Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from *Bacillus thuringiensis*

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Cyt2Aa1 is a cytolytic protein produced by *Bacillus thuringiensis* subsp. *kyushuensis*. Penetration of the toxin into membranes has been studied to learn more about membrane-insertion mechanisms and transmembrane-pore formation. The haemolysis assay of Cyt2Aa1 showed a steep and sigmoidal dose–response curve, indicating that toxin aggregation or oligomerization is required for pore formation. Studies of the effect of temperature on pore formation and fluorimetric studies of acrylodan-labelled toxin suggest that toxin inserts into the membrane before oligomerizing to form a pore. Low temperature neither inhibited membrane binding nor closed pores that have been formed, but markedly inhibited oligomerization of the toxin molecules. When toxin-treated red blood cells at 4 °C were transferred to a toxin-free solution at 37 °C, no significant increase in haemolysis was observed. This result suggests that membrane-bound toxin could not diffuse laterally and interact with other molecules to form a pore. From these results, we propose that Cyt2Aa1 binds and inserts into the membrane as a monomer. Oligomerization occurs when toxin molecules have bound in close proximity to each other and pores are formed from large oligomers.

Key words: *Bacillus thuringiensis*, Cyt2Aa1, cytolytic toxin, haemolysis, membrane pore, pore formation.

INTRODUCTION

Cytolytic (Cyt) δ-endotoxins produced by some strains of *Bacillus thuringiensis* are primarily toxic to Dipteran insects *in vivo*, but are cytolytic to a broad range of cells, including RBCs (red blood cells) *in vitro* [1,2]. These toxins are believed to lyse cells by forming an oligomeric transmembrane pore based on a β-barrel structure [3]. Evidence suggests that the proteolytically activated toxin molecules approach the membrane and bind as monomers [4–6]. The effect of Cyt1Aa (produced by *Bacillus thuringiensis* subsp. *israelensis*) on fluid secretion from malpighian tubules [4,5] showed an inverse relationship between toxin concentration and time of failure of toxin-treated tubules at low toxin concentrations. However, this time for onset of toxin-induced secretion could not be reduced even at very high toxin concentrations. Extraction of membrane-bound Cyt1Aa with Triton X-100 and analysis by sucrose density gradient centrifugation demonstrated that the toxin molecules are in the monomeric form when low concentrations of toxin are bound to the membrane [6]. Toxin aggregates appeared only when higher concentrations of toxin were bound [6]. These findings suggest that only when a critical number of toxin molecules become associated in one place does the complex begin to form a pore.

The rate of pore formation by Cyt δ-endotoxins decreases as the temperature is lowered [5]. At least four possible steps in pore formation could be affected by low temperature: (i) membrane binding, (ii) conformational change during insertion, (iii) oligomerization or (iv) pore opening/closing. Maddrell et al. [5] demonstrated that Cyt1Aa could bind to the membrane at 0 °C, but could not form a pore. There is no clear evidence to suggest which of the remaining three possible steps is affected by low temperature. Cyt2Aa, produced by *Bacillus thuringiensis* subsp. *kyushuensis*, has similar biological properties to those of Cyt1Aa. The present study describes further investigation into the pore-forming mechanism of Cyt2Aa1 (PDB accession number 1CBY).

MATERIALS AND METHODS

Plasmids and bacterial strain

Plasmid pUC18Cyt2Aa1-wt was constructed by cloning the full-length *cyt2Aa1* gene [7] into plasmid pUC18 [8] between the *Sac*I and *Sph*I site. The mutant Cyt2Aa1-L189C was constructed by site-directed mutagenesis and subcloned into plasmid pUC18. Both plasmids were transformed into *Escherichia coli* JM109 for expression.

Toxin preparation

Cyt2Aa1 toxins (wild-type and mutant) were extracted from *E. coli* cells by sonication. Typically, 1 litre of overnight culture induced by 1 mM IPTG (isopropyl β-D-thiogalactoside) was pelleted by centrifugation at 10 000 ***g*** for 5 min at 4 °C. The cell pellet was then resuspended in 200 ml of ice-cold gradient buffer (50 mM Tris, pH 7.5, and 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 and 1 min off) in an MSE sonicator using the 10 mm sonication probe at maximum power. The inclusions were sedimented by centrifugation at 10 000 ***g*** for 10 min at 4 °C and washed three times with 100 ml of ice-cold distilled water and then centrifuged again at 10 000 ***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. Toxin inclusions were solubilized at 1–2 mg/ml by incubation at 37 °C for 1 h in 50 mM Na_2CO_3, pH 10.5. Solubilized toxin was then

Abbreviations used: Cyt, cytolytic; PEG, poly(ethylene glycol); RBC, red blood cell.

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separated from insoluble material by centrifugation at 13 000 ***g*** for 5 min and stored at − 20 °C in 1 ml aliquots. For proteolytic processing, the solubilized material was mixed with 1 % (w/w) proteinase K and incubated at 37 °C for 1 h. Concentrations of solubilized toxins were determined by either A_{280} measurement based on the method of Cantor and Schimmel [9] or the method of Bradford [10] using the Bio-Rad protein assay kit and BSA as standard. It was estimated that an A_{280} of 1.0 was equivalent to 34.7 μM or 1.015 mg/ml [11].

Haemoglobin-release assay

Haemoglobin-release assays were performed as described for *Clostridium septicum* α toxin [12]. Human RBCs; National Blood Service, Cambridge, U.K.) were washed three times in PBS buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl and 2.7 mM KCl, pH 7.4), resuspended in PBS to the concentration of 1 % (v/v) and pre-incubated at the specified temperature (4–37 °C). The cells were then exposed to proteolytically processed Cyt2Aa1 at various concentrations (0.25–10 μg/ml) and incubated at the required temperature. Samples (1 ml) were removed at the indicated times and unbroken cells and cell debris were removed from these samples by centrifugation at 13 000 ***g*** for 30 s. The haemoglobin-containing supernatant was removed and haemoglobin release was quantified at A_{540} using an Ultrospec 3000 (Pharmacia Biotech). The supernatant from 1 % (v/v) RBCs treated with 0.1 % (v/v) Triton X-100 was used as a 100 % haemolysis control and the supernatant from untreated 1 % (v/v) RBCs was used as a 0 % lysis blank.

RBC light-scattering assay

Human RBCs were washed three times in PBS. The cells were removed from the buffer by centrifugation at 1000 ***g*** for 3 min at room temperature (20–25 °C). The cell pellets were then diluted to 10 % (v/v) in the same buffer and used within 8 h. A 0.1 ml sample of 10 % (v/v) RBCs was added to 0.9 ml of PBS buffer containing processed toxin at various concentrations and pre-incubated at 25 °C for 10 min. Light scattering by RBCs was recorded immediately and regularly over 1 h by measuring the absorbance at 595 nm at 25 °C. A 1 % (v/v) RBC suspension treated with 0.1 % (v/v) Triton X-100 was used as a 100 % haemolysis control.

Pore formation in PEG [poly(ethylene glycol)]-stabilized RBCs

Human RBCs (1 %, v/v) equilibrated into PBS containing 10 % (v/v) PEG1000 were exposed to 10 μg/ml processed Cyt2Aa1 and incubated at room temperature for 15 min. The samples were then centrifuged at 1000 ***g*** for 3 min at 4 °C and the supernatant was removed. PBS buffer (without PEG1000), equilibrated at either 4 °C or 37 °C, was added to the RBC pellets which were then incubated at either 4 °C or 37 °C. Samples of 1 ml were removed from the suspension at various times after removal of PEG1000. Haemolysis was assessed by haemoglobin release as described in the previous section.

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 and 1 mM EDTA, pH 7.4) overnight at 4 °C with constant stirring. The RBC ghosts were then removed by centrifugation at 15 000 ***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 and resuspended in 10 ml of PBS. The concentration of RBCs in human blood has been reported to be approx. 5×10^9 cells/ml [13]. Therefore the concentration of RBC ghosts from this preparation was estimated to be 5×10^9 ghosts/ml, since the ghosts were resuspended in a volume of PBS equal to the volume of the fresh blood used.

Preparation of membrane-bound toxin

Human RBC membranes (5–20 × 10^8 ghosts/ml) were incubated with processed toxin (20 μg/ml) in PBS buffer at 4–37 °C for 1 h. The samples were then centrifuged at 24 000 ***g*** for 15 min at 4 °C and the supernatant removed. The pellets were washed twice with PBS buffer and centrifuged as above. Final pellets were stored at 4 °C for subsequent analysis.

SDS/PAGE and immunoblotting

SDS/PAGE was performed as described by Laemmli and Favre [14] using a discontinuous buffer system as modified by Thomas and Ellar [15]. Protein samples were solubilized in 1 × protein loading buffer at room temperature in order to preserve any oligomeric forms of the membrane-bound toxin (Cyt2Aa1 toxin oligomers are stable at room temperature in SDS-containing solutions; B. Promdonkoy and D. J. Ellar, unpublished work) before being applied to a 15 % (w/v) polyacrylamide separating gel with a 5 % (w/v) polyacrylamide stacking gel. Proteins separated by SDS/PAGE were electrophoretically transferred on to nitrocellulose membranes with a Bio-Rad Transblot semi-dry transfer apparatus according to the manufacturer's instructions. Protein bands were detected using a rabbit polyclonal antibody prepared by subcutaneous injection of purified Cyt2Aa1 protoxin. Peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody.

Fluorimetry of acrylodan-labelled toxin

Cyt2Aa1-L189C was labelled with acrylodan as described in [16]. Labelled toxin (1–40 μg) was incubated with 1 ml of RBC membranes (5×10^8 ghosts/ml) at room temperature for 1 h. The toxin-treated membranes were washed twice with PBS/1 mM EDTA. Final pellets were resuspended in 3 ml of PBS/1 mM EDTA and held in a plastic tube made opaque by wrapping with aluminium foil before fluorimetric analysis. Emission spectra were recorded with a Jasco FP-777 spectrofluorimeter using an excitation wavelength of 365 nm, excitation and emission bandpasses of 2 nm and a scanning interval of 1 nm. Spectra were corrected for background fluorescence of the respective buffer and RBC membrane suspensions.

RESULTS AND DISCUSSION

Dose–response curves of Cyt2Aa1

Haemolysis dose–response curves were studied to investigate the correlation between Cyt2Aa1 concentration and haemoglobin release. The steep and sigmoidal dose–response curve seen in Figure 1 suggests that lysis is accompanied by some co-operative effect between toxin molecules. This co-operation may be the aggregation or oligomerization of the toxin molecules to form the pore. To investigate whether oligomerization occurred only on or in the membrane as suggested by Maddrell et al. [4,5], light-scattering assays of RBCs exposed to different concentrations of

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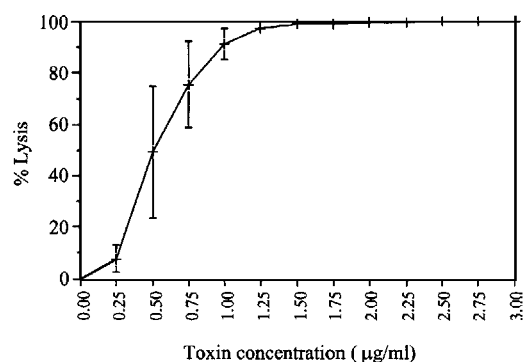

Figure 1 Dose–response curve of RBC lysis by Cyt2Aa1

Different toxin concentrations (0–3.0 μg/ml) were incubated with 1 ml of 1 % (v/v) human RBCs in PBS at room temperature for 1 h. The extent of haemolysis was calculated from the absorption of haemoglobin at 540 nm using the supernatant from a 1 % (v/v) RBC sample treated with 0.1 % (v/v) Triton X-100 as a 100 % lysis control and the supernatant from an untreated 1 % RBC sample incubated at room temperature for 1 h as the blank. Results are means ± S.E.M. ($n = 4$). The un-processed Cyt2Aa1 (protoxin) and the mutant Cyt2Aa1-L189C showed no haemolytic activity in this experiment.

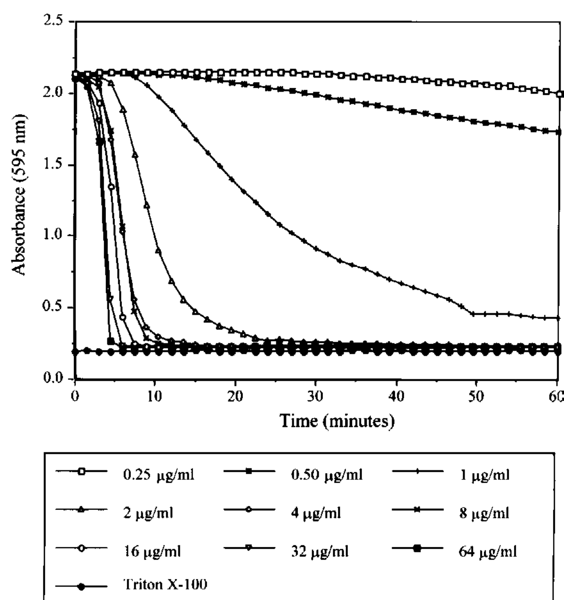

Figure 2 Haemolysis time course of Cyt2Aa1

Cyt2Aa1 (0.25–64 μg) was prepared in 0.9 ml of PBS and left at room temperature for 10 min. A 0.1 ml sample of 10 % (v/v) human RBCs in PBS was then added to the toxin solution. The turbidity of the samples (light-scattering from RBC at 595 nm) was measured for 1 h at 25 °C using PBS as a blank and a sample of 1 % human RBC treated with 0.1 % Triton X-100 as the positive control.

processed toxin were carried out. Figure 2 shows that, at low toxin concentrations (0.25–8.0 μg/ml), the haemolysis rate increased upon increasing the toxin concentrations. However, with solutions containing high toxin concentrations (16–64 μg/ml), the haemolysis rate was unchanged, suggesting that aggregation or oligomerization occurred only on or in the membrane.

Effect of temperature on pore formation by Cyt2Aa1

Figure 3 shows that a very similar high rate of haemolysis occurred at 37 °C and 25 °C. At 15 °C, haemolysis occurred slowly in the first 10 min, but increased markedly after that and reached 100 % in 15 min. Haemolysis was very slow at 4 °C and reached less than 10 % after 1 h. When the incubation time at 4 °C was increased to 3 and 18 h, haemolysis increased to 20 % and 53 % respectively (results not shown). These results indicate that pore formation is slowed, but not prevented by low temperature.

To test whether or not the toxin can bind to the membrane at low temperatures, the processed toxin was incubated with RBCs at 4 °C before being transferred to 37 °C. Figure 4 shows that haemolysis occurred rapidly and reached 100 % within 10 min when the RBC and toxin were incubated at 37 °C. Only 10 % haemolysis occurred after incubation at 4 °C for 1 h, but when a sample was transferred to 37 °C, haemolysis occurred almost immediately. However, if an identical sample of the cells was first separated from the toxin-containing solution before resuspension in the toxin-free solution at 37 °C, only a further 10 % haemolysis occurred over the period of the experiment (45 min). One interpretation of these results is that toxin molecules bind to the membrane at 37 °C, but not at 4 °C. Thus when the toxin-treated cells at 4 °C were transferred to toxin-free solution at 37 °C, no

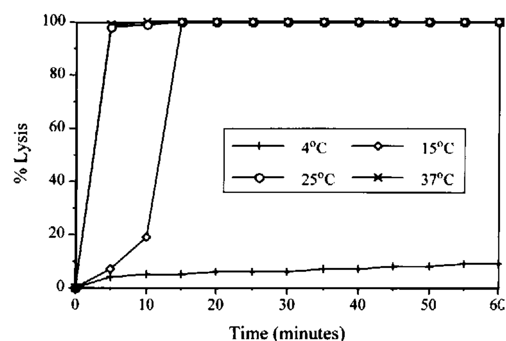

Figure 3 Haemolytic activity of Cyt2Aa1 at different temperatures

Cyt2Aa1 (150 μg) was mixed with 15 ml of 1 % (v/v) human RBCs in PBS, pre-incubated at 4 °C, 15 °C, 25 °C and 37 °C. The samples were then incubated at the same pre-incubation temperature. The extent of haemolysis was assessed every 5 min for 1 h by measuring the absorbance of the released haemoglobin at 540 nm.

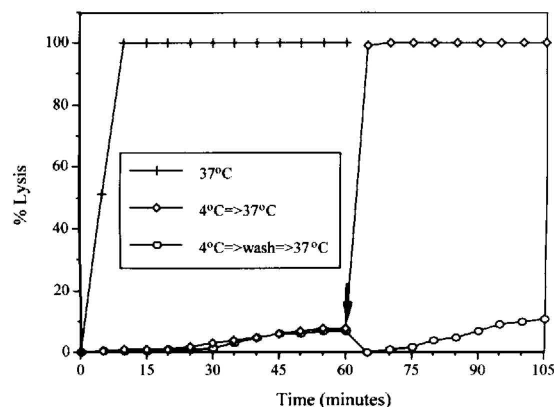

Figure 4 Effect of temperature on the haemolytic activity of Cyt2Aa1

Cyt2Aa1 (200 μg) was mixed with 20 ml of 1 % (v/v) human RBCs in PBS pre-incubated at 4 °C. The samples were incubated at 4 °C (two tubes) or 37 °C (+), and the extent of haemolysis was measured every 5 min. After incubation for 1 h, one tube was transferred from 4 °C to a 37 °C water bath (◇) and the other tube (from 4 °C) was centrifuged at 1000 ***g*** for 3 min at 4 °C and the supernatant was removed. The cell pellet from this tube was resuspended in 10 ml of PBS pre-incubated at 37 °C, and the sample was incubated in a 37 °C water bath (○). The haemolysis of both samples was followed for another 45 min. The arrow indicates the time when the samples were shifted from 4 °C to 37 °C.

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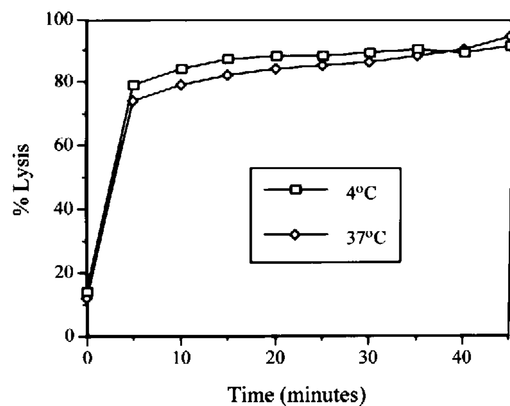

Figure 5 Effect of low temperature on Cyt2Aa1 pore formation

Cyt2Aa1 (100 μg) was mixed with 10 ml of 1 % (v/v) human RBCs in PBS containing 10 % (v/v) PEG1000. After incubation at room temperature for 15 min, the samples were centrifuged at 1000 ***g*** for 3 min at 4 °C. The cell pellets were resuspended in 10 ml of PBS (without PEG1000) pre-incubated at 4 °C or 37 °C. The samples were left at these temperatures and the haemolysis was measured every 5 min for 45 min. The haemolysis at zero time was measured before removing the PBS buffer containing PEG1000.

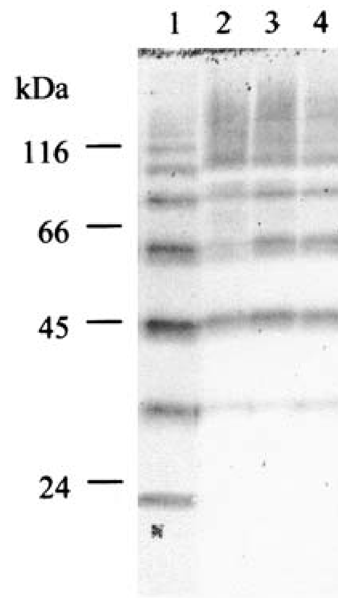

Figure 6 Immunoblot of membrane-bound Cyt2Aa1 toxin at different temperatures

Cyt2Aa1 (10 μg) was incubated with 1 ml of human RBC membranes (5×10^8 ghosts/ml) in PBS for 1 h at 4 °C, 15 °C, 25 °C and 37 °C (lanes 1–4 respectively). The membranes were pelleted and washed twice with PBS at 4 °C. The final pellets were dissolved in 30 μl of 1 × sample buffer and left at room temperature for 20–25 min before separation by SDS/PAGE (5 %, 15 % gels).

pores were formed because of the absence of toxin in solution. The slight haemolysis observed for this sample (Figure 4) was probably caused by a few pores that had already formed at 4 °C. It is possible that the toxin specifically interacted with a lipid microdomain that was 'frozen' at 4 °C. However, the bulk lipid of RBCs is known not to undergo any thermal phase transitions at 4 °C [17]. It is also unlikely that the toxin oligomer itself has a temperature-dependent conformational change that is inhibited at 4 °C. If this was the cause, haemolysis of toxin-treated cells at 4 °C should have been markedly increased when the cells were moved to toxin-free solution at 37 °C. The same observation argues against the possibility that the pore is closed at 4 °C. One alternative explanation for our findings is that the conformational change in the toxin that accompanies membrane binding could be different at 4 °C and 37 °C, with the latter favouring the oligomerization and the former not. It is possible that a toxin that is partially membrane-embedded at 4 °C may be unable to undergo the change to the oligomerization-competent state upon raising the temperature to 37 °C.

To confirm that the pore is not closed at 4 °C, two processed toxin samples of 100 μg were incubated with 10 ml of 1 % (v/v) RBC in PBS containing 10 % (v/v) PEG1000 at room temperature for 15 min. It was assumed that the pores had been formed under these conditions since complete haemolysis was observed in the absence of osmotic protectant (PEG1000). Most of the RBCs remained intact in the presence of PEG1000 with less than 20 % lysis. The two samples were then centrifuged at 1000 ***g*** for 3 min at 4 °C, and the supernatant was removed. The two cell pellets were resuspended in 10 ml of PBS (without PEG1000), pH 7.4, that had been pre-incubated at either 4 °C or 37 °C. The two samples were then maintained at these temperatures and haemoglobin release from both samples was measured every 5 min. Figure 5 shows that haemolysis was not significantly different at 4 °C and 37 °C confirming that the pore is not closed at 4 °C.

To determine whether or not low temperature inhibits toxin binding, the toxin bound to the membrane at different temperatures was analysed by SDS/PAGE. Figure 6 suggests that the total amount of the toxin bound at varying temperatures is very similar. Densitometry of the blot showed that all tracks contained comparable amounts of bound toxin. However, at 4 °C, most of the bound toxin molecules were found as low-molecular-mass oligomers (monomer–pentamer), whereas, at higher temperatures, higher-molecular-mass oligomers were dominant. This result suggests that one of these high-molecular-mass oligomers can form pores in the membrane. The finding that only 10 % haemolysis occurred at 4 °C, although the toxin could bind to the membrane very effectively at this temperature, indicates that low temperature inhibits the oligomerization step that is required to form pores in the membrane. Combining these binding data with the lack of any significant increase in haemolysis from the toxin-treated cells (at 4 °C) after they were moved to toxin-free solution at 37 °C, suggests further that toxin molecules already bound to the membrane cannot diffuse laterally to interact with each other and form a pore. If the membrane-bound toxin could diffuse laterally, the pores would have been formed after the toxin-treated cells (at 4 °C) were moved to toxin-free solution at 37 °C, as reported for *Clostridium septicum* α toxin [12] and perfringolysin O [18].

Toxin insertion monitored by fluorimetry of acrylodan-labelled toxin

The mutant Cyt2Aa1-L189C has low activity and shows a haemolytic end point of 32 μg/ml for unlabelled toxin and 64 μg/ml for acrylodan-labelled toxin [16]. Du et al. [19] demonstrated that only the C-terminal half of Cyt2Aa1 from residue Ile^{150} to Ser^{228} is protected after proteolysis of the membrane-bound toxin. $Leu^{189} \rightarrow$ Cys is located in the middle of β6, which is likely to insert into the membrane [3]. After labelling this residue with the polarity sensitive probe acrylodan, it was possible to examine whether or not it could insert into the membrane at concentrations lower than those needed to form a pore. Figure 7 shows that a green–blue shift occurred at every concentration used. Cyt2Aa1-L189C can therefore bind and partition into the membrane even at 2 μg/ml, which is a concentration 32-fold

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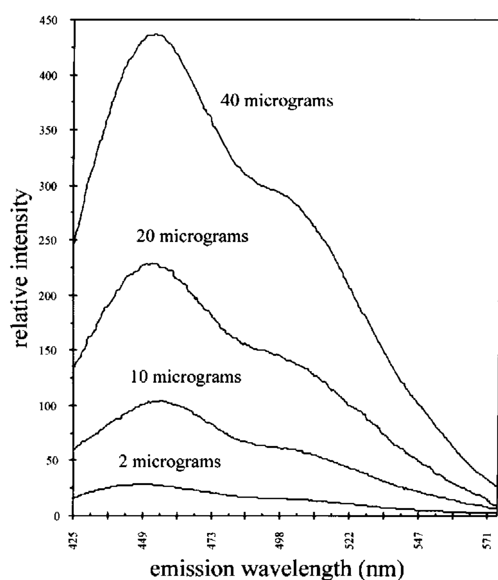

Figure 7 Emission spectra of acrylodan-labelled Cyt2Aa1-L189C

The labelled toxin (2–40 μg) was incubated with 1 ml of human RBC membrane (5×10^8 ghosts/ml) in PBS for 1 h at room temperature. The membranes were pelleted and washed twice with PBS. The final pellets were resuspended in 3 ml of PBS, plus 1 mM EDTA, and emission spectra were determined using an excitation wavelength of 365 nm. The maximum emission wavelength of the labelled toxin in PBS/EDTA was 518 nm.

lower than its haemolysis end-point. When using the labelled toxin at 1 μg/ml, the emission signal was indistinguishable from the background. These data suggest that insertion of the toxin into the membrane occurs before oligomerization to form a pore.

In summary, Cyt2Aa1 binds and inserts into the membrane as a monomer. Oligomerization occurs when toxin molecules have bound in close proximity to each other and pores are formed from large oligomers. There is no clear evidence to indicate the precise number of toxin molecules required to form a pore. The Cyt δ-endotoxin pore diameter of 1–2 nm was determined using osmotic protectants with known viscometric radii [20]. Assuming strands β5, β6 and β7 of Cyt2Aa1 oligomerize to form a closed β-barrel, Li et al. [3] estimated that the number of monomers required to form a barrel of the expected pore size would be about four to six. However, oligomers of approx. 400 kDa were found from the Triton X-100-extracted fraction of insect cell membranes treated with activated Cyt1Aa [6]. If the 400 kDa complex can form a pore and the complex solely comprises activated Cyt1Aa toxin, the pore would contain 18 toxin molecules.

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