Assembly of flammutoxin, a cytolytic protein from the edible mushroom *Flammulina velutipes*, into a pore-forming ring-shaped oligomer on the target cell

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Flammutoxin has been previously isolated as a cardiotoxic and cytolytic polypeptide of 22 or 32 kDa from the fruiting bodies of the edible mushroom *Flammulina velutipes*. In the present study, we purified flammutoxin as a single haemolytic protein of 31 kDa and studied the mode of its cytolytic action. (1) Flammutoxin caused efflux of potassium ions from human erythrocytes and swelling of the cells before haemolysis. (2) Flammutoxin did not lyse human erythrocytes in the presence of non-electrolytes with hydrodynamic diameters of > 5.0 nm, although it caused leakage of potassium ions and swelling of the cells under the same conditions. (3) Experiments including solubilization of cell-bound toxin with 2% (w/v) SDS at 20 °C and subsequent Western

immunoblots showed that flammutoxin formed a band corresponding to 180 kDa under the conditions where it lysed erythrocytes. (4) Electron microscopy of flammutoxin-treated human erythrocytes revealed the presence of a ring-shaped structure with outer and inner diameters of 10 and 5 nm, respectively, on the cells. (5) A ring-shaped toxin oligomer of the same dimensions was solubilized from the toxin-treated human erythrocytes with 2% (w/v) SDS at 20 °C and isolated by a sucrose-gradient ultracentrifugation. These data indicated that flammutoxin assembles into a ring-shaped oligomer possessing a hydrophilic pore of 4–5 nm on target cells.

INTRODUCTION

Several cytolytic proteins have been isolated from the basidiocarps of both toxic and edible mushrooms [1–7]. Lin et al. [8,9] isolated a cytolytic and cardiotoxic 22 kDa protein from the edible mushroom *Flammulina velutipes*, and designated it flammutoxin. They reported that flammutoxin caused lysis of mammalian erythrocytes, swelling of Ehrlich ascites tumour cells, and electrocardiographical changes in parenterally administrated animals (although it not toxic by oral administration) [9]. Thus flammutoxin seems to be a pore former in light of the present knowledge, although little has been elucidated of the mode of its cytolytic action. Bernheimer and Oppenheim [10] later purified a haemolytic protein of 32 kDa from the same mushroom, and referred to it as flammutoxin despite the large difference in molecular mass between the two. They assumed that the flammutoxin of Lin et al. would have been proteolytically derived from their 32 kDa protein [10].

In the present study, we purified the haemolytic principle of *F. velutipes* in the presence of protease inhibitors, and studied the mode of its haemolytic action. A water-soluble 31 kDa protein obtained as a single haemolytic protein from the mushroom showed characteristic features of pore formers, including assembly into a ring-shaped oligomer on target cells.

MATERIALS AND METHODS

Purification of flammutoxin

To minimize proteolysis of flammutoxin, all purification steps were carried out at 0–4 °C, and haemolytically active fractions were treated with protease inhibitors at each step. Human erythrocytes were used for the haemolytic assay. Fresh basidiocarps of *F. velutipes* (960 g, wet weight) were homogenized in 1

litre of 20 mM sodium phosphate buffer, pH 7.2, supplemented with 1 mM PMSF, 2 mM 1,10-phenanthroline and 10 mM EDTA. The homogenate was centrifuged for 60 min at 12000 g, and the haemolytically active fraction in the supernatant was precipitated with (NH₄)₂SO₄ at a concentration corresponding to 60% saturation. The precipitates were dialysed against 20 mM sodium phosphate buffer (pH 7.2), and applied to a DEAE-Cellulofine A-500 column (diameter 4 cm, height 30 cm). Haemolytically active fractions were eluted with 20 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl, and were dialysed against 10 mM potassium phosphate buffer, pH 6.8. The haemolytically active DEAE-Cellulofine A-500 fraction was adsorbed on to a hydroxyapatite column (diameter 2.6 cm, height 10 cm). Haemolytically active fractions were eluted with 100 mM potassium phosphate buffer (pH 6.8), and dialysed against 10 mM potassium phosphate buffer, pH 6.8. (NH₄)₂SO₄ was added to the hydroxyapatite fraction to 20% saturation and was adsorbed on to a Bakerbond WPHI-C3 column (diameter 1.6 cm, height 9 cm). Haemolytic fractions were eluted with a descending linear gradient of (NH₄)₂SO₄ (20–0% saturation), and were dialysed against 10 mM Tris/HCl buffer, pH 7.2. The Bakerbond WPHI-C3 fraction was concentrated using a Diaflo membrane YM10 (Amicon, Denver, MA, U.S.A.) and passed through a gel-filtration column of Toyopearl HW-50S (diameter 1.6 cm, height 100 cm). Active fractions of monomeric flammutoxin were collected and stored at –80 °C until use.

Haemolytic assay

The haemolytic assay was performed as described previously [11]. Erythrocytes were centrifuged at 600 g for 10 min, and the packed cells were suspended at 1% (v/v) in 10 mM Tris/HCl buffer, pH 7.2, containing 140 mM NaCl (TBS). Flammutoxin

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samples were incubated with erythrocytes (approx. 3×10^7 cells) in 200 μ l of TBS at 25 or 37 °C for 30 min. After centrifugation for 10 min at 600 *g*, 100 μ l of the supernatants were withdrawn and assayed spectrophotometrically at 550 nm with a reference at 630 nm, using a MTP32 microplate reader (Corona Electric Co., Katsuda, Japan). 100% lysis was defined as the absorbance of the supernatants obtained from the osmotically lysed cells.

Efflux of potassium ions from flammutoxin-treated erythrocytes

Human erythrocytes were washed five times by centrifugation for 5 min at 600 *g* to remove extracellular potassium ions. The washed erythrocytes (approx. 6×10^8 cells/ml) were incubated with flammutoxin (0.3 μ g/ml) at 25 °C, and were centrifuged for 1 min at 4 °C and 5000 *g*. A small portion of the supernatants were withdrawn for haemolytic assay. The rest of the supernatants were centrifuged at 18000 *g* for 20 min to remove erythrocyte membranes, if any. The supernatants were dried on a hot plate at 70–80 °C, decomposed at 550 °C, and dissolved in a portion of 0.02 M HCl. The atomic absorption spectrophotometric assay for potassium was performed at the wavelength of 766.5 nm with a Hitachi Z-8100 Polarized Zeeman Atomic Absorption Spectrophotometer (Hitachi, Tokyo, Japan).

Estimation of the functional diameter of membrane pores

Toxin-induced lysis of human erythrocytes was assayed at 25 °C in the Tris-buffered isotonic solution that contained non-electrolytes [i.e. poly(ethylene glycol)s and neutral sugars] of different sizes at a concentration equivalent to 40 mOsm. The total osmotic pressure of the extracellular space was adjusted to 295 mOsm by changing the concentration of NaCl. The values for hydrodynamic diameters of the non-electrolytes were taken from the reports of Scherrer and Gerhardt [12] and Sabirov et al. [13], where the hydrodynamic diameters were calculated on the viscosity of non-electrolyte solutions.

Electron microscopy for toxin-treated erythrocytes and the isolated toxin complex

Negative staining of flammutoxin-treated erythrocytes was performed according to a previously described method [14], except that thin carbon film was used as a support. Human erythrocytes (3×10^8 cells/ml) were incubated with flammutoxin (50 μ g/ml) at 37 °C for 30 min. Lysed erythrocytes were placed on to a carbon-coated grid, washed briefly with 5 mM phosphate buffer (pH 7.2) and stained negatively with 1% (w/v) sodium phosphotungstic acid, pH 7.2. Specimens were examined under a JEOL 2000ES electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV. The negatively stained images of the toxin-treated cells were subjected to computer-aided optical filtering to give clear images of ring-shaped molecular arrangements on the cells. The Fourier transform of a selected field of the original images was taken using a Luzex-F real-time image analyser (Nireco, Tokyo, Japan). Background noise was filtered by a mask to pass the ring-shaped intense area of the original spectrum. The final image was obtained by re-transforming such masked areas with the same equipment.

Electron microscopy for isolated flammutoxin complexes was performed as described above, except that the toxin-complex fraction obtained from sucrose-density-gradient ultracentrifugation was dialysed against 10 mM Tris/HCl buffer, pH 7.2, containing 0.1% (w/v) SDS overnight at 4 °C before negatively staining.

Anti-flammutoxin serum

Female New Zealand White rabbits were injected subcutaneously with flammutoxin (1 mg) emulsified in Freund's complete adjuvant (Difco Laboratory, Detroit, MI, U.S.A.), followed by the injections of the toxin (0.5 mg) emulsified in Freund's incomplete adjuvant (Difco Laboratory) every 10 days for 8–10 times. The titres of the sera were monitored using Ouchterlony's immunodiffusion. When titres of the sera reached ≥ 256 fold, the animals were anaesthetized and killed for preparation of antisera.

Assembly of flammutoxin into a large-molecular-sized complex on erythrocytes

Complex formation of flammutoxin on erythrocytes was assayed essentially as described previously [15]. Mixtures of flammutoxin (0.5 μ g) and erythrocytes (1.5×10^8 cells) were incubated in 1 ml of TBS at 25 °C for 30 min, unless otherwise stated. Toxin-erythrocyte complexes were collected by centrifugation at 16000 *g* for 20 min at 4 °C and suspended in 5 mM sodium phosphate buffer (pH 7.2) to lyse intact cells. The toxin-erythrocyte complexes were washed twice by centrifugation at 16000 *g* for 20 min, solubilized in a small portion of 2% (w/v) SDS at 20 °C for 5 min, and subjected to SDS/PAGE using a linear gradient gel of 3–20% (w/v) acrylamide. The solubilized toxin was not heated at 100 °C in the presence of 2% (w/v) SDS before electrophoresis. Protein bands in the gel were directly stained with Coomassie Brilliant Blue R-250, or they were electroblotted on to a nitrocellulose sheet, followed by immunostain using specific antiserum raised against flammutoxin and horseradish peroxidase-conjugated Protein A. 4-Chloro-1-naphthol and H_2O_2 were used as the substrates for horseradish peroxidase.

Separation of the toxin complex from erythrocyte membranes by sucrose-density-gradient ultracentrifugation

Human erythrocytes (3×10^{10} cells in 100 ml of TBS) were exposed to flammutoxin (1 mg of protein) at 25 °C for 30 min. Erythrocytes were collected by centrifugation at 16000 *g* for 20 min at 4 °C and suspended in 5 mM sodium phosphate buffer (pH 7.2) to lyse intact cells. Lysed cells were washed twice by centrifugation at 16000 *g* for 20 min, and packed by ultracentrifugation using a Beckman SW40Ti rotor at 35000 rpm for 1 h at 4 °C. The packed cell membranes were solubilized with 2% (w/v) SDS at 20 °C for 2–3 min, and were applied to a 10–40% (w/w) linear sucrose gradient in 10 mM Tris/HCl buffer, pH 7.2, containing 0.1% (w/v) SDS. Centrifugation was performed using a Beckman SW40Ti rotor at 32000 rpm for 19 h at 4 °C. Fractions were collected from the bottom of the tubes, and subjected to electron microscopy and Western immunoblot as described above.

Miscellaneous

Protein concentration was assayed as described by Bradford using BSA as a standard [16]. The N-terminal amino acid sequence of flammutoxin was analysed using a PSQ-2 Protein Sequencer (Shimadzu, Kyoto, Japan) [17]. Isoelectric focusing was performed at 4 °C in a LKB 8101 column with a carrier ampholyte of pH 3–10 (LKB-Pharmacia, Uppsala, Sweden) at a constant voltage of 600 V for 72 h. The amino acid composition of flammutoxin was determined using a Hitachi HPLC 655 Orthophenylaldehyde System (Hitachi, Tokyo, Japan): One milligram of flammutoxin was dried under reduced pressure, and hydrolysed in 4 M methanesulphonic acid at 105 °C for 24, 48 or

72 h. The contents of cysteine and tryptophan were determined spectrophotometrically according to the methods of Schram et al. [18] and Goodwin and Morton [19] respectively. The osmotic pressures of the non-electrolyte solutions were measured at 25 °C using a Micro-Osmometer Model 3MO (Advanced Instruments, Needham Heights, MA, U.S.A.).

Materials

Basidiocarps of *F. velutipes* were purchased from a farm and stored at -40 °C before use. DEAE-Cellulofine A-500 and hydroxyapatite were from Seikagaku Co. (Tokyo, Japan). Bakerbond WP HI-C3 was from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.). Poly(ethylene glycol)s were purchased from Wako Chemicals (Osaka, Japan). Unless otherwise stated, chemicals used in this study were of the highest grade commercially available.

RESULTS

Purification and some properties of flammutoxin

A single haemolytic protein from the basidiocarps of *F. velutipes* was purified by successive column chromatography using DEAE-Cellulofine A-500, hydroxyapatite, Bakerbond HI-C3 and Toyopearl HW-50S as described in the Materials and methods section. The molecular size of the purified protein was determined to be 31 kDa on SDS/PAGE under non-reducing and reducing conditions (Figure 1; lanes 1 and 2 respectively). The molecular mass of the purified protein was also estimated to be 30 kDa by high-performance gel-permeation column chromatography using a TSKgel G3000SW column (results not shown). These data suggested that the haemolytic protein was isolated from the mushroom as a monomer of 31 kDa. It should be noted that a portion of the purified protein did not enter the stacking gel when heated at 100 °C in the presence of SDS and 2-mercaptoethanol before electrophoresis (Figure 1, lane 2). These results

Figure 1 Purified haemolytic protein from the basidiocarps of *F. velutipes*

Purified haemolytic protein (2 µg) was subjected to SDS/PAGE without heating (lane 1) or after heating at 100 °C for 3 min (lane 2) in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Protein bands were stained with Coomassie Brilliant Blue R-250. Positions of molecular-mass markers are indicated in kDa (k) on the right-hand side.

Figure 2 Haemolytic activity of flammutoxin (FTX) towards the erythrocytes of various animals

Human (●), rabbit (△), dog (□), horse (▲) and sheep (○) erythrocytes were incubated with flammutoxin (0.05–300 µg/ml) at 25 °C for 30 min, and haemolysis was assayed as described in the Materials and methods section. Average values obtained from five independent experiments were plotted.

indicated that the protein partially aggregated upon the heating at 100 °C.

The haemolytic protein exhibited a single pI of 4.6 on analysis using a carrier ampholyte of pH 3–10. Analysis of the amino acid composition of the protein indicated that Thr, Ser, Glx and Asx were the abundant residues (results not shown). The N-terminal amino acid sequence of the protein was determined for the initial 29 residues: Pro-Gln-Val-Lys-Thr-Ser-Trp-Glu-Asp-Leu-Ala-Asn-Leu-Gly-Trp-Trp-Pro-Ile-Gln-Gln-Val-Tyr-Glu-Lys-Ala-Asn-Thr-Ala-Glu-. A similarity search on the DDBJ nucleic acid sequence database (release 29) indicated that the purified haemolysin has no similar sequence at the N-terminus with any other proteins so far registered.

We assayed the haemolytic activity of the purified haemolysin towards erythrocytes from several mammalian species. As shown in Figure 2, the haemolysin lysed rabbit, human and dog erythrocytes in a dose-dependent manner at concentrations of between 0.1 and 1 mg of protein/ml. The concentrations of the toxin required to induce 50% haemolysis were determined to be 0.20, 0.23 and 0.82 mg/ml for rabbit, human and dog cells respectively (Figure 2). In contrast, horse and sheep erythrocytes were not substantially lysed by the toxin at higher concentrations of up to 512 mg/ml (Figure 3 and results not shown).

We designated the purified haemolysin flammutoxin, because it was indistinguishable from the flammutoxin of Bernheimer and Oppenheim [10] in terms of the apparent molecular size (Figure 1) and spectrum of haemolytic action on mammalian erythrocytes of various species (Figure 2).

Pore-forming properties of flammutoxin

Morphological change was monitored for single cells of flammutoxin-treated human erythrocytes under a phase contrast microscope. When human erythrocytes were exposed to flammutoxin (0.3 µg/ml) at 25 °C, intact disc-shaped erythrocytes became swollen round-shaped cells with clear edges within 2–3 min, and thereafter the swollen cells released haemoglobins (Figures 3A–3C). Microscopic observation also revealed that once leakage of haemoglobins from the swollen cells started, it proceeded

Figure 3 Morphological changes of human erythrocytes upon exposure to flammutoxin

Human erythrocytes (0.5%) were exposed to flammutoxin (0.3 $\mu\text{g}/\text{ml}$) in TBS at 25 °C for 0 (A), 10 (B) or 30 min (C, D) in the absence (A–C) or presence (D) of poly(ethylene glycol) 6000 at a concentration equivalent to 40 mOsm. The total osmotic pressure of the extracellular solution was adjusted to 295 mOsm. Photographs (A–C) were taken of the same field to show the morphological changes of single cells of the toxin-treated erythrocytes. Arrowheads indicate the same cells.

Figure 4 Flammutoxin-induced leakage of potassium ions and haemoglobins from human erythrocytes in the absence or presence of poly(ethylene glycol) 6000

Leakage of potassium ions (solid lines) and haemoglobins (dotted lines) from human erythrocytes were monitored after exposure of the cells to flammutoxin (0.3 $\mu\text{g}/\text{ml}$) at 25 °C in the absence (●, ○) or in the presence (▲, △) of poly(ethylene glycol) 6000 at a concentration equivalent to 40 mOsm, as described in the text. The total osmotic pressure of the extracellular space was adjusted to 295 mOsm. Representative data obtained from three independent experiments are illustrated. Abbreviation: meq, milli-equivalent.

rapidly and finished within a few seconds (results not shown). Since swelling of cells is generally caused by the permeabilization of cell membranes, we assayed toxin-induced leakage of potassium ions (a representative of small molecules and ions) from human erythrocytes and lysis of the cells. As shown in Figure 4, approx. 70 % of the intracellular potassium ions leaked from the

cells within 2 min after addition of the toxin, whereas onset of haemolysis was observed after a 10 min or longer incubation time under the same conditions (Figure 4). The immediate efflux of potassium ions and the delayed onset of haemolysis suggested pore formation by flammutoxin, leading to osmotic burst of human erythrocytes.

To study pore formation by flammutoxin, we assayed leakage of potassium ions from toxin-treated human erythrocytes and lysis of the cells in the presence of poly(ethylene glycol) 6000, a possible osmotic protectant, at a concentration equivalent to 40 mOsm (see below). Although flammutoxin caused no lysis of human erythrocytes in the presence of poly(ethylene glycol) 6000, it induced leakage of > 80 % of the intracellular potassium ions from the cells within 2 min under those conditions (Figure 4). Microscopic observation showed that toxin-treated human erythrocytes were swollen but not lysed in the presence of poly(ethylene glycol) 6000 (Figure 3D). The toxin-treated, swollen cells were immediately lysed when collected by centrifugation, and resuspended in ice-cold TBS without osmotic protectant (results not shown), indicating that poly(ethylene glycol) 6000 served as a osmotic protectant. Furthermore, poly(ethylene glycol) 6000 caused neither morphological change of intact human erythrocytes nor efflux of potassium ions from the cells (results not shown). These data suggested that flammutoxin caused colloid osmotic burst of human erythrocytes through pore formation, and that the functional diameter of the pore was smaller than the hydrated diameter of haemoglobin but larger than that of a potassium ion. The toxin-induced efflux of potassium ions from human erythrocytes was slightly accelerated by the presence of poly(ethylene glycol) 6000 (Figure 4). This may be due to the difference between the osmotic pressure endowed by the extracellular poly(ethylene glycol) 6000

Figure 5 Flammutoxin (FTX)-induced lysis of human erythrocytes in the presence of poly(ethylene glycol)s of different molecular sizes

Human erythrocytes (0.5%, v/v) were incubated with flammutoxin (0.025–200 µg/ml) at 25 °C for 30 min in the absence (○) or presence (●) of ethylene glycol, or poly(ethylene glycol)s 200 (△), 300 (▲), 400 (□), 600 (■), 1000 (▽), 1500 (▼), 2000 (◇), 4000 (◆), 6000 (★) or 20000 (☆) as described in the Materials and methods section. Ethylene glycol and poly(ethylene glycol)s were used at concentrations equivalent to 40 mOsm.

(40 mOsm) and that endowed by the intracellular haemoglobins (33 mOsm).

To estimate the functional diameter of the membrane pore formed by flammutoxin, we assayed toxin-induced lysis of human erythrocytes in the presence of poly(ethylene glycol)s with different molecular diameters (Figure 5). Since intracellular haemoglobin gives an osmotic pressure of approximately 33 mOsm [20], we added the non-electrolytes to the extracellular space at a concentration equivalent to 40 mOsm. If the hydrodynamic diameters of the non-electrolytes are larger than that of the membrane pore formed by flammutoxin, they would suppress the toxin-induced lysis by counterbalancing the osmotic pressure endowed by the intracellular haemoglobin. Toxin-induced haemolysis was not significantly affected by the addition of ethylene glycol and poly(ethylene glycol)s 200–600 to the extracellular space (Figure 5). However, the toxin-induced haemolysis was partially inhibited by poly(ethylene glycol)s 1000–4000 in accordance with their sizes, and it was entirely suppressed by poly(ethylene glycol)s 6000 and 20000 (Figure 5). The hydrodynamic diameters of poly(ethylene glycol)s 4000 and 6000 have been previously estimated to be 3.8 and 5.0 nm respectively [12,13]. These data indicated that flammutoxin formed hydrophilic pores with a functional diameter of 3.8–5.0 nm in the cell membrane of human erythrocytes. Similar protection experiments using neutral sugars of different sizes showed that flammutoxin lysed human erythrocytes in the presence of dextran 4, whereas it caused no haemolysis in the presence of dextran 500 (results not shown; the hydrodynamic diameters of dextrans 4 and 500 were estimated to be 3.5 nm and 28 nm respectively [12,13]). Flammutoxin-induced haemolysis was not affected by the addition of glucose, mannitol, sucrose, raffinose or stachyose to the extracellular space (results not shown).

Horse and sheep erythrocytes were insensitive to the lytic action of flammutoxin (Figure 2). Therefore, we tested the pore-forming activity of flammutoxin towards horse and sheep erythrocytes. Neither swelling of cells nor efflux of potassium ions took place upon exposure of horse cells to flammutoxin at higher concentrations (i.e. 5, 20 and 50 µg/ml) at 25 °C for 30 min (results not shown). These data suggested that flam-

mutoxin failed to form pores in the cell membranes of horse and sheep erythrocytes.

Assembly of flammutoxin into a ring-shaped, pore-forming complex on erythrocytes

We performed electron microscopy of flammutoxin-treated human erythrocytes to study the molecular arrangement of the toxin on the cells. Human erythrocytes were exposed to the toxin (50 µg/ml) at 25 °C for 30 min, and the toxin-treated cells were stained negatively with 1 % (w/v) sodium phosphotungstic acid and subjected to electron microscopy. The image showed the presence of many ring-shaped structures on the cells (Figure 6A). To eliminate the overlapping of many ring-shaped images, some areas of the original image were selected and subjected to optical filtering. The processed image exhibited the ring-shaped structure (Figure 6B), whose outer and inner diameters were approx. 10 and 5 nm respectively. In contrast, neither osmotically lysed human erythrocytes nor toxin-treated horse erythrocytes carried such a ring-shaped structure (results not shown).

To elucidate whether or not flammutoxin forms pore-forming complex(es) on the surface of target membranes, cell-bound toxin was solubilized with 2 % (w/v) SDS at 20 °C, and analysed by SDS/PAGE and Western immunoblot. The solubilized toxin was not heated at 100 °C before electrophoresis, to avoid aggregation of the toxin. When human, rabbit and dog erythrocytes were incubated with flammutoxin (0.5 µg/ml) at 25 °C for 30 min, an immunostained band corresponding to approx. 180 kDa was detected as well as the 31 kDa band of monomeric toxin (Figure 7B). In contrast, the 180 kDa band was not stained when horse and sheep erythrocytes were used, although the 31 kDa band of monomeric toxin was stained well (Figure 7B). Intrinsic membrane proteins of these erythrocytes were not stained under these conditions (Figure 7B). Direct staining with Coomassie Brilliant Blue R-250 clearly showed the 180 kDa band as well as the 31 kDa band, when human erythrocytes were exposed to a higher concentration of the toxin, i.e. 20 µg/ml (Figure 7A). Concerning the sensitivity of the Western immunoblot method, the 180 kDa band was visible even when human

Figure 6 Ring-shaped structures observed on the flammutoxin-treated human erythrocytes

(A) Flammutoxin-treated human erythrocytes, negatively stained with 1% (w/v) sodium phosphotungstic acid, were subjected to electron microscopy as described in the Materials and methods section. (B) The same field after optical filtering and contrast enhancement shows ring-shaped images on the toxin-treated cell. Arrowheads indicate typical ring-shaped structures. Each bar represents 50 nm.

erythrocytes were exposed to flammutoxin at 0.1 $\mu\text{g/ml}$ (Figure 7C), or the minimal concentration of the toxin to lyse the cells (Figure 2). Some pore-forming cytolysins such as staphylococcal α -haemolysin show higher haemolytic activity at a lower pH [21,22], so we tested the effect of the extracellular pH on assembly of flammutoxin as well as on its haemolytic activity. When human erythrocytes were used as the target cells, toxin binding and assembly took place more efficiently as the extracellular pH was lowered to between pH 5 and 8 (Figure 7D). Flammutoxin also exhibited higher haemolytic activity towards human erythrocytes at lower extracellular pH (results not shown). In contrast, when horse erythrocytes were employed, flammutoxin showed neither complex formation nor haemolytic activity in between pH 5 and 8, although it bound to the cells efficiently at lower pH (Figure 7D and results not shown). These results suggested that flammutoxin assembles into a 180 kDa pore-forming complex on

the toxin-sensitive erythrocytes, whereas it remains a monomer on the toxin-insensitive erythrocytes.

To elucidate the molecular architecture of the 180 kDa complex of flammutoxin, we isolated and characterized the toxin complex from the toxin-treated human erythrocytes. Cell-bound toxin was solubilized with 2% (w/v) SDS at 20 °C and fractionated by ultracentrifugation using a 10–40% (w/w) sucrose gradient. Fractions obtained from the ultracentrifugation were subjected to SDS/PAGE and Western immunoblot. As shown in Figure 8, the solubilized toxin was fractionated into two portions, fractions I and II, on the sucrose gradient. Fraction I contained the 180 kDa complex but few membrane proteins, whereas fraction II included membrane proteins and the toxin monomer (Figure 8). Electron micrographs of fraction I showed the presence of a ring-shaped structure, whose outer and inner diameters were 10 and 5 nm respectively (Figure 9). Thus the dimensions of the isolated ring-shaped structure coincided with those of the ring-shaped structure observed on the toxin-treated human erythrocytes, suggesting that the toxin complex of 180 kDa corresponds to the ring-shaped structure observed on the toxin-treated human erythrocytes. To analyse the protein composition of the ring-shaped complex, the protein band corresponding to 180 kDa in the polyacrylamide gel was electroblotted to a PVDF sheet and subjected to protein sequencing. Proline was detected as the single amino acid (> 95%) in the first cycle of the protein sequencing, and the determined N-terminal amino acid sequence for the first eight residues (Pro-Gln-Val-Lys-Thr-Ser-Trp-Glu-) was identical with that of flammutoxin. These data clearly indicated that the ring-shaped toxin complex consisted of only flammutoxin molecules.

DISCUSSION

In the present study, we isolated flammutoxin as a single haemolytic protein of 31 kDa from *F. velutipes* and demonstrated its pore-forming ability. Our data indicated that when flammutoxin binds to the toxin-sensitive erythrocytes such as human, rabbit and dog erythrocytes, it assembles into a ring-shaped oligomer possessing a hydrophilic pore of 3.8–5.0 nm. Since haemoglobins have a diameter of 6.2 nm (i.e. twice the Stoke's radius) [23] and do not pass the pore, they make an osmotic imbalance between outside and inside the erythrocytes unless macromolecules with an effective diameter of > 5 nm are present in the extracellular space. Consequently, influx of water and other molecules with diameters of < 5.0 nm occurs to counterbalance the colloid-osmotic pressure endowed by haemoglobins (33 mOsm) [20], leading to swelling and ultimate burst of the cells. When poly(ethylene glycol) ≥ 6000 was present extracellularly, flammutoxin-treated swollen erythrocytes remained unlysed for at least several hours (i.e. due to the Donnan effect). However, they were immediately lysed when transferred into ice-cold TBS without the osmotic protectants (results not shown). Assembly of flammutoxin into the 180 kDa complex took place on human erythrocytes irrespective of the presence of the osmotic protectants (results not shown). Thus the membrane pore formed by flammutoxin existed stably in the cell membrane and functioned in a metabolic energy-independent manner.

The pore-forming oligomer of flammutoxin was isolated as a ring-shaped structure by the sucrose-gradient ultracentrifugation, where a major portion of the solubilized ring-shaped oligomers sedimented faster than the solubilized membrane proteins of human erythrocytes. The large sedimentation velocity of the oligomer fraction (i.e. fraction I in Figure 8) may be interpreted as aggregation of the ring-shaped oligomers. In fact, electron

Figure 7 SDS/PAGE (A) and Western-immunoblot analysis (B–D) of assembly of flammutoxin (FTX) into a large-molecular-sized complex on mammalian erythrocytes

(A) Direct stain of the toxin complex with Coomassie Brilliant Blue R-250: Human (Hu) or horse (Ho) erythrocytes (RBC) (1.5×10^8 cells) were incubated with flammutoxin ($20 \mu\text{g}$) in 1 ml of TBS at 25°C for 30 min. Cell-bound toxin was solubilized and subjected to SDS/PAGE. (B) Complex formation of flammutoxin on the erythrocytes from various animals. Erythrocytes (1.5×10^8 cells) from human, rabbit (Ra), dog (Do), horse or sheep (Sh) were incubated with the toxin ($0.5 \mu\text{g}/\text{ml}$). Cell-bound toxin was solubilized and subjected to Western immunoblot as described in the Materials and methods section. (C) Dose-dependent formation of the 180 kDa complex on human erythrocytes. Human erythrocytes (1.5×10^8 cells) were incubated with flammutoxin at between 0.05 – $2 \mu\text{g}/\text{ml}$ at 25°C for 30 min. The Western immunoblot for the cell-bound toxin was performed as described in the Materials and methods section. (D) Effect of extracellular pH on the complex formation. The Western immunoblot for the formation of the toxin complex on human and horse cells was performed as described in the Materials and methods section, except for the use of various pH values. The following buffers were used: 20 mM acetate buffer for pH 5 and 6, 20 mM Tris/HCl buffer for pH 7 and 8, and 20 mM glycine/NaOH buffer for pH 9 and 10. These buffers were supplemented with 130 mM NaCl. Filled and open arrowheads indicate the positions of the 180 kDa toxin complex and the monomer toxin respectively. Positions of molecular-mass markers are indicated in kDa (k) on the right-hand side.

microscopy of the fraction I revealed the presence of clusters of the ring-shaped oligomers as well as single ring-shaped oligomers (results not shown). Aggregates of the ring-shaped toxin oligomers would have been dissociated to single ones when fraction I was subjected to SDS/PAGE or when it was dialysed against Tris/HCl buffer (pH 7.2) containing 0.1 % (w/v) SDS before electron microscopy. Clusters of the ring-shaped structures were also observed on the toxin-treated human erythrocytes (Figure 6). Although the apparent size of the toxin oligomer (i.e. 180 kDa) suggests a hexameric structure, the details of its fine structure remain to be elucidated. In addition to the pore-forming complex of 180 kDa, another band corresponding to 60 kDa was occasionally detected when cell-bound toxin was analysed by the Western-immunoblot analysis. The 60 kDa complex, probably dimeric toxin, clearly has no pore-forming activity, because it was formed on horse erythrocytes (Figure 7D) as well as on

human cells (results not shown). No other toxin complex of between 60 and 180 kDa was detected by Western-immunoblot analysis for the cell-bound toxin, suggesting that toxin complexes of intermediate sizes would have been dissociated to toxin monomers during solubilization of the cell-bound toxin with 2 % (w/v) SDS.

It should be noted that flammutoxin did not assemble into pore-forming oligomers on horse and sheep erythrocytes, although it bound to these toxin-insensitive cells as efficiently as to human erythrocytes (Figures 7B and 7D). The cell specificity of pore-forming cytotoxins is attributed to the presence of specific receptors, because the presence of high-affinity binding sites on susceptible cells has been suggested for α -haemolysin from *Staphylococcus aureus* and aerolysin from *Aeromonas hydrophila* [24,25]. In fact, Buckley and his co-workers [26] have detected a specific receptor for aerolysin by using a sandwich Western

Figure 8 Isolation of the 180 kDa complex by sucrose-gradient ultracentrifugation

Human erythrocytes (3×10^{10} cells) were incubated with flammutoxin (FTX; 1 mg) in 300 ml of TBS at 25 °C for 30 min. Cell-bound toxin was solubilized with 2% (w/v) SDS at 20 °C and fractionated by ultracentrifugation using a 10–40% (w/w) sucrose linear gradient as described in the Materials and methods section. Small portions of the fractions (I and II) were withdrawn and subjected to (A) SDS/PAGE, followed by staining with Coomassie Brilliant Blue R-250, or (B) Western immunoblot. Filled and open arrowheads indicate the positions of the 180 kDa toxin complex and toxin monomer respectively. Positions of molecular-mass markers are indicated in kDa (k) on the right-hand side.

Figure 9 Electron micrographs of the isolated ring-shaped complex

The complex-containing fraction I was negatively stained with sodium phosphotungstic acid and subjected to electron microscopy as described in the Materials and methods section. Arrowheads indicate the ring-shaped structures. The bars represent 100 nm in (A) and 50 nm in (B–D).

blotting (i.e. an assay for specific toxin binding to the blotted membrane proteins), and purified the receptor as a novel glycosylphosphatidylinositol-anchored protein from the cell membranes of rat erythrocytes. The receptor may concentrate aerolysin on the cell surface and facilitate oligomerization of the toxin and/or insertion of the toxin molecules into lipid bilayer of cell membrane. In this context, the failure of flammutoxin assembly on horse and sheep erythrocytes would be attributable to dysfunction of flammutoxin-specific receptors on these cells, i.e. the putative receptor binds flammutoxin but fails to promote oligomerization of the bound toxin and/or insertion of the toxin into the lipid bilayer. However, our preliminary attempts to identify a specific receptor by using sandwich Western blotting have not yet detected the presence of a flammutoxin receptor on human and horse cells (T. Tomita, T. Noguchi and Y. Hashimoto, unpublished work). Alternatively, oligomerization of flammutoxin and insertion of the toxin into the lipid bilayer would be strongly influenced by certain membrane lipid(s) or the lipid composition of the erythrocyte membrane. Analysis of the lipid compositions of cell membranes of human and horse erythrocytes is currently in progress.

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