel conductances are as yet unknown.

Data published by us as well as others [2,8,10] have shown that ion channels created by Cry1 toxins are cationic in nature at alkaline pH. A common feature among selective sodium, potassium and calcium ion channels is that concentric rings of negative charges invariably line the pore [23]. As shown in Fig. 4A, the hydrophilic surface of α4 would provide two concentric rings of negative charges; one near the pore opening (glu128 and glu129) and one in line with glu129 halfway down the helix. Helix 5 does not fit this criteria as it only contains a single negatively charged residue (asp174) on the hydrophilic side.

Using sugars and polyethylene glycol molecules of different sizes as osmotic protectants, a calculated pore size between 10 and 20 Å has been estimated for Cry1 toxins [24], which compares well to that of β-barrel, non-selective channels made by bacterial porins [23]. However, numerous planar lipid bilayer experiments have demonstrated that when reconstituted in artificial membranes, Cry toxin channels are selective to potassium [2,8–10]. Taking into consideration the van der Waals interaction between the two helices, we estimate the cross-sectional dimension of the α4α5 hairpin to be approximately 15 Å × 23 Å. By examining and comparing intrahelical distances and the approximate pore size required for $K^+$ flow, a simple working model for a potassium-selective channel [23] containing a minimal number of toxin molecules can be produced (Fig. 4). In this model, the aqueous pore is lined by four identical α4 helices each contributed by an integrated α4α5 hairpin. The diameter of the pore, as represented by the thick-lined circle, would be approximately 6 Å. This figure is consistent with the estimated size of the water-filled pore of the 5-$HT_3$ receptor, a channel which discriminates poorly between monovalent cations and which is twice the diameter of a highly selective potassium channel [25,26]. It is important to stress that this model represents a minimal number of toxin molecules as it has been shown that the pore size increases with time as measured by the passage of increasing larger, nonspecific molecules [24]. Further studies are needed to confirm the dual role of α4 in membrane permeation and in regulating the flow of cations through newly formed *Bt* toxin ion channels.

*Acknowledgements:* The authors wish to thank A. Mazza and S. Rivest for expert technical help. This work was supported in part by a grant from the Natural Sciences and Engineering Research Council of Canada to J.L.S.

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