

Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis*

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To investigate the membrane pore structure of Cyt2Aa1 toxin from *Bacillus thuringiensis*, 14 single-cysteine substitutions of the toxin were constructed. Five of these mutants (L172C, V186C, L189C, E214C and L220C) yielded characteristic products when processed by proteinase K; other mutants were degraded by this enzyme. Mutants that yielded characteristic proteolysed products and wild-type toxin were labelled with polarity-sensitive acrylodan (6-acryloyl-2-dimethylaminonaphthalene) at the thiol group of cysteine residues. A green–blue shift in the emission spectra was observed with all labelled toxins on transfer from an aqueous solution into a solution containing membranes or liposomes from red blood cells. These results suggested that the label moved into the hydrophobic environment of the membrane or became buried within hydrophobic regions of the protein oligomers. Digestion of membrane-bound labelled toxin with proteinase K did not cause a significant decrease in emission

intensity from any of the labelled mutants. This suggests that L172C, V186C, L189C, E214C and L220C are inserted into the membrane and are therefore protected from proteolysis. In contrast, a marked decrease in emission intensity was observed when membrane-bound labelled wild-type toxin was digested with proteinase K. This suggests that Cys-19 does not insert into the membrane. Fluorimetric analysis of delipidated pore complexes suggests that L172C, V186C, L189C and E214C point towards the lipid in the membrane, whereas L220C is either within the hydrophobic environment of the protein oligomers or exposed to the membrane lipids. Most of the Cys-19 from wild-type molecules is enclosed within the hydrophobic pockets of the protein oligomers.

Key words: acrylodan labelling, Cyt2Aa1 toxin, emission spectrum.

INTRODUCTION

Bacillus thuringiensis is a Gram-positive spore-forming bacterium that forms insecticidal protein toxin crystals during sporulation [1]. The toxins are divided into two major families (Cry and Cyt) on the basis of sequence similarities [2]. The Cyt toxin family is distinct from the Cry family in a number of ways [1,3]. Only the Cyt toxins cause the cytotoxicity of a variety of eukaryotic cells and red blood cells (RBCs) *in vitro*, even though both activated Cyt and Cry toxins can form pores in lipid bilayers [4,5].

The amino acid sequences of nine Cyt δ -endotoxins [2] show a high degree of sequence similarity and are therefore believed to adopt a similar structure in both the soluble and membrane-inserted states. The structure of one of these toxins has been solved in aqueous solution [6] but little is known about the toxin structure in the membrane-inserted state. Cyt δ -endotoxins are thought to form a β -barrel pore via the insertion of β 5, β 6 and β 7 into the membrane. This assumption is based on the structure of Cyt2Aa [6] together with the characterization of Cyt1Aa mutants [7] and the proteolysis of membrane-bound Cyt1Aa and Cyt2Aa [8].

Until the X-ray crystallographic structure of Cyt δ -endotoxin in the membrane-inserted state becomes available, alternative methods have to be employed to study the pore structure. Here we describe the use of polarity-sensitive acrylodan (6-acryloyl-2-dimethylaminonaphthalene) to label the toxin in an attempt to probe the pore architecture of Cyt2Aa in a biological membrane. Acrylodan gives different emission spectra in hydrophobic and hydrophilic environments [9]. It can therefore be used as a probe to determine whether a labelled toxin residue is in the hydrophobic zone of the lipid bilayer or within a hydrophilic environment. This technique has been used successfully with α -haemolysin [10], streptolysin O [11,12] and perfringolysin O [13]. In the present study, single cysteine mutants were introduced

into different parts of the active core of the Cyt2Aa toxin. The emission spectra of the acrylodan-labelled toxin in solution, membrane-inserted and delipidated states were analysed to determine which part of the toxin molecule had inserted into the membrane and to probe the orientation of the labelled residues.

MATERIALS AND METHODS

Plasmids and oligonucleotides

pMSV1Cyt2Aa1 (wild type) is based on the pALTER plasmid (Promega) and carries the full-length *cyt2Aa1* gene from *B. thuringiensis* subsp. *kyushuensis* [14]. Plasmid pUC18 (Promega) was described by Yanisch-Perron et al. [15]. Mutagenic oligonucleotides were produced with a Pharmacia LKB Gene Assembler Plus by the Oligonucleotide Synthesis Facility (Biochemistry Department, Cambridge University, Cambridge, U.K.). The oligonucleotide sequences are shown in Figure 1.

Site-directed mutagenesis

Site-directed mutagenesis was performed with the Altered Sites II *in vitro* Mutagenesis System (Promega) as described by the manufacturer. Single-stranded template was prepared with R408 helper phage. The mutagenesis reactions were used to transform the mismatch repair-deficient *Escherichia coli* strain ES1301 *mutS* by electroporation [16]. Transformed cells were grown overnight in 5 ml of Luria–Bertani medium containing 100 μ g/ml ampicillin. Plasmid DNA was then isolated and retransformed into *E. coli* JM109 cells. The transformation mixture was spread on Luria–Bertani agar plates containing 100 μ g/ml ampicillin. Transformants obtained were analysed for introduced mutations by digestion with restriction endonucleases followed by DNA sequencing.

Abbreviations used: Bti, *Bacillus thuringiensis* subsp. *israelensis*; RBC, red blood cell.

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Protein preparation

Cyt2Aa1 inclusions were released from *E. coli* cells by sonication. Typically, 1 litre of overnight culture induced by 1 mM isopropyl β -D-thiogalactoside was pelleted by centrifugation at 10000 *g* for 5 min at 4 °C. The cell pellet was then resuspended in 200 ml of ice-cold gradient buffer [50 mM Tris/HCl (pH 7.5)/10 mM KCl] plus 0.01% (v/v) Triton X-100. The bottle containing resuspended cells was immersed in ice and sonicated for six cycles (1 min on/1 min off) in an MSE sonicator with the use of the 10 mm sonication probe at maximum power. The inclusions were sedimented by centrifugation at 10000 *g* for 10 min at 4 °C and washed three times with 100 ml of ice-cold distilled water followed each time by centrifugation at 10000 *g* for 10 min at 4 °C. The inclusions were finally resuspended in 10 ml of distilled water and stored at -20 °C in 1 ml aliquots. The mixtures of Cyt2Aa1 crystals and spores were harvested from *B. thuringiensis* as described by Thomas and Ellar [17]. Spores and crystals were resuspended in gradient buffer and separated on discontinuous sucrose gradients (1.9, 2.1 and 2.4 M) by centrifugation in a Beckman L5-50B ultracentrifuge for 16 h at 85000 *g* and 4 °C. The crystal band at the interface between sucrose concentrations 1.9 and 2.1 M was harvested, washed three times in ice-cold distilled water and stored in 1 ml aliquots at -20 °C.

Inclusions and crystals were solubilized at 1–2 mg/ml δ -endotoxin by incubation at 37 °C for 1 h in 50 mM Na₂CO₃, pH 10.5. Solubilized toxin was then separated from insoluble material by centrifugation at 13000 *g* for 5 min and stored at -20 °C in 1 ml aliquots. For proteolytic processing, the solubilized material was mixed with 0.1–10% (w/w) proteinase K and incubated at 37 °C for 30–60 min.

Concentrations of solubilized Cyt2Aa1 were determined by either the measurement of A_{280} [18] or the method of Bradford [19] with the Bio-Rad protein assay kit and BSA as standard. An A_{280} of 1.0 is equivalent to 34.7 μ M or 1.015 mg/ml [20].

Labelling with acrylodan

Mutant proteins were selectively derivatized on single thiol groups with acrylodan as described by Valeva et al. [10]. Before labelling, proteins were transferred into degassed PBS/1 mM EDTA by using PD10 columns (Pharmacia). A 5-fold molar excess of acrylodan was then added and the mixture was incubated at room temperature for 2 h. Dithiothreitol was then added to 5 mM to stop the labelling reaction. Acrylodan that had not reacted with protein was removed with a PD10 column. Successful removal of the unbound acrylodan was verified by the absence of fluorescence at 365 nm at the dye front of UV-transilluminated SDS gels. The extent of labelling was determined by measuring A_{280} and A_{365} in 6 M guanidinium chloride, pH 6.5. The ϵ_{280} of Cyt2Aa1 was calculated from sequence data [20]. The ϵ_{365} of acrylodan in guanidine was determined as 1.57×10^4 M⁻¹·cm⁻¹. The relative A_{280} of acrylodan in guanidinium chloride was found to be 61% of that at 365 nm. This value was used to correct the total A_{280} of the labelled proteins for calculating the protein concentration.

Haemolysis assay *in vitro*

Haemolysis assays in microtitre plates were performed as described by Thomas and Ellar [17] except that a 4% (v/v) suspension of human blood (National Blood Transfusion Service) in haemolysis buffer (50% (v/v) PBS/2.25% (w/v) glucose/0.05% gelatin) was used. Toxin samples were diluted in 2-fold serial dilutions with haemolysis buffer. Aliquots (0.1 ml) of the diluted toxin samples were mixed with 0.1 ml of diluted blood cells and

left at room temperature. The end point of haemolysis was judged after 24 h as the last well at which coloration of the liquid was still visible.

Preparation of multilamellar vesicles

Multilamellar phospholipid liposome vesicles were prepared as described by Thomas and Ellar [17], with modification. The lipid mixture of egg-yolk phosphatidylcholine, cholesterol and stearylamine in a molar ratio of 4:3:1 in chloroform/methanol (2:1, v/v) was added to a round-bottomed flask and mixed by swirling. The solvent was removed under vacuum with a LabPlant rotary evaporator; the resulting lipid film was flushed with nitrogen. An appropriate buffer was then added and the lipid was resuspended by gentle swirling of the flask until complete. The liposomes were stored at -20 °C in 0.5 ml aliquots.

Preparation of RBC ghosts

Human RBC ghosts were prepared by lysis of 10 ml of fresh human blood in 1 litre of ice-cold hypotonic lysis buffer [5 mM sodium phosphate (pH 7.4)/1 mM EDTA] overnight at 4 °C with constant stirring. The RBC ghosts were then removed by centrifugation at 15000 *g* for 20 min at 4 °C. The supernatant was removed and the ghosts were washed three more times with lysis buffer. The ghosts were then washed once more with PBS, pH 7.4, and resuspended in 10 ml of PBS, pH 7.4. The concentration of RBCs in human blood has been reported to be approx. 5×10^9 cells/ml [21]. Therefore the concentration of RBC ghosts from this preparation was estimated to be 5×10^9 ghosts/ml because the ghosts were resuspended in the same volume as that of the fresh blood used.

Fluorimetry

RBC membranes (1 ml; 5×10^8 ghosts/ml) or liposomes (200 μ g/ml total lipid) were incubated with 10 μ g of acrylodan-labelled toxin at room temperature for 1 h. The toxin-treated membranes or liposomes were washed twice with PBS/1 mM EDTA. Final pellets were resuspended in 3 ml of PBS/1 mM EDTA and kept in a plastic tube made opaque by wrapping with aluminium foil, before fluorimetric analysis. Emission spectra were recorded in a Jasco FP-777 spectrofluorimeter with an excitation wavelength of 365 nm, excitation and emission bandpasses of 2 nm and a scanning interval of 1 nm. For each labelled toxin, samples of both monomeric protein in PBS/1 mM EDTA and oligomeric protein in the membranes were examined. Spectra were corrected for background fluorescence of the respective buffer and suspensions of RBC membranes or liposomes.

Delipidation

The liposomes and RBC membranes carrying acrylodan-labelled toxin were solubilized with 2% (v/v) Triton X-100. To separate toxin oligomers from lipids, the samples were subjected to gel filtration on a PD10 column that had been equilibrated with PBS/1 mM EDTA plus 0.1% (v/v) Triton X-100. The toxin oligomers were eluted with the same solution as that used in the equilibration step. The samples were stored in an opaque tube to avoid photobleaching.

Digestion of membrane-bound toxin with proteinase K

Human RBC membranes (5×10^8 ghosts/ml) were incubated with acrylodan-labelled toxin (10 μ g/ml) in 1 ml of PBS at room temperature for 1 h. The toxin-treated membranes were cen-

trifuged and washed twice with PBS buffer as described previously. Final pellets were resuspended in 1 ml of PBS containing 0.1 mg/ml proteinase K and incubated at 37 °C for 1 h. The samples were centrifuged and washed as above. The membrane pellets were resuspended in 3 ml of PBS/1 mM EDTA and emission spectra were recorded as described above.

RESULTS AND DISCUSSION

Oligonucleotide design for cysteine mutagenesis

The polarity-sensitive fluorophore acrylodan can be attached to the thiol group of cysteine [10]. Cyt2Aa contains a single cysteine residue (Cys-19), which should be cleaved from the toxin during proteolytic activation, leaving the active toxin lacking cysteine [20]. To permit the labelling of Cyt2Aa with acrylodan, single-cysteine substitutions were first introduced in selected parts of the molecule. Evidence suggested that β -strands β 5, β 6 and β 7 insert into the membrane to form a pore, leaving α -helices α A, α B, α C and α D outside the membrane [6,8]. Therefore residues in β 5 (Ser-166, Tyr-169 and Leu-172), β 6 (Val-186, Leu-189 and Glu-192), β 7 (Glu-214, Met-217, Leu-220 and Val-223), α E (Val-202) and a residue in the random coil next to the C-terminus of β 7 (Leu-226) were selected for cysteine substitution. In addition, two cysteine mutants (A61C in α A and V111C in α C) that had previously been constructed were included (M. Adams, personal communication). Residues located on opposite sides of the β -sheets were selected in an attempt to identify the side facing the lipid in the pore structure. It is not clear whether α E inserts into the membrane or remains outside. Proteolytic studies of membrane-bound Cyt2Aa [8] demonstrated that α E could not be removed from the membrane, suggesting either that it is protected by insertion or that there is no

proteolytic site in this region. Mutation V202C was chosen to investigate these alternatives. Residues Ala-61, Val-111 and Leu-226 were chosen to represent regions that might not partition into the membrane. Cysteine substitutions were performed by site-directed mutagenesis with single-stranded DNA pMSV1-Cyt2Aa1 (wild type) as a template and the oligonucleotides shown in Figure 1. A restriction endonuclease site was also introduced or abolished to facilitate mutant identification. The resulting mutant DNA species from the mismatch repair-deficient *E. coli* ES1301 were extracted and transformed into *E. coli* TG1. Plasmids from selected colonies of each mutant were isolated and analysed by restriction endonuclease digestion and DNA sequencing. Plasmids containing the designed mutation were used to transform acrylodan-producing *Bacillus thuringiensis* subsp. *israelensis* (Bti) IPS 78/11 cells [22] by electroporation [23]. The toxin genes were also subcloned into pUC18 at *Sac*I and *Sph*I sites and cloned into *E. coli* JM109 for expression studies.

Expression of Cyt2Aa1 cysteine mutants

Bti IPS 78/11 cells containing the desired genes were grown on CCY agar plates [24] at 30 °C until sporulation occurred (approx. 72 h). When colonies from these plates were examined microscopically, large cytoplasmic toxin crystals were clearly seen for the mutants A61C, V111C, V186C and L220C. Other mutants and the wild type produced none or very small crystals (results not shown). Analysis by SDS/PAGE and immunoblotting showed similar results to those observed microscopically (results not shown).

Cyt2Aa1 toxins (wild type, I150A and T221A mutants and revertants of I150A) are highly expressed in *E. coli* JM109 and TG1 under the control of the *LacZ* promoter in the pUC18 plasmid (D. J. Ellar, unpublished work). It was hoped that cysteine mutants would give similar results with this system. The toxin genes were therefore digested from pMSV1, ligated into pUC18 by using both *Sac*I and *Sph*I restriction sites and transformed into *E. coli* JM109. Analysis of cultures induced with isopropyl β -D-thiogalactoside showed that, with the exception of Y169C, the mutant toxins were expressed at wild-type levels and contained inclusions similar to the wild-type toxin (results not shown).

Purification and solubilization

Crystals of A61C, V111C, V186C and L220C were purified from Bti IPS 78/11 by sucrose-density-gradient centrifugation. The wild-type and other cysteine mutant proteins were purified from *E. coli* JM109 as inclusion bodies. Crystals from wild-type and cysteine mutants could be solubilized in 50 mM Na₂CO₃, pH 10.5, in the absence of a reducing agent, demonstrating that the introduced cysteine residues do not form disulphide bonds during the formation of crystals or inclusions. The major difference between toxins purified from Bti cells and *E. coli* was the stability of the protoxins after solubilization. Toxins purified from *E. coli* remained intact (full length) after solubilization, whereas those purified from Bti cells gave nicked products on solubilization at 37 °C (results not shown). Koni and Ellar [20] demonstrated that the nicked products did not occur in the Bti cell but are probably the result of the residual activity of *B. thuringiensis* proteases associated with the purified crystal. Nicked products were not observed when the crystals were solubilized at 4 °C [20].

Proteolytic processing

Cyt2Aa1 is produced as an inactive protoxin [20]; proteolytic processing at the N-terminus and C-terminus is required to

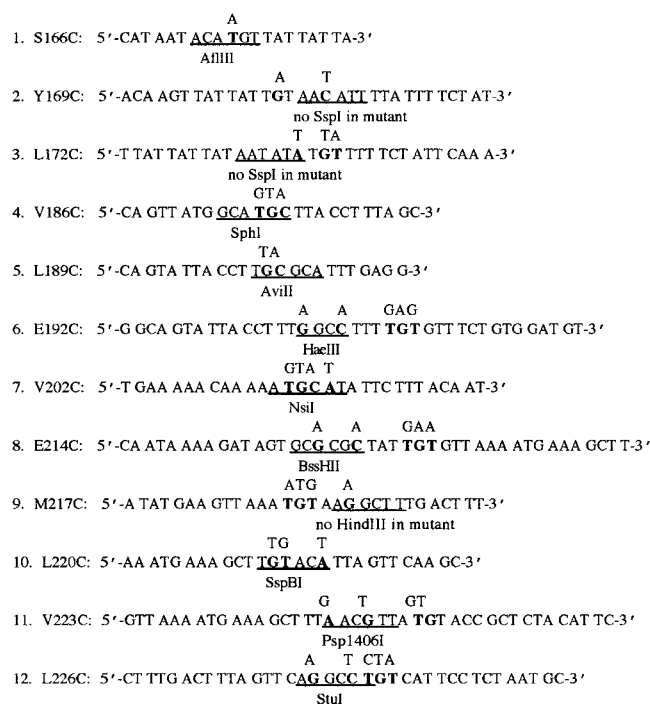


Figure 1 Oligonucleotides used in cysteine mutagenesis

The substituted nucleotides are shown in bold with the original nucleotides (in the wild type) above each sequence. The restriction endonucleases used in the screening of the mutants are shown under each sequence.

produce the active toxin form [6,20]. Solubilized toxins (wild type and mutants) were incubated with 1% (w/w) proteinase K at 37 °C for 1 h and analysed by SDS/PAGE (results not shown). From the results of this analysis the processed toxins could be divided into three groups on the basis of their susceptibilities to digestion with proteinase K. Toxins in the first group (A61C, V111C, Y169C, V202C, M217C, V223C and L226C) were digested almost completely. The second group (S166C, L172C and E192C) were slightly resistant to digestion with proteinase K but were completely digested when 10% (w/w) proteinase K was used (results not shown). The third group (V186C, L189C, E214C and L220C) produced two major products of approx. 22 and 23 kDa with 1% (w/w) proteinase K. However, a single band of approx. 22 kDa, similar to the activated wild type, was obtained from these mutants when 10% (w/w) proteinase K was used (results not shown).

Labelling with acrylodan

Five of the cysteine mutants that produced characteristic proteinase K products (L172C, V186C, L189C, E214C and L220C) and the wild type were selected for labelling with acrylodan. The mutant L172C was found to be degraded more quickly than the other four mutants selected; it was therefore processed with 0.5% (w/w) proteinase K for 30 min at 37 °C. The other four mutants and wild-type toxin were processed with 1% proteinase K for 1 h at 37 °C. The extent of labelling, estimated by measuring both the tryptophan and acrylodan absorbance of the labelled toxins, was 50%, 50%, 40%, 85%, 69% and 44% for wild type, L172C, V186C, L189C, E214C and L220C respectively. Mutants L189C and E214C showed a high efficiency of labelling in comparison with L172C, V186C and L220C. This variation can be explained by the positions of the mutation in the molecule. L172C and V186C are buried within the molecule, which might make them less accessible to acrylodan [6]. In contrast, E214C is present on the surface, making it freely accessible. L189C and L220C are partly exposed but surrounded by different neighbouring residues. These residues might be responsible for the difference in labelling efficiency. In addition, if the overall conformation of the molecule is changed by the mutation, this might alter the accessibility of the mutated residues and hence their labelling efficiency.

Surprisingly, the proteinase K processed wild-type toxin could be labelled with acrylodan. In theory the single cysteine (Cys-19) in Cyt2Aa1 (wild type) is eliminated by processing and therefore should not be labelled. The labelling could be explained if not all molecules have been completely processed under the conditions used. This explanation was confirmed by the finding that wild-type toxin was no longer labelled by acrylodan if the toxin was activated by using 10% (w/w) proteinase K for 1 h at 37 °C (results not shown).

It was felt to be important to obtain results on the fluorescence of the toxin labelled in regions predicted to lie outside the membrane. For this purpose we attempted to label the A61C, V111C, and L226C mutants. Unfortunately the experiments (results not shown) indicated that these three mutants are extremely sensitive to protease digestion. In an attempt to overcome this, we tried to activate the mutants by using a more gentle protocol [0.1% (w/w) proteinase K at 37 °C for 30 min and 1 h]. Even with this protocol, A61C and V111C showed no haemolytic activity and L226C showed very low haemolytic activity in comparison with the wild type or another protease-sensitive mutant L172C. For these reasons the mutants could not be labelled and we were therefore unable to determine their

Table 1 Haemolytic activity of labelled and unlabelled toxins on 2% human RBCs

Haemolytic end points were recorded after 24 h at room temperature. The toxin concentrations were measured before proteolytic processing. The actual concentrations of activated toxins might be lower than shown, especially for those that are highly susceptible to digestion with protease. Abbreviation: n.d., not determined because the activated product was not stable and therefore could not be labelled with acrylodan.

Toxin (Cyt2Aa1 variant)	Haemolytic end point ($\mu\text{g/ml}$)	
	Unlabelled	Labelled
Wild type	0.25	0.5
A61C*	> 250	n.d.
V111C*	> 250	n.d.
S166C*	> 250	n.d.
Y169C†	—	—
L172C*	4	4
V186C	0.25	0.25
L189C	32	64
E192C*	> 250	n.d.
V202C*	> 250	n.d.
E214C	1	0.25
M217C*	> 250	n.d.
L220C	0.25	0.25
V223C*	64	n.d.
L226C*	64	n.d.

* Proteinase-K-sensitive mutants. These mutants were activated by treatment with 0.5% (w/v) proteinase K at 37 °C for 30 min. Other mutants and the wild type were activated by treatment with 1% (w/v) proteinase K at 37 °C for 1 h.

† Y169C showed very low expression and therefore no toxin was available to test the haemolytic activity.

fluorescent properties. We shall continue to search for protease-resistant equivalent mutants in future experiments.

Haemolytic activity of labelled and unlabelled toxins

Haemolytic activities of the labelled and unlabelled toxins are shown in Table 1. Y169C showed very low expression in both *B. thuringiensis* and *E. coli* and the amount of toxin that could be purified was not sufficient to perform haemolytic assays. As stated above, the proteinase-K-activated products of mutants A61C, V111C, S166C, E192C, V202C, M217C, V223C and L226C were unstable and therefore could not be labelled. The activities of these toxins were also difficult to analyse because the precise concentration of the toxin remaining was difficult to determine. However, mutants V223C and L226C retain some haemolytic activity. Mutant L172C, although sensitive to digestion with proteinase K, showed relatively high activity. It is possible that the actual haemolytic activity of this mutant is not different from the wild type because the concentration of L172C remaining after activation by proteinase K must have been considerably lower than the values shown in Table 1.

Mutants V186C and L220C have haemolytic activities comparable to that of the wild type and therefore the residues in these positions might not be important for pore formation. Multiple alignment of Cyt δ -endotoxins indicates that the cysteine residue at the aligned position of V186 (in Cyt2Aa) is conserved in Cyt1Aa, Cyt1Ab and Cyt1Ba.

Mutant E214C showed approximately one-quarter the activity of the wild-type toxin. This residue is conserved in all Cyt2 toxins but is replaced by asparagine or serine in Cyt1 toxins. This residue might not have a role in membrane binding or insertion steps but might be required to stabilize the pore structure by

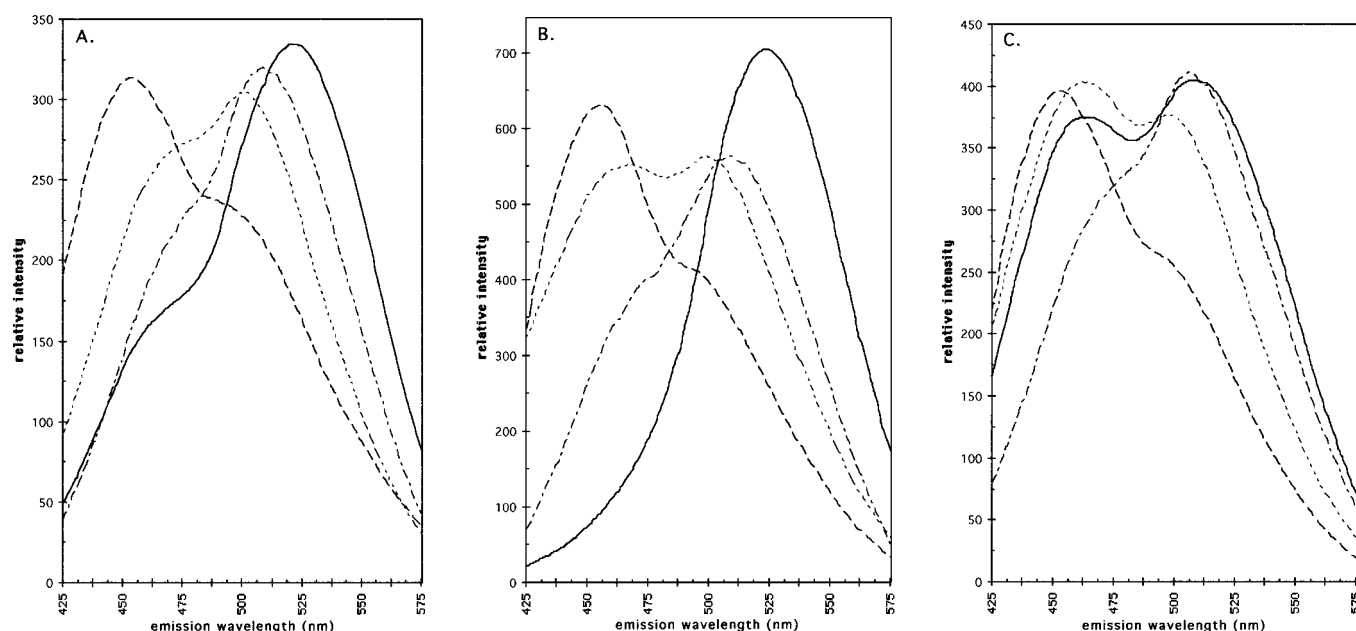


Figure 2 Emission spectra of acrylodan-labelled toxins: L172C (A), L220C (B) and wild type (C)

Emission spectra were scanned from 425 to 575 nm with excitation at 365 nm. Solid line, soluble toxin in PBS plus 1 mM EDTA; broken line, toxin pore complex in RBC membrane; dotted line, toxin pore complex extracted from the membrane by Triton X-100; dot-dashed line, soluble toxin in PBS plus 0.1% Triton X-100. The emission spectra for mutants V186C, L189C and E214C were very similar to those for the mutant L172C and are therefore not shown.

forming hydrogen bonds or salt bridges with residues from other sheets. The difference in activity might reflect the difference in bond characteristics resulting from replacement of the cysteine residue.

Mutant L189C showed the most severe effect on haemolytic activity (Table 1). This position maps to the centre of $\beta 6$ [6]. From the multiple alignment, the residue in this position is not identical in all Cyt toxins but is conserved as a highly hydrophobic residue (Leu, Val or Ile). Mutating the hydrophobic residue to cysteine, which is polar and less hydrophobic, might interfere with the formation of the proposed β -barrel, therefore decreasing the stability of the pore structure. Although this mutant was shown to bind and insert into the membrane, its ability to form a pore was severely decreased (results not shown).

Acrylodan is a small uncharged molecule (molecular mass 225.29 Da) with a negligible effect on toxin activity when linked to streptolysin O [11] or α -hemolysin [10]. In the present study, labelling with acrylodan slightly decreased the haemolytic activity of the wild-type and L189C toxins. In the wild type, acrylodan was linked to Cys-19 which should not be involved in membrane pore formation. Acrylodan might therefore affect only the conformational rearrangement that occurs on the binding of toxin to the membrane. In L189C the hydrophobic balance could be further affected when labelled with acrylodan, resulting in even less activity.

E214C is the only mutant whose haemolytic activity increased when the mutant was labelled with acrylodan. The residue in this position maps to $\beta 7$, which is believed to be inserted into the membrane to form the β -barrel [6,8]. This residue might have a role in stabilizing the pore structure, as mentioned above. Labelling with acrylodan might increase the ability of the toxin to form hydrogen bonds or salt bridges, which in turn could result in an increased stability of the barrel.

Table 2 Summary of emission peaks from acrylodan-labelled toxins in different media

Emission spectra were scanned from 425 to 575 nm with excitation at 365 nm. PBS/EDTA, soluble toxin in PBS plus 1 mM EDTA; RBCm, toxin pore complex in RBC membrane; delipidated, toxin pore complex extracted from the membrane by Triton X-100; Triton X-100, soluble toxin in PBS plus 0.1% (v/v) Triton X-100.

Toxin (Cyt2Aa1 variant)	Emission peak (nm)			
	PBS/EDTA	RBCm	Delipidated	Triton X-100
L172C	522	454	501	508
V186C	526	457	501	510
L189C	518	451	500	510
E214C	520	453	501	513
L220C	523	455	468	509
			501	
Wild type	508	453	463	505
	463		498	

Fluorimetry of acrylodan-labelled toxins

The emission spectra for the mutants L172C and L220C and the wild-type toxins in solution, in the membrane and in delipidated forms, are shown in Figure 2. The spectra for the mutants V186C, L189C and E214C were very similar to those of the mutant L172C and are therefore not shown. A summary of the maximum emission of the toxins in solution, in the membrane and in the delipidated state for each toxin is shown in Table 2. In a hydrophilic environment (PBS solution), the acrylodan emission spectrum for all mutants showed a single major peak at

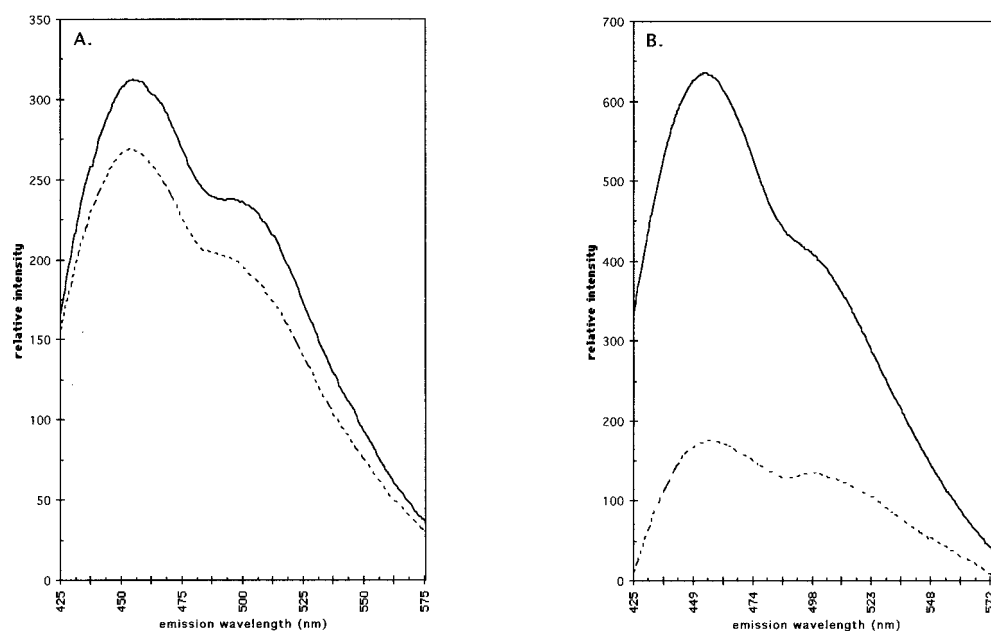


Figure 3 Emission spectra of acrylodan-labelled toxins in RBC membrane before digestion with proteinase K (solid line) and after digestion with proteinase K (dotted line)

(A) Mutant L172C; (B) wild type. The spectra for mutants V186C, L189C, E214C and L220C were very similar to those of mutant L172C and are therefore not shown.

approx. 518–526 nm. The slight difference in maximum emission peaks from these mutants might have been caused by the difference in the positions of the labelled residues within the toxin molecule. Unlike the mutants, the labelled wild type showed two emission peaks, at 463 and 508 nm, in PBS. This suggests that there might be two forms of the toxin in solution, both monomeric and dimeric forms, that might have resulted from an incomplete proteolytic activation, as discussed above. Wild-type Cyt2Aa1 contains only a single cysteine residue at position 19, which is located in a random coil near the N-terminus of the protoxin [6]. This residue might be packed in the hydrophobic cavity on the dimer interface and it is therefore possible that labelling at this location might represent the emission peak at 463 nm. The peak at 508 nm could originate from the monomer in which the label is more exposed to the solution.

In the presence of RBC membranes, Cyt2Aa1 undergoes oligomerization and pore formation. In this condition, the emission peak of all labelled toxins including the wild type is shifted to 451–457 nm (green to blue shift). Similar results were obtained with multilamellar liposome vesicles instead of RBC membranes (results not shown). These results indicated that the labelled probe had moved into a more hydrophobic environment. To check that the observed green–blue shifts were not caused by mutual interaction of the acrylodan molecules attached to neighbouring toxin monomers within the oligomers, labelled toxins were mixed with a 10-fold excess of unlabelled wild-type toxin (4 μ g of labelled plus 40 μ g of unlabelled) before oligomerization on the membranes. The spectra of these samples were indistinguishable from those obtained with labelled toxins alone (results not shown).

Previous work [6,8] has suggested that only the C-terminal half of the toxin is inserted into the membrane. Therefore the shift with the mutants is thought to result from the movement of the label probe into a more hydrophobic zone of the membrane. However, in wild-type toxin, acrylodan was linked to Cys-19,

which is believed to remain outside the membrane [8]. Presumably in this case the label is located in a relatively hydrophobic pocket within the protein oligomer rather than being inserted into the membrane. To confirm that Cys-19 is not inserted into the membrane, the membrane-bound labelled mutants and the wild type were digested with proteinase K. The results demonstrate that L172C, V186C, L189C, E214C and L220C are membrane-protected because their emission intensities were not significantly decreased when digested with proteinase K (Figure 3). In contrast, the emission intensity of the labelled wild type was decreased markedly after digestion with proteinase K, confirming that Cys-19 is not protected by membrane. Strong evidence that this protection is due to membrane penetration rather than ‘burial’ in the interface between the protein and the membrane surface can be seen from the results of the haemolysis assays. Thus mutants V186C and L220C have a haemolytic activity comparable to that of the wild type, indicating that they form a normal transmembrane pore. Mutant L172C, although sensitive to digestion with proteinase K, also showed relatively high activity. Furthermore, it is likely that the actual haemolytic activity of this mutant and the wild type are not different, because the concentration of L172C remaining after activation by proteinase K must have been considerably lower than that shown in Table 1. We intend to obtain more direct evidence for this penetration in the future by performing fluorescence transfer experiments with the acrylodan-labelled mutants and vesicles containing fluorescent or quencher groups.

The green–blue shift observed from the membrane-bound labelled mutants might be caused by a direct interaction of the label with membrane lipids or as a result of the label becoming embedded within the hydrophobic protein environment, which might arise from contact with neighbouring toxin protomers. To test which of these is the cause of the emission shift, membrane-bound toxins were solubilized in Triton X-100 and the toxins and lipids were separated by gel filtration. The emission spectra

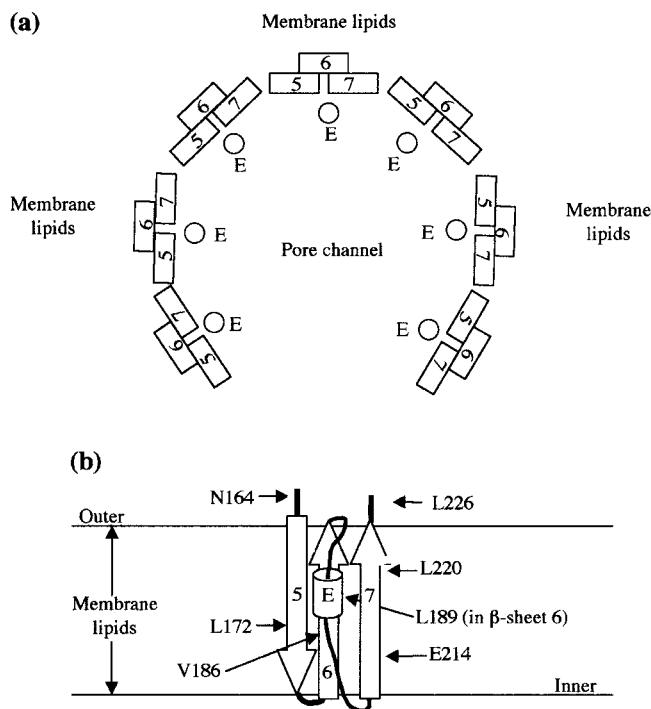


Figure 4 Proposed model for pore structure

Top view (a) and side view (b) of a proposed model for pore structure, showing the regions suggested to be inserted in the membrane together with the approximate locations of each of the mutated residues Leu-172, Val-186, Leu-189, Leu-220 and Glu-214. The three β -strands ($\beta 5$, $\beta 6$ and $\beta 7$) are labelled 5, 6 and 7; E denotes α -helix E as described by Li et al. [6]. The inner (cytoplasmic) and outer surfaces of the plasma membrane are indicated.

obtained from delipidated toxins were reversed to various extents depending on the position of the label within the molecule (Figure 2). From these results it seems likely that L172C, V186C, L189C and E214C, and hence β -strands $\beta 5$, $\beta 6$ and $\beta 7$, make direct contact with membrane lipids because the spectra of the delipidated form reversed towards the hydrophilic state.

Because the emission spectra of delipidated toxins were determined in PBS plus 0.1% (v/v) Triton X-100, the spectrum of soluble toxin alone (never mixed with lipid) was also determined in the presence of 0.1% (v/v) Triton X-100 to represent the spectrum of toxin in the monomeric state. These experiments showed that the differences between the monomer spectra in the PBS solution with and without Triton X-100 are caused by interaction of the label with the detergent. Consequently the observed differences between spectra of the toxins in a monomeric state and an oligomeric state (both in the presence of 0.1% Triton X-100) are caused by the altered protein conformation on insertion into the membrane to form pores.

Delipidated L220C showed two emission peaks, at 468 and 501 nm (Figure 2B). This result suggests that the location of the label varies between direct contact with the lipids and a relatively hydrophobic location within the protein complex. It could be argued that one of the two peaks observed in the delipidated L220C pore complex might have resulted from an irreversible conformational change of the toxin when exposed to the high concentration of Triton X-100 (2%, v/v) used when extracting the pore complex from the lipid membrane. To verify whether this is so, 2% (v/v) Triton X-100 was added to the soluble labelled toxin and the mixture was left at room temperature for

the same duration that was used in the extraction process. The emission spectrum of the solubilized toxin in 2% (v/v) Triton X-100 was not different from that in 0.1% (v/v) Triton X-100 (results not shown). The results indicate clearly that the use of 2% (v/v) Triton X-100 in the extraction process did not cause an irreversible conformational change.

The behaviour of the label in the wild type was different from that of the mutants (Figure 2C). Soluble toxin in PBS solution showed two major peaks, which might indicate the existence of two different forms, as mentioned earlier. In the presence of detergent [0.1% (v/v) Triton X-100] the toxin showed a single major peak, which suggests that most of the toxin molecules are in a similar hydrophobic environment. On membrane insertion and pore formation, the wild type shows only a single peak (Figure 2C). This suggests that the label at this N-terminal location might move into the hydrophobic environment of the protein oligomer or lie on the membrane surface covered by hydrophobic residues from the oligomer. In either location the label could thus yield a similar blue shift in emission even though no insertion into the lipid bilayer has occurred. After the lipids were removed from the pore complex of the wild type, the fluorescence spectrum indicated that a substantial proportion of label still remained within a relatively hydrophobic environment. We interpret this result to indicate that, after oligomerization, Cys-19 is buried within a hydrophobic region of the protein oligomer. The minor wild-type peak representing a more hydrophilic label environment observed in the delipidated complex could represent Cys-19 from monomer that inserted into the membrane but did not form a pore.

Li et al. [6] proposed that Cyt δ -endotoxins form a pore based on a β -barrel structure. The pore is thought to be oligomeric, with each monomer contributing $\beta 5$, $\beta 6$ and $\beta 7$ to form the barrel. A possible structure of the monomer based on these experiments is shown in Figure 4. The model shows the regions suggested to be inserted in the membrane, together with the approximate locations of each of the mutated residues L172, V186, L189, L220 and E214. It is not known at present whether each of these residues faces the lipid bilayer or the pore lumen; however, the results presented here suggest that all five labelled residues might face the lipid phase.

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