



Fig. 2. Western blot analysis of lysates extracted from *E. coli* cells harboring the pME4B vector (lane 2), pME4D (lane 3), or the mutant plasmids-K123A, Y125A, N128A, S130A, Q135A, R136A, R136K, R136Q, R136D, Q139A, and E141A (lanes 4-14, respectively)-showing the 65-kDa CryII toxin and small molecular fragments that cross-reacted with the CryIIA antibodies. Lane 1 represents the molecular mass standards.

charged and five polar amino acids in helix 4 (Fig. 1B) were substituted with alanine. Most of the targeted residues, Tyr-125, Asn-128, Gln-135, Arg-136 and Gln-139, but not Lys-123, Ser-130 and Glu-141, are located at the hydrophilic surface (see Fig. 1C).

Expression of the mutant toxins in *E. coli* was controlled by the *tac* promoter. Upon addition of IPTG to mid-exponential phase cultures, all mutant toxins were predominantly produced as sedimentable inclusion bodies. Lysates were analyzed by SDS-PAGE and immunoblotting, and the protein expression level of all mutant derivatives was found to be comparable to the wild type. The 65-kDa expressed mutant proteins specifically cross-reacted with antibodies raised against the CryIIA toxin (see Fig. 2). However, two relatively intense immuno-reactive bands of ca. 50 kDa and ca. 35 kDa were detected in all mutant lysates. This indicates that the expressed mutant proteins are rather sensitive to proteolytic degradation.

The solubility of mutant protein inclusions in comparison to the wild-type inclusion was assessed using a carbonate buffer, pH 9.0. The amount of 65-kDa soluble proteins in the supernatant was compared with those of the proteins initially used, in order to determine the percentage of protein solubilisation. All of the mutant inclusions were found to be soluble to some extent in this buffer, giving less than 20% solubility, which resembles closely the wild-type inclusions under similar conditions.

To determine the effect of mutations on toxicity, *E. coli* cells that expressed each type of the mutant toxin were tested for their relative biological activity towards *Aedes aegypti* larvae. All of the assays were carried out in ten replicates for each sample and repeated three times; the mortality data recorded after a 24-hour incubation are shown in Fig. 3. Interestingly, only the R136A mutation resulted in a total loss of larvicidal activity, while alanine substitutions at seven other positions (K123A, Y125A, N128A, S130A, Q135A, Q139A

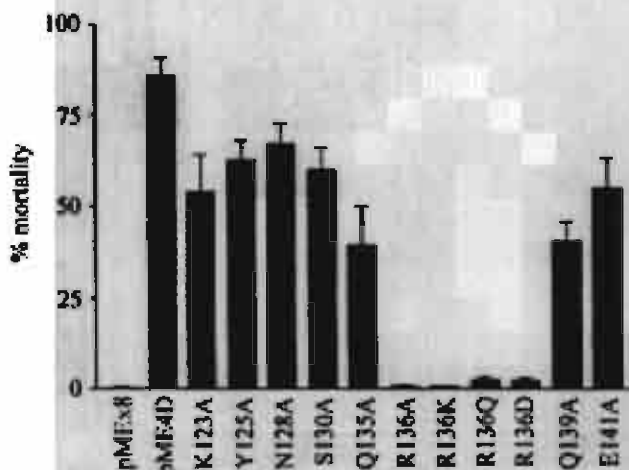


Fig. 3. Mosquito-larvicidal activities of *E. coli* cells expressing the CryIIA wild-type toxin (pME4D), or its mutants-K123A, Y125A, N128A, S130A, Q135A, R136A, R136K, R136Q, R136D, Q139A and E141A-against *Aedes aegypti* larvae. Error bars indicate standard error of the mean from the three independent experiments.

and E141A) still retained over 50% of the wild-type activity. When this critical arginine residue at position 136 was converted to aspartate, glutamine or even to the most conserved residue for positively charged side chain, *i.e.* lysine, all R136 mutants (R136D, R136Q and R136K) were shown to be nontoxic to mosquito larvae (see Fig. 3). These results could imply the requirement for a specific structure of the positive side chain at this position. Perhaps Arg-136, which is likely to face the pore lumen, could interact with an aqueous environment, and somehow stabilize the functional pore. However, the precise function of this residue remains to be elucidated.

Protein expression levels and solubility of the inclusions suggested that the complete loss of toxicity observed for the R136A mutant is least likely to be caused by misfolding of the protein. Taken together, our results indicate that Arg-136 is a critical residue involved in CryIIA toxin activity. The data further support our previous findings that Arg-158 in $\alpha 4$ played a crucial role in toxicity of the 130-kDa Cry4B toxin, since the single alanine substitution at this residue almost completely abolished its activity towards mosquito larvae (Sramala *et al.*, 2001). In addition, results reported by other workers revealed that an arginine residue at position 131 in $\alpha 4$ is important for toxicity of both the lepidopteran-specific CryIAa and CryIAC toxins (Kumar and Aronson, 1999; Masson *et al.*, 1999). Two other negatively charged residues (Glu-129 and Asp-136) of CryIAa were also shown to be critical in the passage of ions through the pore (Masson *et al.*, 1999).

Comparisons of structural models among CryIIA, Cry4B, and CryIAa suggest that, although Arg-136 of CryIIA is located on the opposite side of helix 4 relative to Arg-158 of Cry4B or Arg-131 of CryIAa, all of these three critical

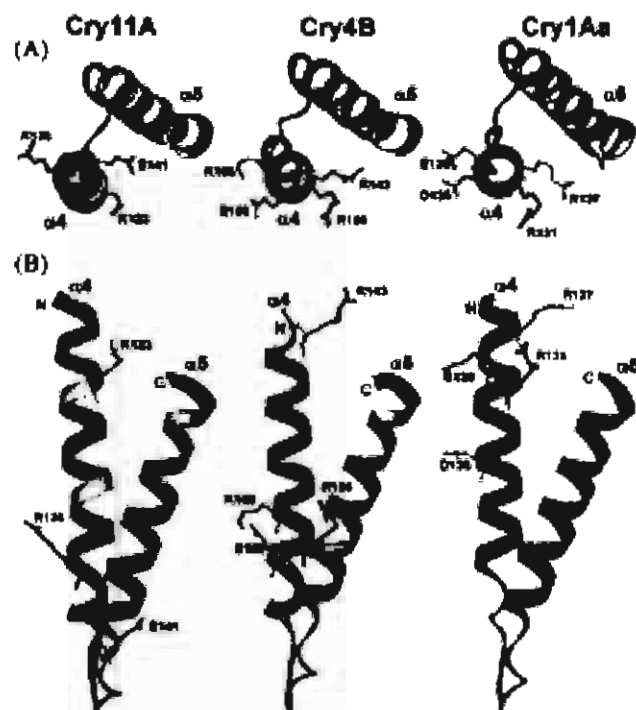


Fig. 4. (A) Top and (B) side views of amino acid arrangement in helix 4 together with the relative position of helix 5 in 3D models of Cry11A and Cry4B built by homology modeling and the Cry1Aa crystal structure. The labeled residues, shown in red and yellow, indicate the critical arginine residues and other charged positions, respectively. The structures were prepared using Wehlab viewer (Molecular Simulations Inc.).

residues are oriented on the side of helix 4, which is furthest away from helix 5 (see Fig. 4A). It should be noted that Arg-136 and Arg-158, in both of the dipteran-specific toxins, are situated near the C-terminal end of helix 4, while Arg-131 of the lepidopteran-specific Cry1Aa toxin is located furthest from the C-terminus of this helix (Fig. 4B). Differences in the location of these critical residues may conceivably reflect the diversity in the channel architecture for each group of insect-specific Cry toxins. Further studies are required to elucidate the role of these positively charged residues in helix 4, to discover whether they are involved in the passage of ions through the pore.

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RESEARCH

Specific Mutations Within the $\alpha 4$ - $\alpha 5$ Loop of the *Bacillus thuringiensis* Cry4B Toxin Reveal A Crucial Role for Asn-166 and Tyr-170

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Abstract

The widely accepted model for toxicity mechanisms of the *Bacillus thuringiensis* Cry δ -endotoxins suggests that helices $\alpha 4$ and $\alpha 5$ form a helix-loop-helix hairpin structure to initiate membrane insertion and pore formation. In this report, alanine substitutions of two polar amino acids (Asn-166 and Tyr-170) and one charged residue (Glu-171) within the $\alpha 4$ - $\alpha 5$ loop of the 130-kDa Cry4B mosquito-larvicidal protein were initially made via polymerase chain reaction-based directed mutagenesis. As with the wild-type toxin, all of the mutant proteins were highly expressed in *Escherichia coli* as inclusion bodies upon isopropyl- β -D-thiogalactopyranoside induction. When *E. coli* cells expressing each mutant toxin were assayed against *Aedes aegypti* mosquito larvae, the activity was almost completely abolished for N166A and Y170A mutations, whereas E171A showed only a small reduction in toxicity. Further analysis of these two critical residues by induction of specific mutations revealed that polarity at position 166 and highly conserved aromaticity at position 170 within the $\alpha 4$ - $\alpha 5$ loop play a crucial role in the larvicidal activity of the Cry4B toxin.

Index Entries: Aromaticity; *Bacillus thuringiensis*; δ -endotoxins; mutagenesis; larvicidal activity; polarity.

1. Introduction

During sporulation, the gram-positive bacterium *Bacillus thuringiensis* (*Bt*) produces intracellular crystalline inclusions consisting of one or more insecticidal proteins known as δ -endotoxins (1). These cytoplasmic inclusions, which are released together with the bacterial spore upon completion of sporulation, are specifically toxic to several orders of insect larvae including Lepidoptera (butterflies and moths), Coleoptera (beetles), Diptera (mosquitoes and flies), and Hymenoptera (wasps and bees) (1-3). The *Bt* δ -endotoxins can be classified into the two families of Cry (crystal) and Cyt (cytolytic) toxins, in accordance with the recently revised nomenclature based on their amino-acid sequence identity (4). For instance,

one of the four major insecticidal proteins produced by *Bt* subsp. *israelensis* has been classified as Cry4B, and is highly active against mosquito larvae of the genera *Aedes* and *Anopheles* (1,4).

The *Bt* δ -endotoxins are present in the native crystalline inclusions as insoluble inactive protoxins. Upon ingestion by susceptible larvae, the protoxin inclusions are solubilized in the larval midgut and activated by midgut proteases. It is believed that the activated toxins first bind to a specific receptor located on apical membranes of midgut epithelial cells of the target insect larvae. Subsequently, the toxins insert into the membrane in an irreversible binding step, and disrupt the permeability of the midgut cell membranes, resulting in a net influx of ions and water that

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leads to osmotic cell lysis (5). However, the exact nature of this toxicity process at the molecular level, especially in the step of membrane insertion and lytic pore-formation, is still not clearly understood. Knowledge of the pore-forming structure within the lipid membranes could therefore advance understanding of the precise mode of action of these toxins, and would facilitate the design of more potent toxins.

To date, tertiary structures of four different Cry toxins, Cry1Aa (6), Cry2Aa (7), Cry3Aa (8), and Cry3Bb (9), have been determined by X-ray crystallography. Despite the differences in their insect specificity and the comparatively low amino-acid sequence identity between these proteins, the structures of all four of them display a high degree of overall similarity. A homology-based 3D model of the 65-kDa activated Cry4B toxin suggests that this mosquitocidal protein would fold into a structure similar to known crystal structures with a three-domain organization (10). The N-terminal domain I is composed of seven α helices in which the relatively hydrophobic helix $\alpha 5$ is encircled by six other amphipathic helices (6–9). That this domain is clearly equipped for membrane insertion and pore formation has been supported by various studies demonstrating that the isolated helical fragment of different Cry toxins is responsible for pore/ion-channel-forming activity (11–13).

A molecular mechanism of membrane insertion and pore formation of the Cry δ -endotoxins has been proposed in an "umbrella model" (14). In this model, the $\alpha 4$ and $\alpha 5$ helices form a helical hairpin to initiate membrane penetration upon specific receptor binding in which a structural rearrangement of the toxin occurs. After insertion of this hairpin, the other helices spread over the membrane surface in a step that is followed by oligomerization of the toxin in the membranes. Currently, this proposed model is supported by a number of experiments demonstrating the functional role of $\alpha 4$ and $\alpha 5$ in the toxicity of different Cry toxins. Evidence suggests that $\alpha 4$ is oriented to face the pore lumen and is involved in channel function, whereas $\alpha 5$ appears to be in contact with the lipid membranes and plays a role in toxin oligomerization (15–17).

In addition, membrane permeation studies found that the $\alpha 4$ -loop- $\alpha 5$ hairpin was extremely active as compared with the isolated helices or their mixtures, indicating that the loop is needed for efficient insertion into the lipid membranes (18).

Previously, we have made proline substitutions in five helices (i.e., $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, and $\alpha 7$) of the 130-kDa Cry4B toxin, and found that helices $\alpha 4$ and $\alpha 5$ are important determinants of larvicidal activity (19,20) and likely to be involved in membrane pore formation. Further investigation by charged-to-alanine scanning mutagenesis in helix 4 revealed that the specific structure for the positively charged side chain of Arg-158 is critically involved in the toxic activity of Cry4B (21). As an extension of the previous studies together with the proposed umbrella model, we hypothesized that when the loop connecting $\alpha 4$ and $\alpha 5$ initially inserts into the membrane, polar or charged residues in this loop may interact with ions and water molecules. These residues could play a critical role in the toxicity of Cry4B. To test this hypothesis, we created a number of Cry4B mutants in which Asn-166, Tyr-170, or Glu-171 in the $\alpha 4$ - $\alpha 5$ loop was altered. The results revealed that polarity of Asn-166 and aromaticity of Tyr-170 residues are essential for larvicidal activity of the Cry4B toxin.

2. Materials and Methods

2.1. Plasmids and Site-Directed Mutagenesis

The recombinant pMU388 plasmid encoding the 130-kDa Cry4B toxin, which has been cloned from *Bt* subsp. *israelensis* into pUC12 vector (22), was used as a template for site-directed mutagenesis. In order to substitute Asn-166, Tyr-170, and Glu-171 with alanine and other amino acids, 13 pairs of complementary mutagenic oligonucleotide primers (Table 1) were designed and purchased from Genset Inc. (Singapore). All mutant plasmids were generated by polymerase chain reaction (PCR) using high-fidelity *Pfu* DNA polymerase according to the procedure of the Quick-Change™ Mutagenesis Kit (Stratagene). Each mutant plasmid was then confirmed in the mutated region by DNA sequencing, using an ABI prism 377 automated sequencer (PerkinElmer).

Table 1
Complementary Primers for Substituting a Code Residue with Different Amino Acids

Primer	Sequence ^a	Restriction site
N166A-f: N166A-r:	A	<i>Pst</i> I
	5'-CGAGAGACTGCAGTTTATTTTAGCGCATTAGTAGGT-3' 5'-ACCTACTAATGCGCTAAAATAAACTGCAGTCTCTCG-3'	
A166C-f: A166C-r:	C	<i>Pvu</i> II
	5'-GACTGCAGTTTATTTTCAGCTGTTTAGTAGGTTATG-3' 5'-CATAACCTACTAAACAGCTGAAAATAAACTGCAGTC-3'	
N166D-f: N166D-r:	D	<i>Pvu</i> II
	5'-CGAGAGACAGCTGTTTATTTTAGCGATTAGTAGG-3' 5'-CCTACTAAATCGCTAAAATAAACAGCTGGTCTCTCG-3'	
N166Q-f: N166Q-r:	Q	<i>Acl</i> I
	5'-CGAGAGACCGCAGTATACTTTAGCCAATTAGTAGG-3' 5'-CCTACTAATTGGCTAAAGTATACTGCGGTCTCTCG-3'	
N166R-f: N166R-r:	R	<i>Xba</i> I
	5'-GAGACCGCAGTTTATTTTCTAGATTAGTAGGTTATG-3' 5'-CATAACCTACTAATCTAGAAAAATAAACTGCGGTCTC-3'	
N166I-f: N166I-r:	I	<i>Bst</i> NI
	5'-GTTTATTTTAGCATCTGGTAGGTTATG-3' 5'-CATAACCTACCAGGATGCTAAAATAAAC-3'	
Y170A-f: Y170A-r:	A	<i>Acl</i> I
	5'-GACCGCAGTATACTTTAGCAACTTAGTAGGTGCTGAATTATTG-3' 5'-CAATAATTCAGCACCTACTAAGTTGCTAAAGTATACTGCGGTC-3'	
Y170D-f: Y170D-r:	D	<i>Bst</i> NI
	5'-GCAGTTTATTTTAGCAACCTGGTAGGTGATGAATTATTG-3' 5'-CAATAATTCATCACCTACCAGGTTGCTAAAATAAACTGC-3'	
Y170R-f: Y170R-r:	R	<i>Nru</i> I
	5'-GCAGTTTATTTTAGCAACTTAGTAGGTGCGGAATTATTG-3' 5'-CAATAATTCGCGACCTACTAAGTTGCTAAAATAAACTGC-3'	
Y170L-f: Y170L-r:	L	<i>Xba</i> I
	5'-GCAACTTAGTAGGTCTAGAAATTATTGTTATTACC-3' 5'-GGTAATAACAATAATTCTAGACCTACTAAGTTGC-3'	
Y170F-f: Y170F-r:	F	<i>Hin</i> FI
	5'-GCAACTTAGTAGGATTCGAATTATTGTTATTACC-3' 5'-GGTAATAACAATAATTCGAATCCTACTAAGTTGC-3'	
Y170W-f: Y170W-r:	W	<i>Bst</i> BI
	5'-GCAGTTTATTTTTCGAACCTTAGTAGGTTGGGAATTATTG-3' 5'-CAATAATTCCTAACCTACTAAGTTGCAAAAATAAACTGC-3'	
E171A-f: E171A-r:	A	<i>Bst</i> NI
	5'-TATTTTAGCAACCTGGTAGGTTATGCATTATTGTTA-3' 5'-TAACAATAATGCATAACCTACCAGGTTGCTAAAATA-3'	

^aMutated nucleotide residues are shown as boldface. Mutated amino acids are shown on top of each pair of primers. Introduced restriction-enzyme recognition sites are underlined.

2.2. Toxin Expression, Solubilization and Proteolytic Activation

The wild-type and $\alpha 4$ - $\alpha 5$ loop Cry4B mutant toxins were expressed in *E. coli* strain JM109 under control of the *LacZ* promoter. Cells were grown in a Luria Broth medium containing 100 $\mu\text{g/mL}$ ampicillin until the OD_{600} of the culture reached 0.3–0.5. After the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, incubation was continued for another 4 h. Protein expression was analyzed by sodium dodecyl sulfate- (13% w/v) polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* cells expressing each mutant Cry4B toxin as cytoplasmic inclusion bodies were harvested by centrifugation, resuspended in distilled water, and disrupted in a French Pressure Cell at 10,000 psi. The crude lysates were centrifuged at 5,000g for 20 min and the pellets obtained were washed 3 times in distilled water by sonication. Protein concentrations were determined by using a protein microassay (Bio-Rad), with bovine serum albumin fraction V (Sigma) as a standard. Protoxin inclusions were solubilized in 50 mM Na_2CO_3 , pH 9.0 and incubated at 37°C for 1 h as previously described (19). After centrifugation for 10 min, the supernatants were analyzed via SDS-PAGE in comparison with the inclusion suspension. To test the stability of the mutant toxins, the solubilized mutant and wild-type toxins were incubated with tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin at a ratio of 1:20, (w/w) enzyme:toxin in 50 mM Na_2CO_3 , pH 9.0, for 16 h.

2.3. Larvicidal Activity Assays

Bioassays were performed as previously described (20), using 2-d old *Aedes aegypti* mosquito larvae reared from eggs that were supplied by the mosquito-rearing facility of the Institute of Molecular Biology and Genetics, Mahidol University, Nakornpathom, Thailand. About 500 larvae were reared in a container (22 $\times$ 30 $\times$ 10 cm) with approx 3 L of distilled water supplemented with a small amount of rat diet pellets. Both the rearing of larvae and the bioassays were done at room temperature (25°C). The assays were done in 1 mL of *E. coli* suspension (10^8 cells suspended in

distilled water) in a 48-well microtiter plate (11.3-mm well diameter; Costar), with 10 larvae per well and a total of 100 larvae for *E. coli* expressing each type of the Cry4B toxin. *E. coli* cells containing the pMEx8 vector were used as a negative control. Mortality was recorded after a 24-h incubation period.

3. Results and Discussion

It has recently been shown that the loop connecting the $\alpha 4$ and $\alpha 5$ helices of Cry1Ac is needed for efficient insertion of the $\alpha 4$ - $\alpha 5$ helical hairpin into lipid membranes (18). As predicted from the homology-based 3D model of Cry4B, the $\alpha 4$ - $\alpha 5$ loop comprises eight amino acids, of which one is charged (i.e., Glu-171) and two of which are polar (i.e., Asn-166 and Tyr-170) (10). Although Asn-166 is not conserved, both Tyr-170 and Glu-171 are structurally conserved among the known Cry toxins (see Fig. 1A,B) with the result that these loop residues could play a crucial role in Cry4B toxicity. In this study, we therefore initially generated three Cry4B loop mutants in which Asn-166, Tyr-170, and Glu-171 were substituted with alanine via PCR-based directed mutagenesis. When each mutant toxin was expressed in *E. coli* upon IPTG induction, all were predominantly produced as sedimentable inclusion bodies, and the protein expression level was comparable to that of the wild-type Cry4B toxin. In addition, all of the purified mutant inclusions were found to be soluble in carbonate buffer, pH 9.0, giving approx 70% solubility, which closely resembles the solubility of wild-type inclusions under similar conditions (data not shown). The 130-kDa solubilized mutant protoxins were also assessed for their proteolytic stability by digestion with trypsin, and all were found to produce two major trypsin-resistant products, of approx 47 kDa and 18 kDa, respectively, that were identical to products of the wild-type toxin (Fig. 2, lanes 1–4). These results suggested that the point mutations in the loop residues of the mutant toxins had no apparent effect on proteolytic processing or protein folding.

To determine an effect of the loop mutations on toxicity, we tested *E. coli* cells expressing each type of the mutant toxin for their relative biologi-

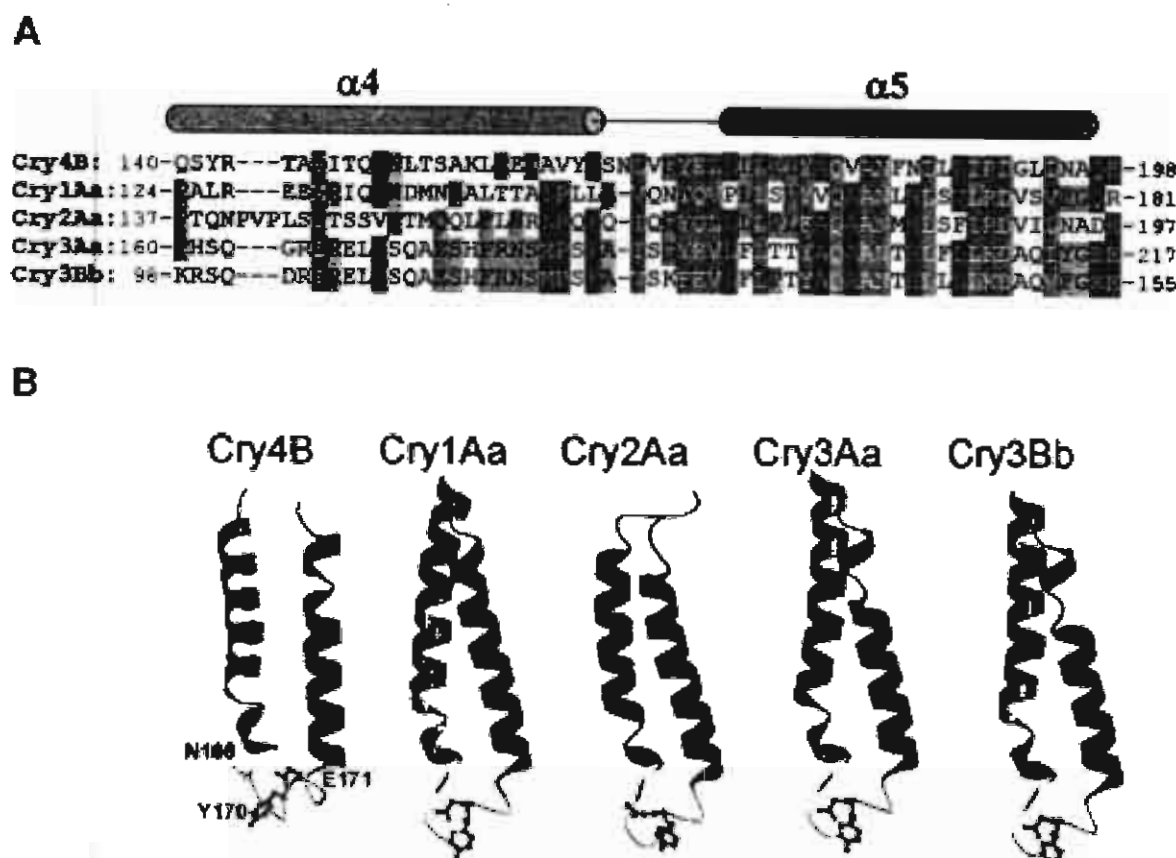


Fig. 1. (A) Multiple sequence alignment of the transmembrane $\alpha 4$ -loop- $\alpha 5$ fragment of Cry4B with those of the known structures of Cry1Aa, Cry2Aa, Cry3Aa, and Cry3Bb toxins. The sequences were aligned using the program CLUSTALX. (B) Side views of the $\alpha 4$ -loop- $\alpha 5$ helical hairpins of a homology-based Cry4B model and Cry1Aa, Cry2Aa, Cry3Aa, and Cry3Bb crystal structures. Grey ribbons represent helix 4 and helix 5. Balls and sticks show the mutated residues in Cry4B comparison with Cry1Aa, Cry2Aa, Cry3Aa, and Cry3Bb. Asn-166 is shown in Cry4B. Two relatively conserved loop-residues, Tyr and Glu or Gln, are shown in all five helical hairpins.

cal activity against 2-d old *A. aegypti* larvae. All bioassays were conducted in 10 replicas for each sample, and were repeated at least three times. The mortality data recorded after a 24-h incubation are shown in Fig. 3. Alanine substitutions of Asn-166 and Tyr-170 almost completely abolished the Cry4B bioactivity, although mutation at Glu-171 produced only a small decrease in larvicidal activity. These results suggested that Asn-166 and Tyr-170 play an important role in the larvicidal activity of the Cry4B toxin.

Further substitution of Asn-166 with Asp, Gln, Arg, Cys, or Ile, revealed that substitutions of Asn-166 with polar amino acids could preserve more than 80% of the toxicity of the wild-type

toxin, whereas substitution with a nonpolar residue (i.e., isoleucine) almost totally abolished the toxicity (see Fig. 3). All of the Asn-166-mutant toxins were tested for toxin stability, which revealed that all produced stable, trypsin-resistant products, as shown for some of these mutants in Fig. 2 (lanes 6 and 7). These results suggested that the polarity at position 166 in the $\alpha 4$ - $\alpha 5$ loop is important for larvicidal activity of the Cry4B toxin. Molecular modeling of a putative toxin-induced pore consisting of six copies of the $\alpha 4$ - $\alpha 5$ helical hairpin of Cry4B (see Fig. 4A) indicated that Asn-166 points toward the pore lumen and could form hydrogen bonds with water molecules. A crucial role in the mechanism of toxicity at this

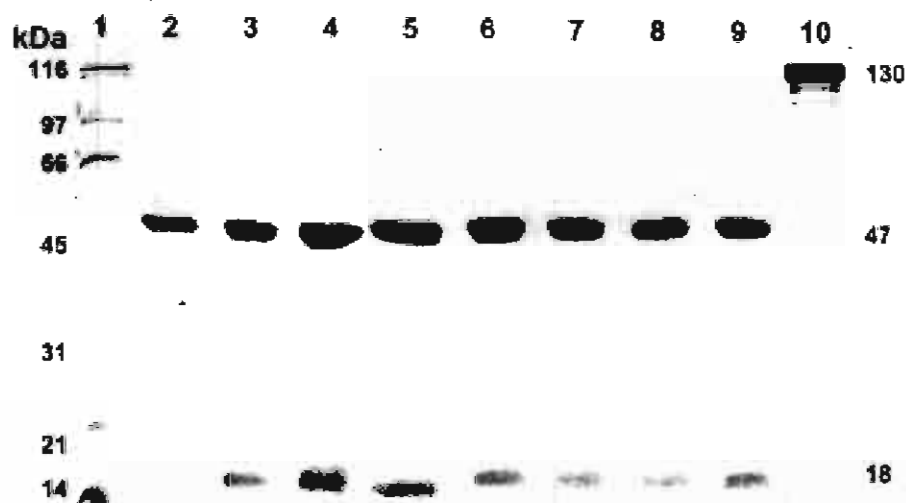


Fig. 2. SDS-PAGE (12.5% gel) analysis of proteolytic processing of Cry4B and its mutant toxins. Lane 1: molecular mass standards, Lanes 2 and 10: trypsin-treated products and the 130-kDa protoxin of wild-type Cry4B, respectively. Lanes 3–9: mutant representatives, consisting of N166A, Y170A, E171A, N166I, N166D, Y170D, and Y170F, respectively, after digestion with trypsin.

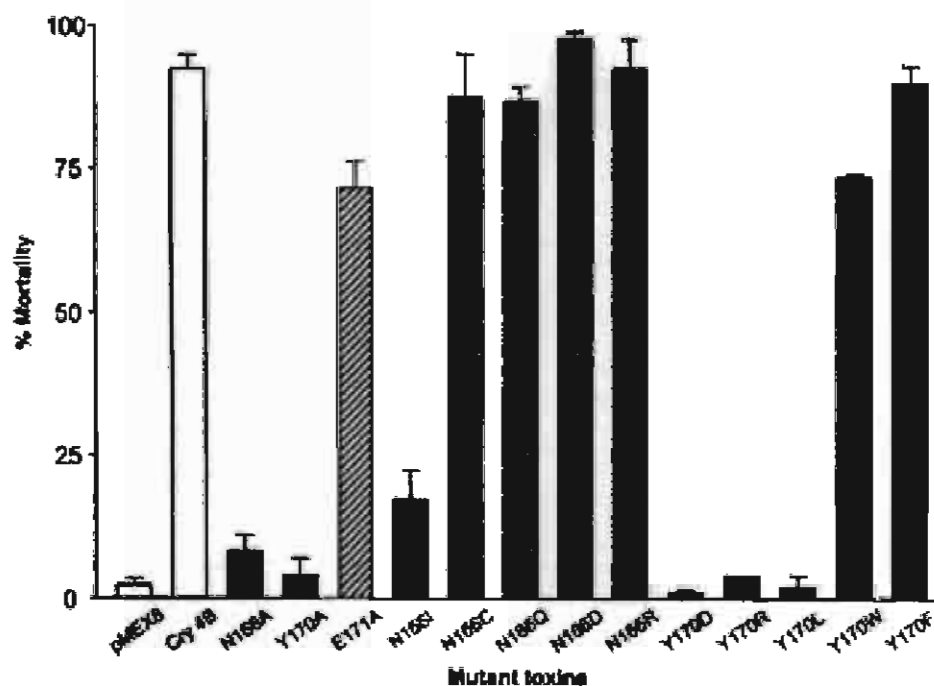


Fig. 3. Mosquito-larvicidal activities of *E. coli* cells expressing wild-type Cry4B toxin or mutant toxins (N166A, Y170A, E171A, N166I, N166C, N166Q, N166D, N166R, Y170D, Y170R, Y170L, Y170W, and Y170F) against *A. aegypti* larvae. Error bars indicate standard errors of the mean from three independent experiments.

critical position could conceivably be the formation of hydrogen bonds with water to stabilize the loop structure, or involvement in the passage of ions through the channel (see Fig. 4A). Whether

these possibilities can be generalized remains to be elucidated.

Tyr-170 was also converted to Asp, Arg, Leu, Trp, or Phe. Interestingly, substitutions of this

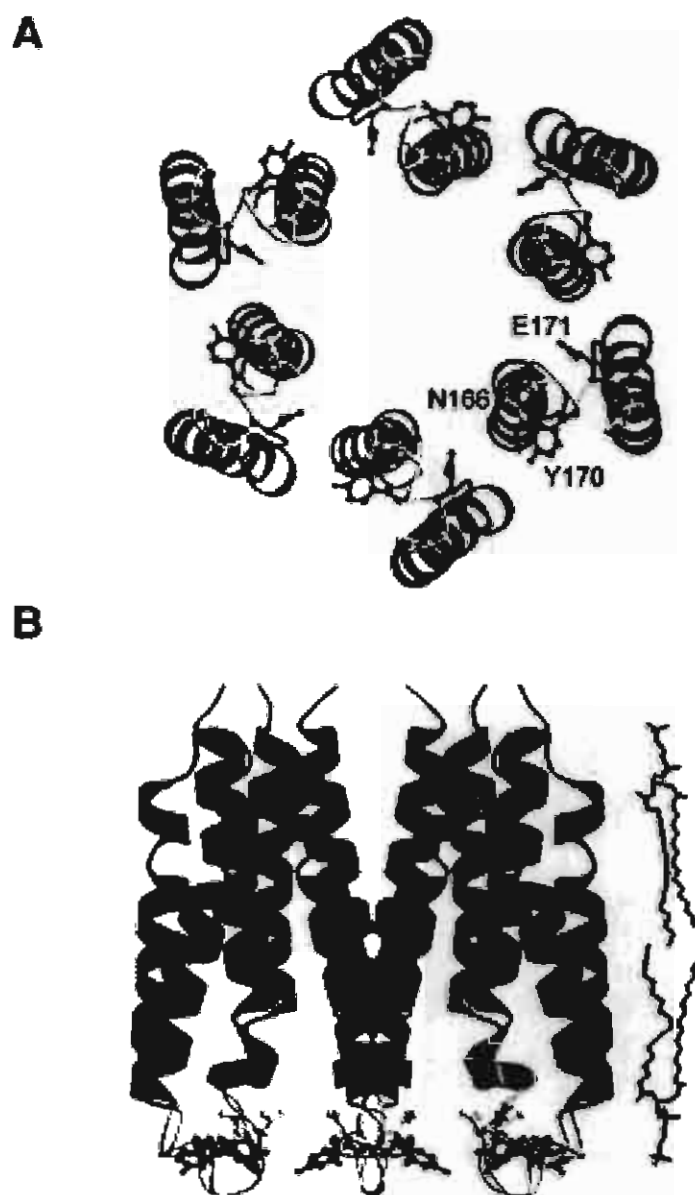


Fig. 4. (A) Bottom view of the model of a putative oligomeric pore consisting of six copies of the $\alpha 4$ - $\alpha 5$ hairpin of the Cry4B toxin. Asn-166, Tyr-170, and Glu-171 are shown as balls and sticks. (B) The Tyr-170 residues are found at the membrane-water interface after oligomerization and pore formation. Rectangular boxes represent the planar lipid-membrane bilayers. Helices 4 and 5 are shown as grey ribbons.

critical tyrosine residue with only the aromatic residues (Trp or Phe) were shown to preserve Cry4B toxicity against mosquito larvae (see Fig. 3). Also, the protein expression levels, inclusion solubility, and proteolytic stability of the three other inactive mutants (Y170D, Y170R, and Y170L) suggested that their observed drastic loss of toxic-

ity is unlikely to be caused by misfolding of the protein. These results, together with the high level of conservation of the tyrosine residue at this position among the Cry toxins, strongly implies a general requirement for an aromatic structure at this crucial position. In several membrane proteins, a variety of roles have been proposed for

the aromatic residues, especially Tyr and Trp in the transmembrane helices, which are preponderantly found at or near the membrane-water interface (23–25). These roles include facilitating translocation of the periplasmic portion of proteins through the membrane, acting as determinants of protein orientation (26), introducing rigidity to the periphery of the transmembrane segments (27), or allowing vertical mobility of the transmembrane helical region with respect to the membranes (28). Considering all of the above, a function for Tyr-170 within the $\alpha 4$ – $\alpha 5$ loop of Cry4B may conceivably be an interaction with the phospholipid head groups for stabilizing the oligomeric pore structure (see Fig. 4B).

In conclusion, our results indicate that the characteristics of polarity and aromaticity for residues at positions 166 and 170, respectively, in the $\alpha 4$ – $\alpha 5$ loop play a crucial role in toxicity of the 130-kDa Cry4B toxin. However, further studies are needed to determine the exact role of these two critical residues in toxin function.

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Ion channels formed in planar lipid bilayers by the dipteran-specific Cry4B *Bacillus thuringiensis* toxin and its $\alpha 1$ – $\alpha 5$ fragment

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Summary

Trypsin activation of Cry4B, a 130-kDa *Bacillus thuringiensis* (Bt) protein, produces a 63-kDa toxin active against mosquito larvae. The active toxin is made of two protease-resistant products of ca. 45 kDa and ca. 20 kDa. The cloned 21-kDa fragment consisting of the N-terminal region of the toxin was previously shown to be capable of permeabilizing liposomes. The present study was designed to test the following hypotheses: (1) Cry4B, like several other Bt toxins, is a channel-forming toxin in planar lipid bilayers; and (2) the 21-kDa N-terminal region, which maps for the first five helices ($\alpha 1$ – $\alpha 5$) of domain 1 in other Cry toxins, and which putatively shares a similar tri-dimensional structure, is sufficient to account for the ion channel activity of the whole toxin. Using circular dichroism spectroscopy and planar lipid bilayers, we showed that the 21-kDa polypeptide existed as an α -helical structure and that both Cry4B and its $\alpha 1$ – $\alpha 5$ fragment formed ion channels of 248 ± 44 pS and 207 ± 23 pS, respectively. The channels were cation selective with a potassium-to-chloride permeability ratio of 6.7 for Cry4B and 4.5 for its fragment. However, contrary to the full-length toxin, the $\alpha 1$ – $\alpha 5$ region formed channels at low dose; they tended to remain locked in their open state and displayed flickering activity bouts. Thus, like the full-length toxin, the $\alpha 1$ – $\alpha 5$ region is a functional channel former. A pH-dependent, yet undefined region of the toxin may be involved in regulating the channel properties.

Keywords: *Bacillus thuringiensis*, δ -endotoxin, truncated protein, planar lipid bilayer, ion channel.

Introduction

Bacillus thuringiensis (Bt) is an endospore-forming bacterium that produces larvicidal proteins (Cry and Cyt δ -endotoxins) as crystalline inclusions during sporulation [1]. These proteins are highly toxic to a wide variety of important insect pests as well as other invertebrates, which explains the extensive use of Bt as a biological control agent. To date, 40

classes of cry gene sequences have been identified based on their deduced amino acid sequence identity [2]. Upon ingestion by susceptible larvae, the proteic crystals are solubilized and proteolytically cleaved in the alkaline mid-gut juice to produce the activated toxins [1]. These, in turn, bind to specific receptors on the mid-gut epithelial cells and form pores in their membranes, resulting in cell swelling and eventually cell lysis due to osmotic imbalance [3,4].

The 130-kDa Cry4A and Cry4B protoxins produced by Bt sub-species *israelensis* are toxic, following solubilization and proteolytic processing in the insects' gut, to *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus* mosquito larvae [5]. These mosquitoes are serious human disease vectors that transmit malaria, filarial parasites, dengue and yellow fever virus. The exact mode of action of Cry4 toxins is not known. It has been proposed that most Cry toxins share a similar folding based on the five highly conserved regions of their primary amino acid sequences. This is supported by the fact that the atomic resolution crystal structures obtained for Cry1Aa [6], Cry2Aa [7], Cry3A [8] and Cry3Bb1 [9] toxins are very similar. *In vitro* proteolysis of the full-length Cry4A and Cry4B protoxins produces activated toxins that are composed of two non-covalently associated fragments resulting from the cleavage of the loops between helices $\alpha 5$ and $\alpha 6$ [5,10], as deduced from three-dimensional models of Cry4A and Cry4B generated by homology modelling (unpublished data). This cleavage is critical for *in vitro* cytolytic activity [10].

Several activated Cry toxins, including Cry1Aa [6,11–14], Cry1Ac [13,15–17], Cry1B [13], Cry1C [18], Cry2A [19], Cry3Aa [16] and Cry3B2 [20], have the ability to form ion channels in receptor-free phospholipid vesicles and planar lipid bilayer membranes. Different Cry toxins have conductances ranging from a few pS to over 1000 pS, suggesting that certain Cry classes might have specific channel characteristics. Since the N-terminal domain of Cry toxins, which is made of seven to eight helices, is thought to play a crucial role in membrane insertion and pore formation [14,21–29], we hypothesized that the first five helices of the Cry4B toxin may constitute the putative pore-forming region responsible for membrane permeabilization by this toxin. This is supported by the fact that Cry4B helices 4 and 5 play an important role in mosquito larvicidal activity [28,30] and that the $\alpha 1$ – $\alpha 5$ fragment of the toxin permeabilizes liposomes [31].

In this study, ion channel formation by the activated Cry4B toxin and its $\alpha 1$ – $\alpha 5$ fragment was investigated. The results show that both form cation-selective channels that have different properties under identical pH conditions. Therefore, while the $\alpha 1$ – $\alpha 5$ region is sufficient to account for the permeabilizing effect of the whole toxin, the overall channel activity of the toxin is also dependent on a gating region that may have different properties depending on the pH condi-

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lions under which the protoxin has been processed and may map for a part of the activated toxin located outside its $\alpha 1-\alpha 5$ region.

Results

Cry4B and $\alpha 1-\alpha 5$ secondary structures

The secondary structures of the 65-kDa activated Cry4B toxin and its $\alpha 1-\alpha 5$ fragment were examined by circular dichroism spectroscopy. Figure 1 shows the CD spectra of Cry4B and the $\alpha 1-\alpha 5$ polypeptide in the 185–280-nm region. Since a negative band at about 222 nm is typical of α -helix content due to the $n\pi^*$ transition, the helical content was interpreted by the molar ellipticity at this wavelength. The activated Cry4B toxin and the $\alpha 1-\alpha 5$ fragment exhibited helical content values of 33% and 61%, respectively. These values are close to those calculated with the Swiss PDB software used to model the full-length toxin and its $\alpha 1-\alpha 5$ peptide [32], i.e. 31% and 63%, respectively.

Channel properties of Cry4B and its $\alpha 1-\alpha 5$ region

Under symmetrical conditions, Cry4B channel activity was observed after about 1 hour following addition of 150–300 nM of the toxin, whereas the channels formed by the $\alpha 1-\alpha 5$ region of the toxin were observed after a few minutes (never more than 30 min) at doses as low as 4.3 nM. Representative current traces of Cry4B and $\alpha 1-\alpha 5$ channels are shown in Figures 2(A) and 3(A) respectively.

The Cry4B toxin, solubilized and activated at pH 10.5, displayed multiple amplitudes with some steps occurring from the closed state, i.e. from $I = 0$, and showed many sub-

conducting states. In all Cry4B experiments, the current-voltage relations were rectilinear (Figure 2(C)), demonstrating the ohmic behaviour of the channels. The channel conductances, obtained by Method 1 described in the Experimental procedures, ranged from 19 ± 4 to 399 ± 44 pS (Table 1). When all the current steps were analysed (Method 3 in Experimental procedures), the conductances were found to be between 27 ± 10 and 248 ± 44 pS (Table 1). Fast kinetic activity was observed in a few experiments at some applied voltages (Figure 2(B)). Ion selectivity was measured by conducting experiments under asymmetrical conditions. The reversal potential, V_R , of the Cry4B channels was -20 ± 2 mV (Table 1). Under the same ionic gradient conditions, the Nernst potential for K^+ , calculated at 25°C, was -28.2 mV, demonstrating the cation selectivity of the channels. The P_K/P_{Cl} permeability ratio was calculated using the Goldman-Hodgkin-Katz equation and the V_R value above. It was equal to 6.7, confirming the cationic selectivity of the Cry4B channels.

Unlike Cry4B, the $\alpha 1-\alpha 5$ fragment of the toxin could not be solubilized in 50 mM Na_2CO_3 buffer, pH 10.5, so 50 mM Tris buffer, pH 8.8, was used instead. In all experiments, some of the $\alpha 1-\alpha 5$ channels remained always open. Their current-voltage curves were rectilinear and their conductances, determined by Method 2 in Experimental procedures, ranged from 148 to 3243 pS (Figure 3(C) and Table 1). The conductances of all the current steps were between 35 ± 4 and 207 ± 23 pS (Table 1). At a 430-nM dose, the $\alpha 1-\alpha 5$ channels had very large conductances (in excess of 650 pS, and often larger than 1000 pS), quite larger than those of Cry4B, which were found to be in the 20–400-pS range, at similar doses, when the full-length toxin had been prepared at pH 10.5 (Table 1). To exclude the possibility that the large

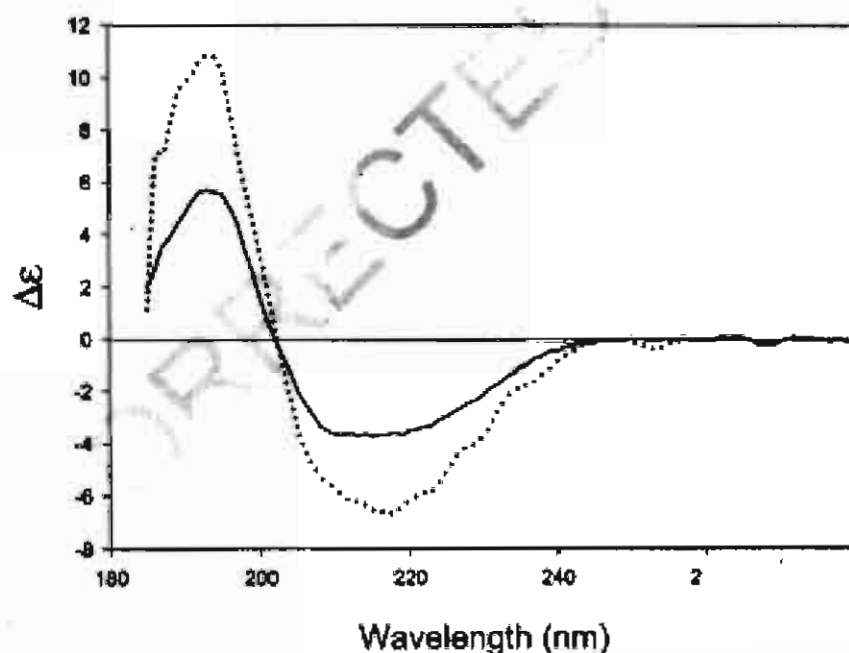


Figure 1. Far UV circular dichroism spectra of the 65-kDa activated Cry4B toxin and its $\alpha 1-\alpha 5$ polypeptide. The CD spectra of Cry4B ($n = 3$) in 10 mM NaH_2PO_4 , pH 8.0, and the $\alpha 1-\alpha 5$ fragment ($n = 5$) in 50 mM Tris, pH 8.8, are shown by dotted and solid lines, respectively. Ellipticity units ($\Delta\epsilon$, vertical scale) are per residue molar ellipticity.

Channels of full-length and truncated Bt toxins

3

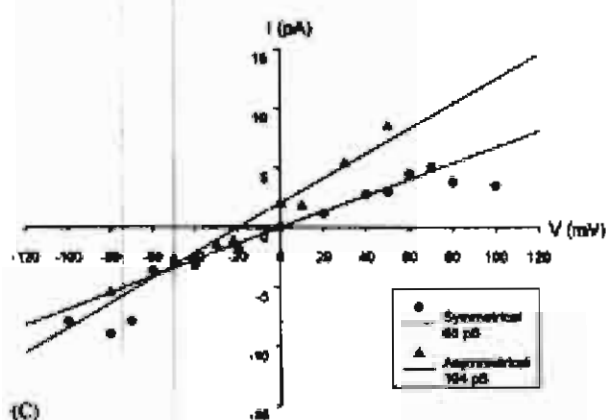
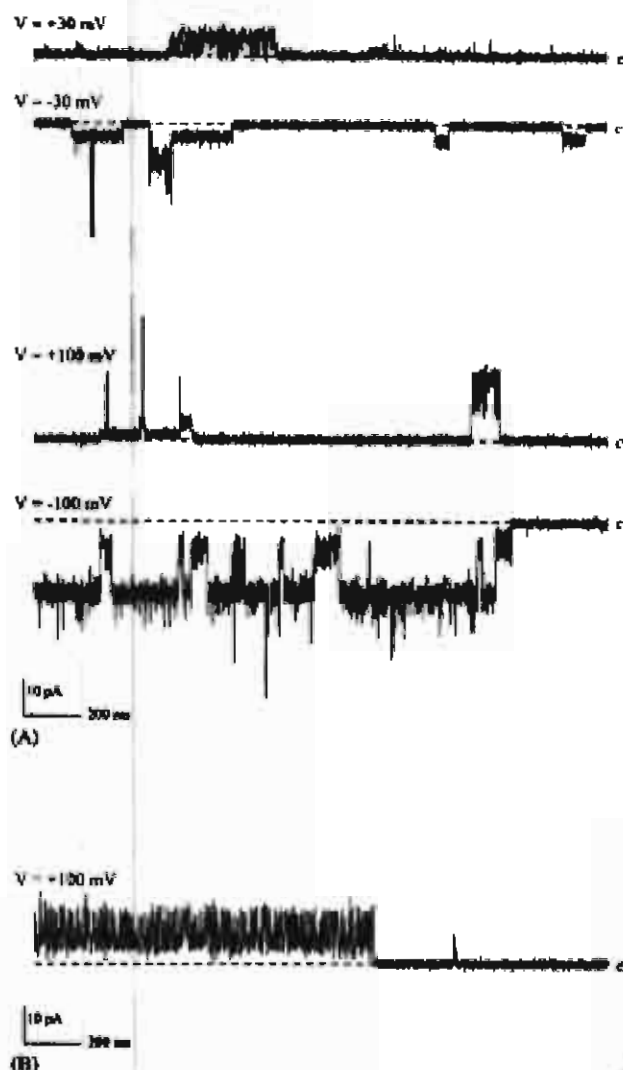


Figure 2. Single-channel current traces of the activated Cry4B toxin. (A) and (B) Representative segments of typical current traces recorded in symmetrical 150 mM KCl solution at various holding voltages, V , as indicated next to the traces. The current levels corresponding to the closed state of all channels are indicated by the letter c and dashed lines. (B) Episode of fast kinetic activity of Cry4B channel under symmetrical conditions. Current-voltage relations obtained from currents recorded first under symmetrical (solid circles) and then under asymmetrical conditions (solid triangles). Data are representative of three experiments. Under asymmetrical conditions, the reversal potential was shifted to -20 mV, which demonstrates the cationic selectivity of the activated Cry4B toxin channels. The conductances measured under each set of conditions are given in the text box shown on the graph. Data points were fitted by linear regression.

$\alpha 1$ - $\alpha 5$ channels corresponded to the superposition of several smaller channels with a very high probability of opening, smaller $\alpha 1$ - $\alpha 5$ polypeptide concentrations were used, i.e. doses from 4.3 to 8.6 nM. Channel activity was still observed under these reduced dose conditions. Two differ-

ent kinetic behaviours were observed in $\alpha 1$ - $\alpha 5$ channels. In some experiments, several channels were active and their open probability was very high (Figure 3(A)). In other experiments, the open probability of the channels was smaller (Figure 3(B)). Fast kinetics was also displayed in

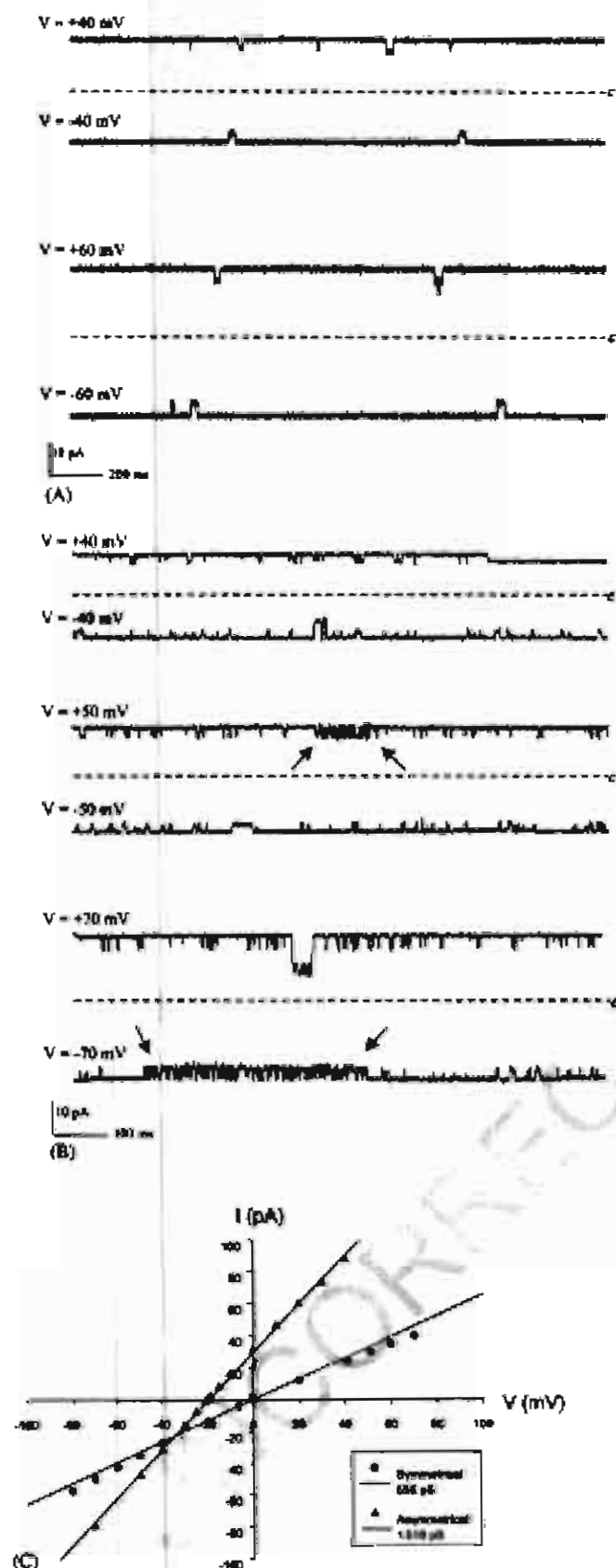


Figure 3. Single-channel current traces of Cry4B's $\alpha 1$ – $\alpha 5$ fragment channels. (A) and (B) Representative segments of typical current traces recorded under 150 mM KCl symmetrical conditions at various holding voltages, V , as shown next to the traces. The current levels corresponding to the closed state of all channels are indicated by the letter c and dashed lines. (A) Representative records of channels displaying a very high open probability. Note that there are at least four identical channels active in this experiment, with at least three channels simultaneously locked in their open states. (B) Representative records showing channel activity with average open probability and flickering behaviour (shown between arrows). At least five channels were active in this experiment, with at least two channels simultaneously locked in their open states. (C) Current–voltage relations obtained from currents recorded first under symmetrical (solid circles) and then under asymmetrical conditions (solid triangles). Data are representative of three experiments. Under asymmetrical conditions, the reversal potential was shifted to -19 mV, which shows the cationic selectivity of the $\alpha 1$ – $\alpha 5$ fragment channels, similar to that of the full-length toxin. The conductances measured under each set of conditions are given in the text box shown on the graph. Data points were fitted by linear regression.

Table 1. Biophysical properties of the ion channels formed by the activated Cry4B toxin and its $\alpha 1$ - $\alpha 5$ fragment.

Channel properties	Activated Cry4B ^{a,b}	$\alpha 1$ - $\alpha 5$ fragment ^{a,b}
Conductances ^c	Calculation method ^d	Calculation method ^e
	19 $\pm$ 4 (n = 4)	148 (n = 1)
	79 $\pm$ 8 (n = 3)	233 $\pm$ 13 (n = 2)
	141 $\pm$ 1 (n = 5)	412 $\pm$ 14 (n = 4)
	399 $\pm$ 44 (n = 3)	681 $\pm$ 13 (n = 4)
		790 $\pm$ 14 (n = 4)
		1014 $\pm$ 16 (n = 5)
		1529 $\pm$ 9 (n = 2)
		1784 (n = 1)
		2444 (n = 1)
		3243 (n = 1)
Minimum and maximum conductances obtained from all observed current steps ^{c,f}	27 $\pm$ 9 (n = 14)	35 $\pm$ 4 (n = 19)
V_R , reversal potential (mV) ^g	248 $\pm$ 44 (n = 14)	207 $\pm$ 23 (n = 19)
P_K/P_{Cl} , permeability ratio	-20 $\pm$ 2 (n = 11)	-17 $\pm$ 1 (n = 12)
	6.7	4.5

^amean $\pm$ SEM^bn indicates the number of experiments^cexperiments were conducted under symmetrical KCl conditions^dconductances were determined using Method 1 (see Experimental procedures)^econductances were determined using Method 2 (see Experimental procedures)^fconductances were determined using Method 3 (see Experimental procedures)^gexperiments were conducted under asymmetrical KCl conditions

three experiments, but not at every applied voltage (Figure 3(B), arrows). Under asymmetrical conditions, the $\alpha 1$ - $\alpha 5$ fragment formed cation-selective channels with V_R equal to -17 ± 1 mV and a P_K/P_{Cl} permeability ratio of 4.5.

When the Cry4B protoxin was solubilized and activated at pH 9.0, it showed on 15% SDS-PAGE the same two major bands at about 47 kDa and 18 kDa as those seen with the toxin prepared at pH 10.5 (data not shown), but its channel activity was different. In all experiments, some of the channels remained always open, i.e. the current never returned to zero. The channels displayed multiple conductances varying from 16 pS to 3301 pS, similar to those observed with the $\alpha 1$ - $\alpha 5$ fragment (data not shown). On the other hand, the selectivity and the kinetic behaviour of the full-length toxin obtained from the Cry4B protoxin prepared at pH 9.0 did not differ from those of the toxin resulting from Cry4B solubilization and activation at pH 10.5 (data not shown).

Discussion

This study clearly demonstrates that the dipteran-specific Cry4B toxin and its $\alpha 1$ - $\alpha 5$ fragment form cation-selective channels in planar lipid bilayers, suggesting that the $\alpha 1$ - $\alpha 5$ region is sufficient to form functional channels like those assembled by the full-length 65-kDa toxin. This concept is supported by previous studies that showed that the N-terminal domains of Cry1Ac, Cry3A and Cry3B2—i.e. the $\alpha 1$ - $\alpha 7$ regions of these toxins—form channels that compare well to those of the full-length toxins [20,33,34]. The present study provides the first evidence that the first five helices of a Cry toxin can function efficiently as ion channels.

The incorporation time of the $\alpha 1$ - $\alpha 5$ fragment into planar lipid bilayers was faster than that of the full-length toxin and lower doses (4.3 nM) were required for the fragment to induce channel activity comparable to that of Cry4B and

other Cry toxins, for which at least 70–150 nM are needed to induce activity. Furthermore, the $\alpha 1$ - $\alpha 5$ channel conductances were generally larger than those of the 65-kDa toxin (Table 1). It is conceivable that the $\alpha 1$ - $\alpha 5$ fragment, which is produced in aggregated form larger than 200 kDa, has a higher oligomerization order than the full-length toxin, which would explain the larger conductances observed. It is also possible that the smaller polypeptide is more flexible and requires a lesser conformational change to insert into the membrane, which would account for its faster integration into the membrane.

Interestingly, the pH conditions used to solubilize and activate the 130-kDa Cry4B protoxin affected both the conductance range of the channels formed by the 65-kDa toxin and their activity. When the protoxin was treated at pH 10.5, the conductance of the resulting toxin was found to be between 19 pS and 399 pS, and the channel currents did return completely to zero, whereas the range of the channel conductances of the 65-kDa toxin produced at pH 9.0 was far wider, i.e. from 16 pS to 3300 pS, and the channels never closed fully.

One simple explanation for the different kinetic behaviour of the full toxin and that of the $\alpha 1$ - $\alpha 5$ polypeptide may be that the residues involved in the transition from the open state to the closed state of the Cry4B channels are located in a region of the toxin that is not mapped by the $\alpha 1$ - $\alpha 5$ polypeptide. Their absence in the truncated protein would therefore account for the tendency of the $\alpha 1$ - $\alpha 5$ channels to remain open. However, when Cry4B was prepared at pH 9.0 rather than pH 10.5—a lower pH, which did not result in toxin truncation as demonstrated by the presence of the two major bands observed at about 47 kDa and 18 kDa on SDS-PAGE—its channel kinetic activity resembled that of the $\alpha 1$ - $\alpha 5$ polypeptide. Thus, it cannot be excluded that the residues involved in gating are located in the $\alpha 1$ - $\alpha 5$ region itself and that, under the reduced preparative pH conditions used for

the full toxin (pH 9.0) and its $\alpha 1$ – $\alpha 5$ fragment (pH 8.8), the capacity of the gating residues to return the channels to the closed state was inhibited, which would explain the similar kinetic behaviour of Cry4B and its $\alpha 1$ – $\alpha 5$ fragment under these conditions. This pH-dependence of the channel activity remains to be elucidated further.

Therefore, our results suggest that the pH at which the protoxin is processed affects the regions of the toxin involved in oligomerization, cluster assembly and gating due to protonation or deprotonation of certain residues. There is evidence that particular stretches in domain 3 of Cry1Aa and Cry1Ac toxins are involved in ion channel regulation [12,35]. Moreover, it has been reported that pH directly affects Cry1C channel selectivity and conductance [18] and that reducing pH promotes unfolding of this toxin [36]. Furthermore, it has been shown recently that large conductances of Cry1C channels are actually due to clusters of channels of smaller conductances [37].

Taken together, our data demonstrate that the major determinant of Cry4B pore formation is the $\alpha 1$ – $\alpha 5$ segment of the putative domain 1 of the toxin, a region that functions as an efficient channel-forming polypeptide without the remainder of the activated protein. Our results also show that Cry4B channel size and gating depends on the pH conditions used for the processing of the protoxin. While it is possible that assembly and gating of the channels are regulated by residues located outside the $\alpha 1$ – $\alpha 5$ region of the toxin, it cannot be excluded that they may be found within the $\alpha 1$ – $\alpha 5$ segment itself, in an area that is sensitive to pH during the processing of the truncated polypeptide and the 135-kDa protoxin. Further work is needed to locate this particular region of the Cry4B protein and to clarify the role of pH in its gating and pore-assembly properties.

Experimental procedures

Toxin preparation

Cry4B proteins and the $\alpha 1$ – $\alpha 5$ polypeptides were expressed as inclusion bodies produced from the cloned *cry4B* and *$\alpha 1$ – $\alpha 5$* genes in *E. coli*. They were partially purified as described earlier [31]. Cry4B was solubilized and then trypsin-activated in 50 mM Na_2CO_3 , pH 9.0 or 10.5, except for secondary structure determination where 10 mM NaH_2PO_4 , pH 9.0, was used instead. Protein concentrations were determined using a Bio-Rad protein quantitative kit with bovine serum albumin (Sigma-Aldrich Corp., St Louis, MO, USA) as a standard. The activated Cry4B toxin was further purified by size-exclusion chromatography using a Superose 12 column (Amersham Biosciences Corp., Piscataway, NJ, USA). The purified fractions were analysed by SDS-15% w/v polyacrylamide gel electrophoresis. Purified Cry4B came out as a monomeric form with its two fragments of 47 kDa and 21 kDa attached together.

Since the $\alpha 1$ – $\alpha 5$ fragment could not be solubilized in 50 mM Na_2CO_3 buffer, pH 10.5, 50 mM Tris buffer, pH 8.8, was used. The solubilized protein was passed through a Centricon-10 (Millipore, Billerica, MA, USA) for concentrating before injection on a Superdex 200 FPLC column (Amersham Biosciences Corp., Piscataway, NJ, USA). The protein was detected in the void volume as an aggregate larger than 200 kDa (data not shown). Prior to use, toxin solutions were vortexed and sonicated for about 1 min to prevent aggregation.

Circular dichroism (CD) spectroscopy

CD spectra of the activated Cry4B toxin and the $\alpha 1$ – $\alpha 5$ polypeptide were measured with a Jasco J-715 spectropolarimeter (Jasco Inc., Easton, MD, USA). They were scanned between 185 nm and 280 nm at room temperature in a rectangular quartz cuvette with a 0.2-mm optical path length. CD measurements of Cry4B ($n = 3$) and its $\alpha 1$ – $\alpha 5$ fragment ($n = 5$) were taken at a rate of 20 nm/min. The concentration of the full-length toxin and its truncated polypeptide was equal to 4.5×10^{-5} M and 3×10^{-5} M, respectively, as determined by far UV absorbance. Spectra were corrected for solvent baseline obtained under the same conditions. The molar circular dichroism $\Delta\epsilon$ at a given wavelength was calculated with the following formula:

$$\Delta\epsilon = (K \times \theta \times M_w) / (c \times N \times d)$$

where K was equal to 3298×10^6 , θ was the measured ellipticity (in millidegrees), M_w was the molecular weight of the sample, c was the sample concentration (in g/ml), N was the number of peptide bonds of the sample and d was the optical path length of the sample cell (in cm). Reference spectra were obtained and scale calibration at 290 nm was performed with camphorsulfonic acid (CSA, Sigma-Aldrich Corp., St. Louis, MO, USA) and the θ value of CSA (1 mg/ml) was measured at the beginning of each experiment, giving $\Delta\epsilon$ (192.5 nm)/ $\Delta\epsilon$ (290.5 nm) = 2.08 [38]. The helical contents of the protein and its fragment were derived from their $\Delta\epsilon$ determined at 222 nm using $\Delta\epsilon$ (222 nm) = 10.6 as the value for 100% helicity [39].

Planar lipid bilayers

Planar lipid bilayers were formed from the 7:2:1 (w/w/w) lipid mixture of PE, PC and Ch [18]. The final concentration was 25 mg/ml dissolved in decane. The lipid bilayer was painted, using a pre-pulled glass pipette dipped in the decane-lipid solution, on a 200- μm orifice drilled in a Delrin cup and pre-treated with the same lipid mixture. Membranes had a typical capacitance of 150–250 pF and remained stable for several hours. Before toxin or polypeptide reconstitution, the bilayer was monitored under non-zero holding voltage conditions for more than 30 min to ensure that no contaminant-induced channel activity was present. Incorporation was performed by adding aliquots of the protein or its fragment directly to the cis chamber, which was stirred using a small magnetic flea until channel activity was observed. The insertion process was facilitated by applying a 100-mV holding potential across the bilayer. Channel activity was detected by the presence of distinct current jumps recorded during test voltage steps applied across the planar lipid bilayer. Activated Cry4B was used at concentrations between 150 nM and 300 nM, whereas the $\alpha 1$ – $\alpha 5$ fragment concentration ranged from 4.3 nM to 430 nM. All experiments were performed at room temperature (22–25°C).

Data recording and analysis

Single channel currents were recorded with an Axopatch-1D patch-clamp amplifier (Axon Instruments, Foster City, CA). Signals were digitized with a Digidata 1200 analogue-to-digital converter using Axoscope 8.0 software (both from Axon Instruments) at a 50-kHz sampling frequency. They were low-pass filtered at 600 Hz and analysed on a personal computer using Axoscope 8.0 software.

Channel conductances were determined as follows. In experiments where channel currents returned to the baseline—i.e. to the current level observed at any particular voltage in the absence of either the toxin or its fragment—the largest amplitude of the current steps observed from, or to, the baseline level was plotted against applied voltage to generate current–voltage relations, or I – V curves (Method 1). In experiments where channel currents never returned to the baseline—i.e. in which at least one channel remained always open—the main channel current was obtained from the amplitude of the smallest current level observable for at least 50% of the duration of the recording at each voltage, and the corresponding I – V curves were obtained (Method 2). Finally, all observable current steps were measured for each voltage and their amplitudes were plotted versus

voltage (Method 3). Using the three methods described above, the channel conductances were then derived from the slopes of the linear regression lines to the data points. The conductances obtained for each individual experiment were then averaged over the number of experiments performed under the same conditions. Results are expressed as means $\pm$ SEMs.

Ion selectivity was measured under 450/150 mM KCl (cis/trans) asymmetrical conditions. The reversal potential, V_R , was obtained as the voltage for which the corresponding linear regression intersected the horizontal axis of the I-V curves, and was compared to the value calculated for K^+ according to Nernst equation [40]. Channel selectivity for K^+ over Cl^- was derived from P_K/P_{Cl} permeability ratios calculated using V_R and the Goldman-Hodgkin-Katz equation [40].

Chemicals and solutions

Phosphatidylethanolamine (PE), phosphatidylcholine (PC) and cholesterol (Ch) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals used were of analytical grade (from various suppliers). Under symmetrical conditions, both the cis and the trans chambers of the bilayer experimental apparatus contained 150 mM KCl, 1 mM $CaCl_2$ and 10 mM Tris-HCl, pH 9.0. For ion selectivity determination, ionic gradients were established with 450 mM KCl on the cis side and 150 mM KCl in the trans side of the bilayer.

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