Biochemical characterization of *Bacillus thuringiensis* cytolytic toxins in association with a phospholipid bilayer

Jinping DU[1*], Barbara H. KNOWLES[2*], Jade LI† and David J. ELLAR[3]‡

*Department of Zoology, Univeristy of Cambridge, Downing Street, Cambridge CB2 3EJ, U.K., † Laboratory of Molecular Biology, Medical Research Council, Hills Road, Cambridge CB2 2QH, U.K., and ‡ Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1GA, U.K.

The interaction of two *Bacillus thuringiensis* cytolytic toxins, CytA and CytB, with a phospholipid bilayer and their structure in the membrane-bound state were investigated by proteolysis using phospholipid vesicles as a model system. A toxin conformational change upon membrane binding was detected by comparing the proteolytic profile of membrane-bound toxin and saline-solubilized toxin. When membrane-bound toxin was exposed to protease K or trypsin, novel cleavage sites were found between the α-helical N-terminal half and β-strand C-terminal half of the structure at K154 and N155 in CytA and at I150 and G141 in CytB. N-terminal sequencing of membrane-protected fragments showed that the C-terminal half of the toxin structure comprising mainly β-strands was inserted into the membrane, whereas the N-terminal half comprising mainly α-helices was exposed on the outside of the liposomes and could be removed when liposomes with bound toxin were washed extensively after proteolysis. The C-termini of the membrane-inserted proteolytic fragments were also located by a combination of N-terminal sequencing and measurement of the molecular masses of the fragments by electrospray MS. Using a liposome glucose-release assay, the membrane-inserted structure was seen to retain its function as a membrane pore even after removal of exposed N-terminal segments by proteolysis. These data strongly suggest that the pores for glucose release are assembled from the three major β-strands (β-5, β-6 and β-7) in the C-terminal half of the toxin.

Key words: CytA, CytB, membrane, pore, proteolysis.

INTRODUCTION

Insecticidal toxins produced by *Bacillus thuringiensis* during sporulation in the form of crystal inclusions are membrane pore-forming proteins [1–3] and have been utilized as bioinsecticides [4–7]. *B. thuringiensis* toxins are divided into five major classes based on their insecticidal activity spectrum and sequence similarity [4]. Among these classes, *B. thuringiensis* Cyt toxins are distinguished from Cry toxins by their specific insecticidal activity to dipteran insects *in vivo*, their broad cytolytic activity to various insect cell lines and erythrocytes *in vitro*, and their ability to insert spontaneously into membranes containing unsaturated phospholipids [8–15].

The amino acid sequences of six Cyt toxins have been determined [16–20]. Of these toxins, CytA was cloned from *B. thuringiensis israelensis* [16] and *B. thuringiensis morrisoni* PG-14 [21], and CytB from *B. thuringiensis kyushuensis* [17]. Ever since the primary structure of the first Cyt toxins became known, various attempts have been made to develop models for their tertiary structure [11,22–27]. These models, based on sequence analysis, hydrophobicity and mutagenesis data, represented cytolytic toxins as proteins containing a helix bundle. Recently, the three-dimensional structure of CytB was determined [28], and found to have a single domain of α/β architecture, consisting of two outer layers of α-helix hairpins wrapped around a mixed β-sheet [28]. Based on this structure, plus the previous work on biochemical characterization of toxin mutants and the results of proteolysis, Li et al. [28] proposed that it is the β-sheets rather than the α-helices that are responsible for membrane binding and pore formation.

Although the toxin structure in aqueous solution may be represented by the crystal structure, practically nothing is known about the toxin structure in the membrane-inserted state. Characterization of the toxin structure in the membrane-bound state is, however, the key to understanding its biological function in a membrane. On the bases of their structures, models of toxin structure in a membrane for some other membrane-pore-forming toxins such as colicin [29,30], bacterial porins [31,32], diphtheria toxin [33], staphylococcal α-haemolysin [34] and anthrax toxin protective antigen [35] have been proposed. In particular, the recent description of the crystal structures of anthrax toxin protective antigen, in both monomeric and heptameric forms, has given us insights into the molecular mechanisms of membrane insertion and the architecture of membrane proteins.

In the present paper, we report work on the characterization of the structure of *B. thuringiensis* cytolytic toxins in the membrane. A conformational change of the toxin upon binding to the membrane was observed and the membrane-insertion structure was identified through proteolysis, protein N-terminal sequencing and MS. The function and topology of the membrane-inserted structure was also investigated using a liposome-entrapped glucose-release assay and 'sided'-proteolysis.

MATERIALS AND METHODS

Toxin purification

CytA and CytB were purified in the form of crystal inclusions from strains of recombinant *B. thuringiensis* IPS78/11 [36] containing the chloramphenicol-resistant plasmid, cam 2027, for CytA [37] and pBYCYTB [17] respectively, by sucrose density gradient ultracentrifugation as described previously [13]. The cytolytic toxin inclusions were kept in distilled water at -20 °C at 1–10 mg·ml^{-1} and were solubilized in the appropriate buffer before use. Protein concentrations of the inclusion suspensions

Abbreviations used: LUV, large unilamellar vesicle; MLV, multilamellar vesicle.

[1]Present address: Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, U.K.
[2]Present address: Natural Environment Research Council, Polaris House, North Star Avenue, Swindon SN2 1EU, U.K.
[3]To whom correspondence should be addressed (e-mail dje1@bioc.cam.ac.uk).

were determined by the Bicinchoninic Acid Protein Assay (Sigma, St. Louis, MO, U.S.A.) according to the manufacturer's instructions. All manipulations during the purification of the toxin were conducted at 4 °C.

Preparation of phospholipid vesicles and toxin binding

Multilamellar vesicles (MLVs) were prepared using the method described by Thomas and Ellar [8] with minor modification. The molar ratio of the lipid mixture (phosphatidylcholine: cholesterol:stearylamine or dicetyl phosphate) was 4:3:1. Small unilamellar vesicles were prepared by bath sonication of MLVs as described by Barenholtz et al. [38]. Large unilamellar vesicles (LUVs) were prepared according to Reeves and Dowben [39].

To make membrane-bound toxin, solubilized toxin was incubated with a liposome suspension, typically in 50 mM Na_2CO_3, pH 8.0, at 37 °C for 30 min. The mixture was then pelleted by centrifugation at 12000 ***g*** for 10 min, and the liposomes were washed with the same buffer twice. The binding was checked by running the samples from both the supernatant and pelleted liposomes on SDS/PAGE.

SDS/PAGE and immunoblotting

Standard Tris/glycine SDS/PAGE was used as described by Thomas and Ellar [40], which was modified from Laemmli [41]. Gel electrophoresis of samples containing peptides of low molecular mass, especially those under 20 kDa , was carried out using the method described by Schägger and von Jagow [42]. Gels comprised a stacking gel of 4% (w/v) acrylamide, a separating gel of 16.5% (w/v) acrylamide and a spacer gel of 10% (w/v) between them.

Immunoblotting was based on Towbin et al. [43]. Proteins separated by SDS/PAGE were electrophoretically transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA, U.S.A.) with a Bio-Rad Transblot semi-dry transfer cell according to the manufacturer's instructions. Protein bands were detected using polyclonal antibody raised against Cyt toxins and peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody.

Proteolysis of solubilized and membrane-bound toxin

For solubilization, purified toxin inclusions were solubilized at about 1 mg·ml^{-1} in 50 mM Na_2CO_3 (pH 10.5)/10 mM dithiothreitol by incubating at 37 °C for 30 min. Freshly prepared protease solution was added to solubilized toxin at 0.01–10% (w/w) and incubated at 37 °C for the required lengths of time.

Proteolysis of membrane-inserted Cyt toxins at sites accessible on the external surface of the liposome membrane was conducted as follows: toxin was incubated with MLVs in 50 mM Na_2CO_3/0.1 M NaCl (pH 8.0), at a toxin:lipid ratio of 1:10 (w/w) for 30 min at 37 °C to ensure the binding of the toxin to the liposomes. The unbound toxin was removed by washing the liposome–toxin complex three times with the same buffer; this was conducted by resuspending the liposome–toxin complex in the buffer and then removing the buffer after the liposomes had been precipitated by centrifugation at 12000 ***g***. The pellet was then resuspended in 50 mM Na_2CO_3 (pH 8.0)/10% (w/w) protease was added before proteolysis at 37 °C for 60 min. After protease treatment, the liposomes were separated from the medium by centrifugation at 12000 ***g*** for 30 min and the pellet was washed with the same buffer three times again to remove the remaining protease and the removable toxin fragments.

Proteolysis of regions of membrane-inserted Cyt toxin that might be accessible on the internal surfaces of the liposomes was conducted by allowing toxin to bind to liposomes containing protease trapped inside and then incubating the toxin-bound liposomes at 37 °C for 60 min. The protease-containing liposomes were made by washing the liposomes containing protease on both sides in suspension buffer and adding protease inhibitor to the external medium.

Proteolysis was terminated by adding ice-cold trichloroacetic acid to the sample to a final concentration of 15% (w/v). The proteolytic fragments were then purified as described previously [44]. The samples of the proteolytic fragments were divided into two aliquots. One aliquot was used for separating the fragments from each other by 16.5% Tris/Tricine SDS/PAGE [42], either for immunoblotting analysis or for N-terminal sequencing, and the other aliquot was used for electrospray MS if required (see below). In the case of the proteolysis of membrane-bound toxin, excess acetone was needed to remove the lipid from the sample.

N-terminal sequencing and electrospray MS of the proteolytic fragments

The proteins or peptides separated by SDS/PAGE were transferred to Pro-Blot membrane (Applied Biosystems, Foster City, CA, U.S.A.), as described above, and the peptides were visualized directly by incubation in 0.1% Coomassie Blue R in 50% methanol/1% acetic acid for 1 min and destained with several changes of 2–5 min each with 50% methanol. After washing the membrane with distilled water and then drying at room temperature, protein bands were excized and subjected to N-terminal sequencing using an ABI 477A Applied Biosystems automated pulse-liquid sequencer.

Electrospray MS was conducted using a BIO-Q quadrupole analyser [45] from VG Instruments (Manchester, U.K.) connected to an Intel computer running on Lab-Base software. Gramicidin-S was used as an internal standard at 2 mg/l. The mass range scanned was calibrated using 20 μM myoglobin (horse heart). For positive ion analysis, the mobile phase and the myoglobin calibration solvent was 50% methanol/1% acetic acid, the needle voltage was 4 kV and samples were made to 50% methanol before analysis. For negative ion analysis, the mobile phase and the myoglobin calibration solvent was 50% acetonitrile, the needle voltage was 3 kV and samples were made to 50% acetonitrile.

In vitro haemolysis assay of the toxins and toxin fragments

Rabbit blood cells were washed with PBS/glucose (2.25% glucose made in 0.5 × PBS buffer) and made up to 2.5% (v/v) in PBS/glucose/gelatin (50 mg of gelatin in 100 ml of PBS/glucose), divided into 2 ml aliquots and left on ice until required. The assay was conducted as decribed previously [40]. Wells that contained no button of red cells at the bottom were recorded as showing haemolysis.

Liposome glucose-release assay

A liposome glucose-release assay was employed [46] with minor modification to investigate the pore-forming segment of the Cyt toxins and its effect on a lipid membrane. The relative glucose-release activities were indicated as a percentage of maximum release, which was obtained by adding 3% Triton X-100 to the glucose-containing liposomes. First, glucose-entrapped liposomes (LUVs) were prepared as described above for normal liposomes except the suspension buffer was replaced with 300 mM glucose/10 mM Hepes (pH 8.0). The LUV suspension was then passed through a Sephadex G-50 (coarse) column and eluted in 150 mM KCl/10 mM Hepes (pH 8.0). The same glucose assay

was used to ensure that the glucose residue in the external medium was completely removed. The assay buffer was composed of 2 mM Mg^{2+}-acetate/1 mM ATP/67 μg/ml hexokinase/370 μg/ml NADP/34 μg/ml glucose-6-phosphate dehydrogenase. The control cuvette contained the same amount of sample to be tested and the reaction buffer with glucose-6-phosphate dehydrogenase replaced by 150 mM KCl/10 mM Hepes (pH 8.0). The absorbance at 340 nm was recorded for 30 min continuously after the sample was added and mixed well.

RESULTS

Proteolysis of solubilized and membrane-bound Cyt toxins

Previous studies [13,15,47] have shown that solubilized protoxins of CytA and CytB can be processed by protease K and by trypsin to smaller peptides. Here we used 10 enzymes, including protease K and trypsin, to probe the protease-sensitivity of the Cyt toxins in the water-soluble and membrane-bound states.

Protease K and trypsin

Membrane-bound Cyt toxins were prepared as described in the Materials and methods section. After protease K (10%, w/w) treatment, the proteolytic fragments purified from both the aqueous supernatant and the liposome pellet fractions were analysed by SDS/PAGE and immunoblotting, and this was compared with the toxins proteolysed in solubilized form in the absence of liposomes (Figure 1). CytA was processed by protease K to a 22 kDa fragment after incubation at 37 °C for 60 min (Figure 1A, lane 6), which was resistant to further proteolysis (results not shown).

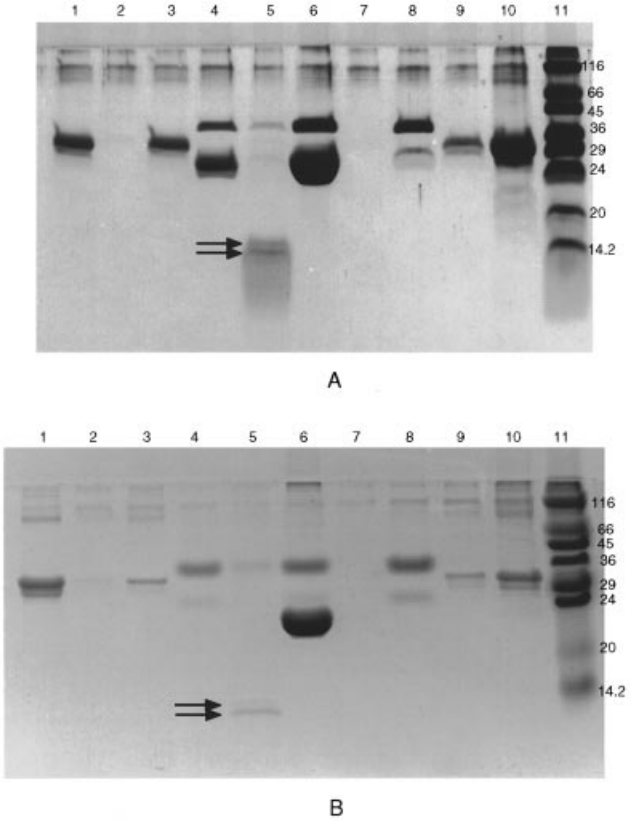

Figure 1 SDS/PAGE (16.5% Tris/Tricine gel) analysis of CytA (A) and CytB (B) with protease K

Lanes 1, solubilized toxin, 10 μg aliquot. Lanes 2 and 3, supernatant and pellet fractions of the toxin–membrane mixture after incubation at 37 °C for 30 min. Lanes 4 and 5, supernatant and pellet fraction of membrane-bound toxin after treatment with protease K (10%, w/w) at 37 °C for 60 min. Lanes 6 and 7, supernatant and pellet fraction of solubilized toxin treated with protease K. Lanes 8, protease K (the same amount as added in lanes 4 and 5, and lanes 6 and 7, incubated at 37 °C for 60 min). Lanes 9 and 10, membrane-bound toxin without protease K added but incubated for the same period of time as a control. Lanes 11 show molecular-mass markers labelled in kDa.

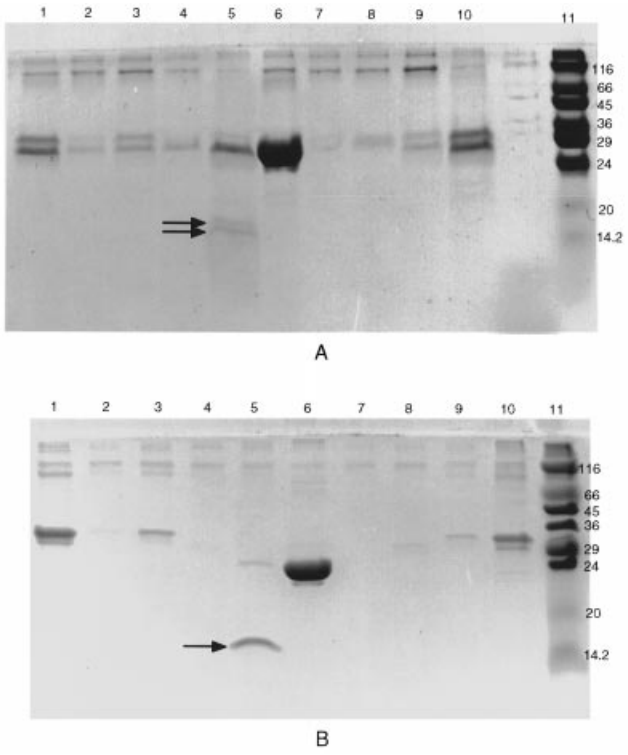

Figure 2 SDS/PAGE (16.5% Tris/Tricine gel) analysis of proteolysis of CytA (A) and CytB (B) with trypsin

Lanes 1, solubilized toxin, 10 μg aliquot. Lanes 2 and 3, supernatant and pellet fractions of toxin–membrane mixture after incubation at 37 °C for 30 min. Lanes 4 and 5, supernatant and pellet fractions of membrane-bound toxin after treatment with trypsin at 37 °C for 60 min. Lanes 6 and 7, soluble and sedimentable fraction of solubilized toxin treated with trypsin. Lanes 8, trypsin (the same amount as added in lanes 4 and 5, and lanes 6 and 7, incubated at 37 °C for 60 min). Lanes 9 and 10, membrane-bound toxin without trypsin added but incubated for the same period of time as a control. Lanes 11 show molecular-mass markers labelled in kDa.

Soluble CytB was processed into two products with slightly different molecular masses at around 21.5 kDa after incubation with 10% protease K (w/w) for 2 h (Figure 1B, lane 6). However, both CytA and CytB responded differently to protease K proteolysis in the membrane-bound state compared with the soluble state. When membrane-bound CytA was incubated with protease K under the same conditions it was cleaved into two much smaller products with molecular masses around 11.5 and 12.5 kDa (Figure 1A, lane 5). Membrane-bound CytB also showed a different proteolytic profile with two fragments of 10.0 and 10.5 kDa formed in the presence of liposomes (Figure 1B, lane 5). No smaller fragments of either CytA or CytB were detected in the supernatant fraction of the membrane-bound toxin after protease K treatment (Figures 1A and 1B, lanes 4).

Similarly, whereas trypsin treatment of both CytA and CytB in the solubilized form for 60 min at 37 °C gave a product of 22 kDa, proteolysis of membrane-bound toxin with trypsin under

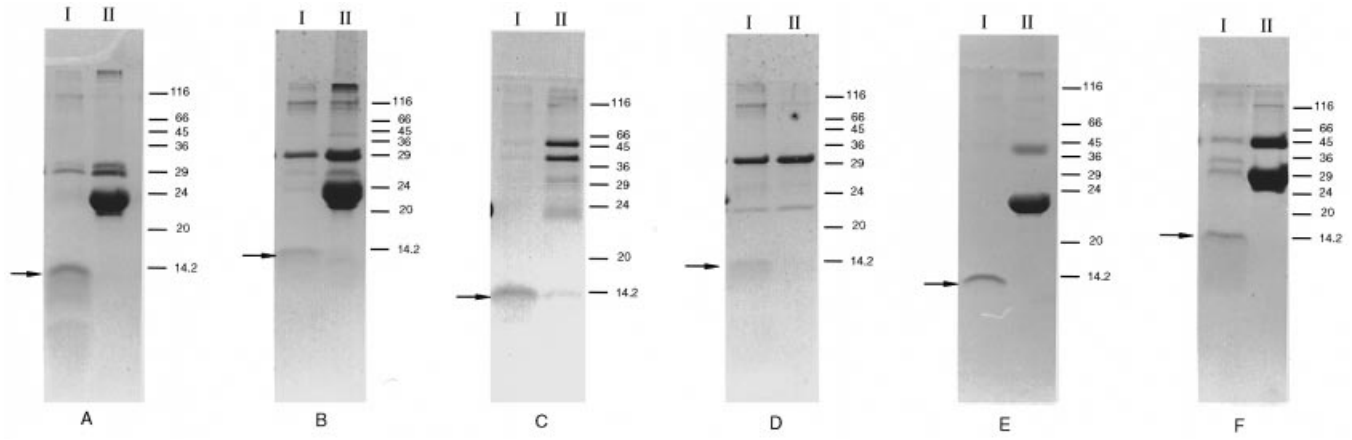

Figure 3 SDS/PAGE (16.5% Tris/Tricine gel) analysis of membrane-bound toxins with six proteases

(**A**) CytB with elastase; (**B**) CytB with nargase; (**C**) CytB with Pronase E; (**D**) CytB with subtilisin; (**E**) CytB with thermolysin; and (**F**) CytA with thermolysin. Lanes I, pellet fraction of membrane-bound toxin after proteolysis at 37 °C for 60 min. Lanes II, supernatant fraction of membrane-bound CytA after proteolysis. The proteolytic fragments of membrane-bound toxin are indicated with arrows.

the same conditions produced two fragments of about 10.5 and 11 kDa with CytA (Figure 2A, lane 5) and one fragment of 12 kDa (Figure 2B, lane 5) with CytB.

Proteolysis with thermolysin, nargase, V8 protease, chymotrypsin, elastase, papain, subtilisin and Pronase E

Of the eight other proteases used for the proteolysis of the membrane-bound toxins, elastase, nargase, Pronase E and subtilisin degraded membrane-bound CytB into a fragment of about 11–12 kDa (Figures 3A–3E, lane I) but no fragments of similar size were generated by these enzymes from membrane-bound CytA (results not shown). Thermolysin produced a fragment of about 12 kDa for both CytA (Figure 3F, lane I) and CytB (Figure 3E, lane I). Papain, V8 protease and chymotrypsin did not yield any smaller proteolytic fragment from either membrane-bound CytA or CytB than a 22 kDa fragment, which was the same as the proteolytic product of the solubilized toxins (results not shown). It should be noted that treatment of solubilized CytA with all these enzymes for the same length of time resulted in one proteolytic species of 20 or 21 kDa, which was very resistant to further degradation by these enzymes (results not shown).

Proteolytic processing and haemolytic activity

It has been reported that protease K-processed CytA and CytB are both haemolytic, whereas unprocessed CytA is less haemolytic and unprocessed CytB is non-haemolytic [15,48]. Whereas the previous work on the haemolytic activities of the Cyt toxins demonstrated a requirement for protease activation of protoxin, no systematic information has yet been gained on the effect of prolonged proteolysis of Cyt toxins on their cytolytic activities. CytA and CytB were therefore treated with protease K, trypsin, thermolysin and chymotrypsin (10%, w/w) for various lengths of time (1–34 h) and used for *in vitro* haemolysis assays with rabbit red blood cells in microtitre plates. The results are summarized in Table 1.

It was known from N-terminal sequencing of the proteolytic fragments that treatment of CytA with protease K for 2 h resulted in cleavage at R30 and V31 [15]. Longer incubations up to 24 h did not significantly change the amounts of the proteolytic products seen by SDS/PAGE (results not shown). However, whereas the 2 h treatment caused only a slight decrease of the haemolytic activity, treatment up to 5 h caused a 4-fold increase in the threshold concentration for haemolytic activity (see (Table 1). Similarly, proteolysis of CytA with trypsin resulted in a striking decrease in its haemolytic activity within 2–5 h, and no haemolytic activity could be detected after 22 h (Table 1), even though it can be shown that two stable proteolytic products of 22.5 and 10.5 kDa remained (results not shown). Therefore, the loss of activity was not due to a reduction in the amount of toxin, but it was more likely that the prolonged proteolysis removed additional amino acid residues from either the N- or C-termini, or from both termini, and that some of these residues are important for membrane binding or pore formation or for overall toxin structure.

Chymotrypsin had little effect on the haemolytic activity of CytA toxin even after treatment for 34 h. Unlike CytA, when treated with protease K, solubilized CytB became slightly more haemolytic. Proteolysis of CytB with trypsin, chymotrypsin and thermolysin had no effect on the haemolytic activity.

Proteolytic sites of solubilized and membrane-bound toxins revealed by N-terminal sequencing and MS of the proteolytic fragments

N-termini of the membrane-protected fragments of CytA and CytB when treated with protease K, trypsin and thermolysin

Three proteolytic cleavage sites were revealed by N-terminal amino acid sequencing when liposome-bound CytA toxin was treated with 10% (w/w) protease K. They are located before amino acid residues S42, E45 and K154 (Table 2). It was shown that with protease K, CytA was split into two main fragments: one was the N-terminal half from amino acid residues S42 or E45, the other was the C-terminal half starting from amino acid residue K154. The apparent molecular mass estimated from SDS/PAGE suggested the former extended to around Q153 and the latter extended to the C-terminus of the complete CytA sequence.

A striking difference is noticeable (Table 2) between the amounts of the proteolytic fragments in the membrane-associated samples after protease K treatment, e.g. K154:E45 = 10:1,

Table 1 Haemolytic activities of protease-processed Cyt toxins

CytA and CytB were processed with proteases for various lengths of time and the proteolytic products were analysed for their haemolytic activities as described in the Materials and methods section. Briefly, rabbit red blood cells were added to a serial dilution of the samples and the haemolysis was recorded after the microtitre plate was incubated at room temperature for 2 h. The concentrations of the proteolysed Cyt toxin samples were based on the initial amount of solubilized toxin.

			Haemolysis end point (μg/ml) at different processing time				
Toxin	Protease	Processing time (h) ...	1	2	5	22	34
CytA	Protease K		0.98	1.95	3.90	250	$> 10^3$
	Trypsin		0.98	1.95	250	$> 10^3$	–
	Chymotrypsin		0.49	0.98	1.95	3.90	3.90
CytB	Protease K		0.98	0.49	0.49	0.49	0.49
	Trypsin		3.90	3.90	3.90	3.90	7.80
	Chymotrypsin		1.95	1.95	1.95	3.90	3.90
	Thermolysin		1.95	1.95	1.95	3.90	3.90

Table 2 N-termini of the proteolytic fragments of liposome-bound CytA and CytB after proteolysis

Liposome-bound CytA and CytB were treated with protease K, trypsin and thermolysin, the liposomes were separated from the medium solution by centrifugation as described in the Materials and methods section. The proteolytic fragments associated with the membrane fraction were purified and N-terminally sequenced. In the case of CytB, the liposomes were washed three times and the amounts of the membrane-associated fragments were compared with the non-washing treatment. –, not tested.

			Amount (pmol)		
Toxin	Proteases	N-terminus	No washing	Washing 3 ×	Molecular mass on gel (kDa)
CytA	Protease K	S42	4	–	12.0
		E45	2	–	12.0
		K154	20	–	11.5
	Trypsin	V31	58	–	10.5, 10.0
		G84	12	–	6.0
		N155	120	–	10.0, 10.5
		E164	34	–	6.0, 10.0
CytB	Protease K	I150	8 (100%)	–	10.5
	Trypsin	H30	41 (49%)	85 (29%)	12.5
		T95	9 (11%)	45 (16%)	12.5
		G141	33 (40%)	160 (55%)	12.0
	Thermolysin	I31	76 (53%)	25 (20%)	12.5
		L146	67 (47%)	100 (80%)	12.0

Table 3 C-terminal determination of protease K proteolytic fragments of membrane-bound CytA and CytB

MS of the membrane-associated fragments of CytA and CytB resulted in 9 (A–I) and 4 (a–d) substances of various molecular masses respectively, which were then matched to a series of calculated molecular masses of the segments based on the N-terminal amino acid sequencing. Therefore the C-termini of the membrane-associated fragments can be located.

Toxin	Toxin fragment detected by MS ($\pm$S.D.)	Cleavage sites revealed by N-terminal sequencing	Fragments and the C-termini (underlined) that match the molecular masses	Matched fragments and their mass
CytA	A: 9711.45 ($\pm$1.5)		42–123: 8927.8	H: 8929.09
	B: 9600.07 ($\pm$2.1)	S42	42–130: 9596.7	B: 9600.07
	C: 9396.11 ($\pm$3.5)		42–131: 9709.9	A: 9711.45
	D: 9285.58 ($\pm$2.1)		45–123: 8613.4	I: 8614.90
	E: 9067.79 ($\pm$4.2)	E45	45–130: 9282.3	D: 9285.58
	F: 9040.77 ($\pm$1.4)		45–131: 9395.5	C: 9396.11
	G: 8946.98 ($\pm$2.6)	K154	154–234: 9073.9	E: 9067.79
	H: 8929.09 ($\pm$0.2)			
	I: 8614.90 ($\pm$1.4)	N155	155–234: 8945.7	G: 8946.98
		D147	–	No match
CytB	a: 10042.95 ($\pm$1.3)		150–237: 10040.51	a: 10042.95
	b: 9895.02 ($\pm$2.1)	I150	150–236: 9893.34	b: 9895.02
	c: 9781.44 ($\pm$0.4)		150–235: 9780.18	c: 9781.44
	d: 9171.25 ($\pm$0.7)		150–229: 9171.25	d: 9171.25

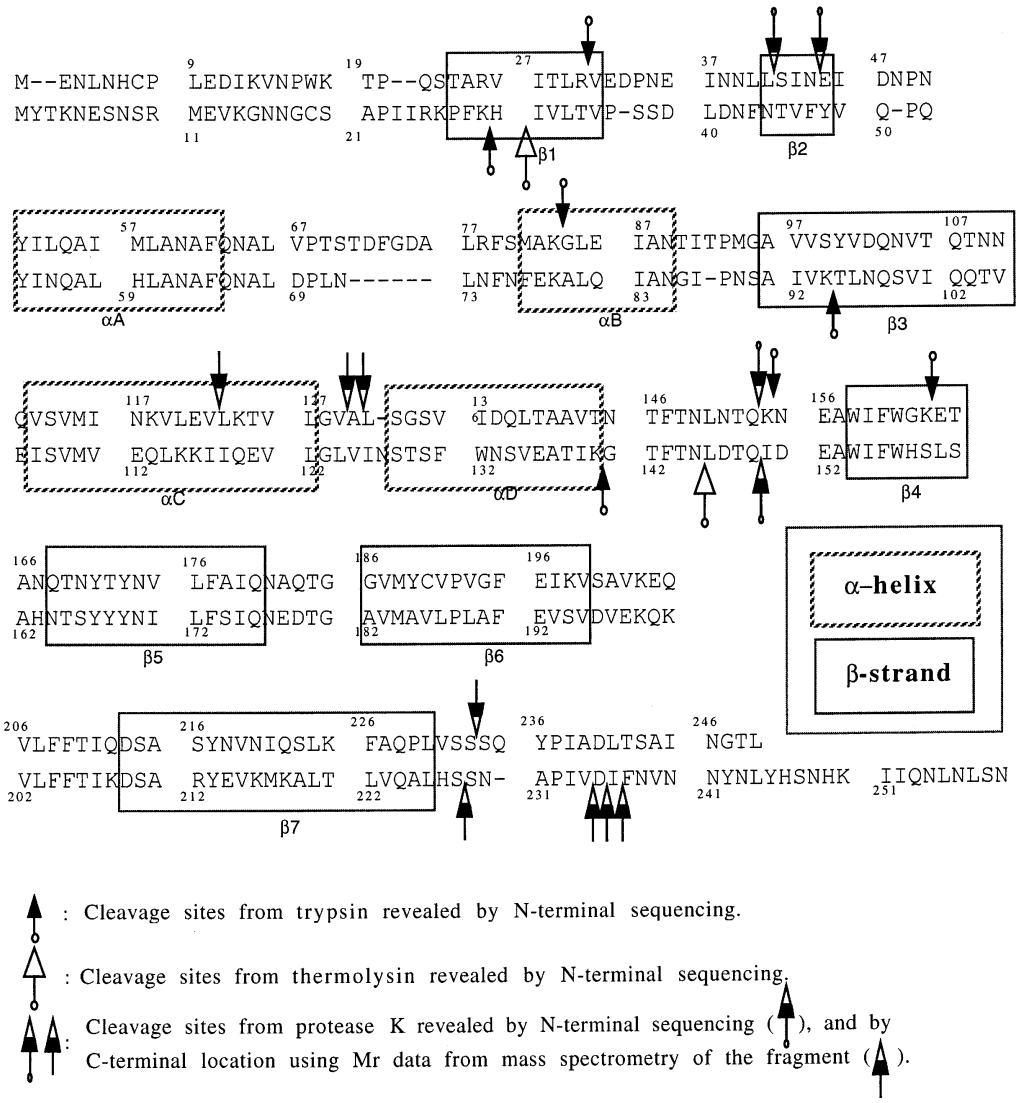

Figure 4 Proteolytic cleavage sites along the sequence alignment of CytA (upper sequence) and CytB (lower sequence)

The secondary structures of the toxins are based on the CytB structure [28] and it is assumed here that CytA has the same tertiary structure.

K154:S42 = 5:1. This suggests that it is the C-terminal CytA cleavage product starting at K154 that is preferentially retained in the membrane.

Proteolysis was also performed with trypsin under the same conditions, and four cleavage sites were discovered before amino acid residues V31, G84, N155 and E164. Of these, a fragment with its N-terminus at amino acid residue N155 comprises most of the protein in the membrane-associated fraction of the sample.

Proteolysis of membrane-bound CytB with protease K produces only a single proteolytic species. This fragment has an N-terminus from amino acid residue I150 with a molecular mass of about 10 kDa estimated from SDS/PAGE. With trypsin, three proteolytic fragments were detected in the membrane-associated fraction; they have N-termini at amino acid residues H30, T95 and G141. With thermolysin, two fragments were found in the membrane fraction with N-termini from I31 and L146, respectively. However, the proportions of these proteolytic products in the membrane-associated fraction were different if the liposomes were washed with buffer three times after separation from the external medium. Both in the experiments with trypsin and thermolysin, washing liposomes with buffer significantly decreases the proportion of the fragments from the N-terminal half of the toxin sequence. It is therefore highly likely that the fragments with N-termini H30 and I31 are not membrane-embedded, but are probably only adsorbed to the membrane surface through ionic interaction with the head groups of the lipid molecules.

C-terminal determination of the fragments by MS

In order to determine the C-termini of the membrane-protected fragments, electrospray MS [45] was employed to reveal the accurate masses of the proteolytic fragments.

Nine components were found with masses ranging from 8614.9 to 9711.45 Da in the CytA proteolytic sample and four were found in the CytB proteolytic sample with masses ranging from 9171.25 to 10042.95 Da (Table 3, column 2). In the case of both CytA and CytB, the molecular masses of almost all fragments

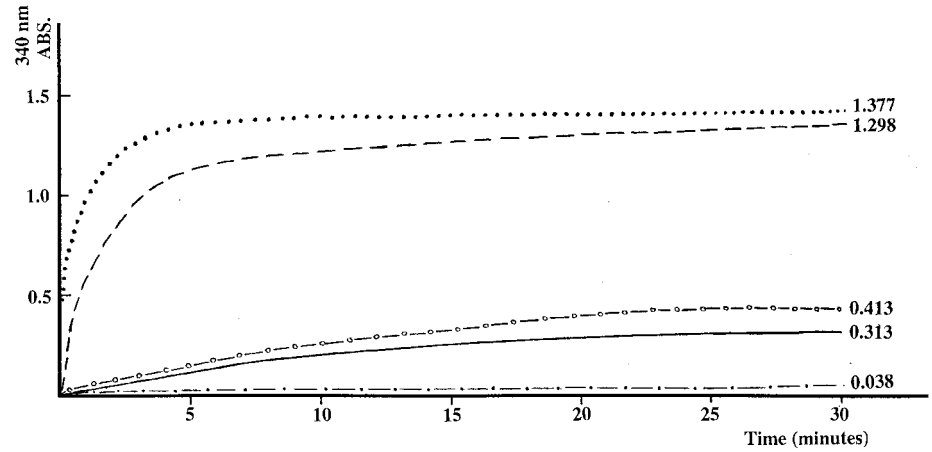

Figure 5 Glucose-release assay of protease-processed CytA

Release of LUV-entrapped glucose with solubilized CytA (---), phospholipid-bound CytA (-○-○-), and protease K-processed phospholipid-bound CytA (——). Absorbance at 340 nm was recorded continuously for 30 min. The maximum release was obtained by adding 3% Triton X-100 (……). Phospholipid vesicles without added toxin were employed as a control (-●-●-). The lines were traced from the original chart recordings. The concentration of solubilized CytA was 50 $\mu g \cdot ml^{-1}$, and the molar concentration in the two other treatments were equivalent to 50 $\mu g \cdot ml^{-1}$. The assay was conducted at room temperature.

(except fragment F in CytA) measured with MS can be matched quite accurately with the molecular mass of a toxin segment with the N-terminus at the proteolytic cleavage site. This revealed more cleavage sites on the C-terminal ends of those proteolytic fragments. For CytA, the major proteolytic fragments from the N-terminal half of the toxin sequence with N-termini starting from S42 or E45 have their C-termini at L123, A130 or L131, whereas other major proteolytic fragments from the C-terminal half of the toxin sequence with N-termini at K154 or N155 both have their C-termini at S234. The molecular-mass matching indicated all four proteolytic fragments of CytB have the same N-terminus at I150 and have their C-termini at A229, D235, I236 and F237 respectively.

The proteolytic cleavage sites along the sequences of CytA and CytB when the membrane-inserted toxins are treated with protease K, trypsin and thermolysin from outside the vesicles are shown in Figure 4. These cleavage sites are concentrated in three main regions. The first proteolysis-susceptible region is between amino acid residues 30 and 45, which is located in the first and second β-strands according to the CytB crystal structure published by Li et al. [28]. The second is around amino acid residue 150, which is in the loop region between the mainly α-helical N-terminal half and the mainly β-sheet C-terminal half of the toxin structure. The third site revealed by molecular-mass matching is near the C-terminal ends of both CytA and CytB, just next to β-strand 7. All three regions are more or less in the same positions in the two aligned sequences, and this is in agreement with the suggestion by Li et al. that CytA and CytB have similar tertiary structure [28]. For proteolysis with protease K, three cleavage sites were also found in α-helix C or between α-helices C and D. The fact that almost all proteolytic cleavage sites were found in the N-terminal halves of the protein sequences and no cleavage sites were detected in any of the three major β-strands strongly suggests that the mainly α-helical N-terminal half and the C-termini of the toxin were exposed to the proteases added from the external medium of the liposomes to which the toxins binds, whereas the segment consisting of the three β-strands in the C-terminal half was protected from proteolysis either because it is protected by insertion into the membrane, or because there are no protease-sensitive sites in this region.

It has been shown in this and previous studies [15,47] that in the absence of a membrane, both CytA and CytB are processed by protease K, trypsin and thermolysin at amino acid residues 29–31. Also, Koni and Ellar [15] showed that both toxins were processed by 10–50% (w/w, enzyme:toxin) of protease K just before amino acid residue 233 for CytA and before 230 and 238 for CytB. This suggests that a conformational change, caused by binding of the toxins to a phospholipid membrane, has on the one hand exposed most of the N-terminal half of the toxin, thus resulting in more proteolytic cleavage sites in this region, but on the other hand has not changed the relative susceptibility of the two ends to proteolysis.

Washing the liposomes with 50 mM Na_2CO_3 (pH 8.0) did not remove all the fragments from the N-terminal halves of the toxin sequences but did significantly reduce the amount in the membrane fraction. This suggests the removable fragments from the N-terminal half of the toxin sequences are not irreversibly bound or inserted into the membrane. Instead, they may merely be attached to the lipid head groups through ionic interactions. Washing the liposomes with buffer of higher salt concentrations could serve to test this hypothesis, but the high-salt buffer disrupts the integrity of the liposome structure and therefore makes it difficult to interpret the proteolysis results from outside the membrane. It also makes the separation of the liposomes from the medium more difficult.

Functionality of the membrane insertion structure

A combination of liposome fusion and glucose-release assay was employed to investigate the possible pore-forming capability of the membrane-embedded fragment and the importance of the mainly α-helical section of Cyt toxin in pore formation.

In this experiment, the pore-forming function of the liposome-embedded structure of the toxin and the whole structure of the toxin were compared with each other by measuring their glucose-

release activities when the toxin- and toxin fragment-embedded liposomes were fused to the liposomes containing glucose. Direct addition of solubilized CytA at a concentration of 50 $\mu g \cdot ml^{-1}$ to the glucose-containing liposome suspension resulted in the immediate release of glucose, with absorbance reaching its highest level within 2 min, which is about 94.3 % of the maximum release (Figure 5). Membrane-bound CytA at the same concentration achieved 30 % glucose release, indicating that fusion of the liposomes brought about the translocation of at least some of the pore structures from the toxin-bound liposome population to the glucose-trapped liposome population, thus causing the release of the trapped glucose. The rate of glucose release was different between solubilized CytA and membrane-bound CytA. Solubilized CytA caused a rapid increase in absorbance in the first 5 min of the reaction, while membrane-bound CytA caused a much slower, more gradual increase in absorbance during the course of the 30 min reaction (Figure 5). The much slower glucose-release activity from the membrane-bound toxin probably reflects the time required for fusion of the pore structure into the liposomes containing trapped glucose.

It is interesting to note that the protease K-processed membrane-bound CytA structure had a similar glucose release effect on liposomes to the unprocessed membrane-bound toxin, both in the time course and the maximum amount of glucose release it caused. Since it has been shown that the membrane-protected CytA fragment from protease K proteolysis is the section that consists mainly of the β-sheet half of the protein with an N-terminus around amino acid residue K154, this result indicates that the β-sheet half of the CytA toxin is the only part that participates in the pore-forming structure, although other parts may play a role in the initial stage of the toxin–membrane interaction.

DISCUSSION AND CONCLUSION

CytA and CytB treated with 10 different proteases in solubilized form produced stable fragments of 20–22.5 kDa. However, when CytA and CytB were first incubated with phospholipid liposomes, new protease-sensitive sites were created for most of the proteases used, resulting in smaller fragments of about 10–12.5 kDa. This suggests that a major conformational change was induced by membrane binding.

The proteolytic cleavage sites are concentrated in three main regions along the Cyt toxin sequences (Figure 4). The first proteolysis-susceptible region is between amino acid residues 30 and 45, which is next to the β-1 strand according to the CytB crystal structure. The second is around amino acid residue 150, which is in the loop region between the mainly α-helical N-terminal half and the mainly β-strand C-terminal half of the toxin structure. The third region is near the C-terminal ends of both CytA and CytB just after β-7. Each of the three regions lies more or less in the same segment in the aligned sequences of the two toxins, which supports the proposal that CytA and CytB have a similar tertiary fold [28]. For protease K, three cleavage sites were also found in helix α-C and between helices α-C and α-D. The fact that almost all proteolytic cleavage sites were found in the N-terminal half of the protein sequences and no cleavage sites were detected in any of the three major β-strands strongly suggests that the mainly α-helical N-terminal half and the C-terminus of the toxin were exposed to the proteases added to the external medium of the liposomes to which the toxins bind, whereas the segment consisting of the three β-strands in the C-terminal half was protected against proteolysis either because it was protected by insertion into the membrane, or because there are no protease-sensitive sites in this region.

In addition, washing the liposomes with 50 mM Na_2CO_3 (pH 8.0) after proteolysis significantly reduced the amount of N-terminal fragments associated with the membrane fraction, suggesting that this part of the sequence is not irreversibly bound or inserted into the membrane, and it is the fragment derived from the C-terminal half of the toxin sequence that is preferentially retained with the liposomes.

Fusion between the liposomes bearing membrane-bound toxin and the glucose-containing LUVs led to steady release of the trapped glucose. This shows that the membrane-embedded toxin structure is in a functional state forming membrane pores. The ability to induce glucose release is undiminished after cleavage of the membrane-bound toxin by protease K, when the C-terminal half of the molecule is preferentially retained in the membrane. Therefore these data strongly suggest that the pores for glucose release are constituted from the part of the molecule comprising the three major β-strands (β-5, β-6 and β-7) in the C-terminal half of the toxin. No conclusive data were obtained on how the three β-strands are arranged in the membrane to form the pore. Our attempts to apply proteases from inside the toxin-bearing liposomes have so far failed to produce novel internal cleavage sites that would show conclusively cleavage of membrane-embedded toxin at the inner membrane surface. This may be because toxin segments exposed to the internal medium of the liposome are not accessible or sensitive to the proteases, because the toxin structure does not penetrate to the inner membrane face, or due to some other technical reason. These attempts are continuing.

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