Assembly of *Staphylococcus aureus* γ-hemolysin into a pore-forming ring-shaped complex on the surface of human erythrocytes

Noriko Sugawara, Toshio Tomita, Yoshiyuki Kamio*

Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumi-dori Amamiya-machi, Aoba-Ku, Sendai 981, Japan

Received 21 April 1997; revised version received 10 May 1997

Abstract Staphylococcal γ-hemolysin consists of Hlg1 (or Luk F) of 34 kDa and Hlg2 of 32 kDa, which cooperatively lyse human erythrocytes. Since γ-hemolysin caused swelling of human erythrocytes prior to lysis, we studied pore-forming nature of the toxin by use of polyethylene glycols as osmotic protectants and determined the functional diameter of the pore. To elucidate the molecular architecture of the membrane pore formed by γ-hemolysin, we solubilized the pore complex with 2% sodium dodecyl sulfate, separated it from erythrocyte membrane proteins by sucrose gradient ultracentrifugation, and observed the isolated complex under an electron microscope. Our data showed that Hlg1 and Hlg2 of γ-hemolysin assemble into a ring-shaped 195 kDa complex in a molar ratio of 1 : 1, which may form a membrane pore with a functional diameter of 2.1–2.4 nm.

Key words: Staphylococcal γ-hemolysin; Bi-component cytolysin; Pore-forming toxin; Complex formation; Ring-shaped structure

1. Introduction

Staphylococcal γ-hemolysin has been isolated as a bi-component hemolysin from the culture fluids of *Staphylococcus aureus* [1,2,2a]. It consists of Hlg1 (or Hγ I) of 34 kDa and Hlg2 (or Hγ II) of 32 kDa, which cooperatively lyse erythrocytes from mammalian species [2]. Previous studies by us and other groups showed that γ-hemolysin shares one component with the staphylococcal bi-component leukocytolytic toxin, leukocidin (i.e., Hlg1 is identical with LukF) [2a–6], and that the genes coding for γ-hemolysin and leukocidin (i.e., *hlg2*, *lukS* and *lukF* = *hlg1*) are located in a cluster on the chromosome of the bacterium [2a,4–8].

Our previous studies have demonstrated that the binding of Hlg1 (or lukF) is a prerequisite for the subsequent binding of Hlg2 to the cells [9,10]. We have also analyzed the cell-bound state(s) of γ-hemolysin by use of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot using specific antisera raised against Hlg1 and Hlg2, and showed that γ-hemolysin forms large-molecular-sized complexes of 150–240 kDa on the cell surface [9,10].

In this paper, we show that staphylococcal γ-hemolysin assembles into a pore-forming, ring-shaped complex which consists of Hlg1 and Hlg2 in a molar ratio of 1 : 1. This report describes the formation of a functional supermolecule by a bi-component cytolysin, whose components are secreted from *Staphylococcus aureus* as separately existing, water-soluble molecules.

2. Materials and methods

2.1. *Staphylococcus aureus* γ-hemolysin

γ-Hemolysin was purified from the culture supernatant of *Staphylococcus aureus* 5R Smith strain as described previously [11].

2.2. Hemolytic assay

Hemolytic assay was performed as described previously [10,12]. 100% hemolysis was defined as the absorbance value obtained when erythrocytes were exposed to 0.5% Triton X-100 at 25 or 37°C for 30 min.

2.3. *Efflux of potassium ions and hemoglobin from the toxin-treated cells*

Human erythrocytes were washed five times by centrifugation at 600×*g* for 5 min to remove extracellular potassium ions. The washed cells (2.5%, v/v) were incubated with Hlg1 (2.5 μg/ml) and Hlg2 (2.5 μg/ml) at 25°C for 5 min, followed by centrifugation at 18000×*g* for 30 s. A small portion of the supernatant was withdrawn for hemolytic assay. The rest of the supernatant was further centrifuged at 18000×*g* for 20 min to remove erythrocyte membranes, if any. The supernatant was dried on a hot plate 70–80°C, decomposed at 550°C overnight, and dissolved in a portion of 0.02 M HCl. Atomic absorption spectro-photometric assay for potassium was performed at the wavelength of 766.5 nm with Hitachi Z-8100 Polarized Zeeman Atomic Spectrophotometer (Hitachi, Tokyo). 100% release was defined as the concentration of potassium ion or hemoglobin from the cell exposed to 0.5% Triton X-100 at 25°C for 30 min. 0% release of potassium ion was defined as the concentration from the cells which were incubated without toxin at 0°C for 5 min.

2.4. *Estimation of the functional diameter of the pore formed by γ-hemolysin*

Toxin-induced lysis of human erythrocytes was assayed at 25°C in the presence of nonelectrolytes (i.e., polyethylene glycols) with different hydrodynamic diameters. The nonelectrolytes were added at a concentration equivalent to 40 mosM, and the total osmotic pressure of the extracellular space was adjusted to 295 mosM. The value for hydrodynamic diameter of the nonelectrolytes were taken from the reports of Scherrer and Gerhardt [13] and Sabirov et al. [14], where the hydrodynamic diameters were calculated on the viscosity of the nonelectrolyte solutions.

2.5. *Complex formation of γ-hemolysin on the cell surface*

This was performed essentially as described previously [9,10,12]. Human erythrocytes (1%, v/v) were incubated with Hlg1 (1–25 μg/ml) and/or Hlg2 (1–25 μg/ml) at 37°C for 30 min. Toxin-erythrocyte complexes were collected by centrifugation at 22000×*g* for 20 min and suspended in 5 mM sodium phosphate buffer (pH 7.4) to lyse intact cells, if any. Toxin-erythrocyte complexes were washed twice by centrifugation at 22000×*g* for 20 min, solubilized in a small portion of 2% sodium dodecyl sulfate (SDS) at 20°C for 1 min, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a linear gradient gel of 3–14% (w/v) acrylamide. Protein bands in the gel

*Corresponding author. Fax: (81) (22) 717-8780.
E-mail: ykamio@biochem.tohoku.ac.jp

Abbreviations: Hlg1, Hlg1 (or Hγ I) component of staphylococcal γ-hemolysin; Hlg2, Hlg2 (or Hγ II) component of γ-hemolysin; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

PII S0014-5793(97)00618-2

were electroblotted onto a polyvinylidene difluoride sheet for 1 h, and immunostained using specific antisera raised against Hlg1 and Hlg2.

2.6. Isolation of toxin complex from erythrocyte membrane by use of sucrose density gradient ultracentrifugation

Erythrocyte-bound toxin was solubilized with 2% SDS at 20°C for 1 min, and applied to a 10–40% (w/w) linear sucrose density gradient in 10 mM Tris HCl buffer, pH 7.2, containing 0.1 SDS. Centrifugation was performed using a Beckman SW40Ti rotor at 32 000 rpm for 19 h at 4°C. Fractions were collected from the bottom of the tubes, and analyzed for toxin complexes. The fractions containing toxin complexes were combined and concentrated to several folds by use of an Amicon Centriflo ultrafiltration membrane (Grave, Danvers, MA, USA) at $800\times g$. The concentrated fraction was diluted with several volumes of 10 mM Tris HCl buffer (pH 7.2) containing 0.1% SDS to reduce the concentration of sucrose, and re-concentrated to several folds by ultrafiltration. Toxin complexes were further purified by the second sucrose density gradient ultracentrifugation as described above except the use of a 10–30% (w/w) linear gradient of sucrose.

2.7. Electron microscopy

Human erythrocytes (1%, v/v) were incubated with Hlg1 (1–20 μg/ml) and Hlg2 (1–20 μg/ml) at 37°C for 15 min. Lysed erythrocytes were placed onto a carbon-coated grid, washed briefly with phosphate buffer (pH 7.4), and stained negatively with 1% (w/v) sodium phosphotungstic acid, pH 7.4. The specimens were examined under a Hitachi electron microscope H-8100 (Hitachi, Tokyo) at an acceleration voltage of 100 kV.

2.8. Miscellaneous

Protein concentration was assayed as described by Bradford using bovine serum albumin as a standard [15]. Osmotic pressure of the nonelectrolyte solutions was measured at 25°C using a Micro-Osmometer Model 3MO (Advanced Instruments; Needham Heights, MA, USA). Polyethylene glycols were purchased from Wako Chemicals (Osaka, Japan).

3. Results and discussion

3.1. Pore-forming properties of staphylococcal γ-hemolysin

We monitored the γ-hemolysin-induced hemolysis for single cells of human erythrocytes under a phase contrast microscope. Intact, disc-shaped erythrocytes became swollen, round-shaped cells with clear edge after the incubation with Hlg1 and Hlg2 of γ-hemolysin for 10 min, and the swollen cells lysed thereafter. Since swelling of cells is generally caused by the permeabilization of cell membranes, we presumed that γ-hemolysin induced colloid osmotic lysis of human erythrocytes through pore formation. To study whether or not γ-hemolysin forms membrane pore, we assayed the leakage of potassium ions and hemoglobin from human erythrocytes in the presence or absence of polyethylene glycol 6000, a possible osmotic protectant. As shown in Fig. 1, toxin-induced hemolysis was suppressed by the addition of polyethylene glycol 6000 to the extracellular space at a concentration equivalent to 40 mosM. In contrast, >80% of the intracellular potassium ions leaked from the cells within 5 min after addition of γ-hemolysin, irrespective of the presence of polyethylene glycol 6000. Morphological study of the toxin-treated human erythrocytes indicated that γ-hemolysin caused swelling of the erythrocytes in the presence of polyethylene glycol 6000, but it did not lyse the cells under the same conditions (data not shown). Intact human erythrocytes showed no morphological change in the presence of the nonelectrolyte. These data suggested that γ-hemolysin formed hydrophilic pores in the cytoplasmic membrane of human erythrocytes, eventually inducing colloid osmotic lysis of the cells. They also suggested that the functional diameter of the pores was less than the hydrated diameter of hemoglobin but larger than that of potassium ion.

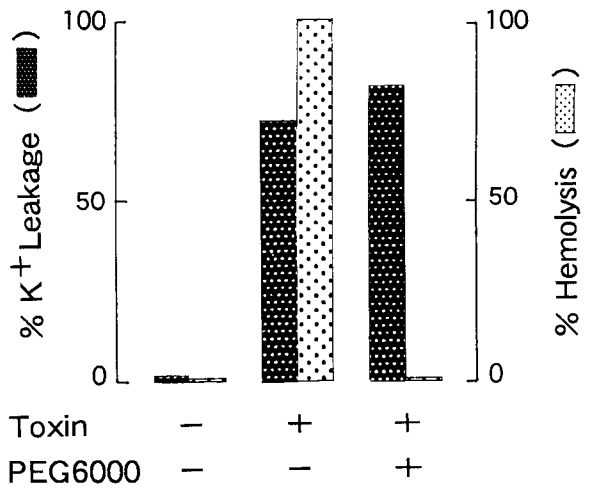

Fig. 1. Toxin-induced leakage of potassium ions and hemoglobins from human erythrocytes in the absence or presence of polyethylene glycol 6000. Human erythrocytes (2.5%, v/v) were incubated with or without Hlg1 (2.5 μg/ml) and Hlg2 (2.5 μg/ml) in TBS at 25°C for 5 min in the absence or presence of polyethylene glycol 6000. The cell suspensions were centrifuged at $18\,000\times g$ for 30 s at 4°C, and the supernatants obtained were assayed for % leakage of potassium ions and hemoglobins as described in Section 2. K^+: potassium ion. PEG6000: polyethylene glycol 6000.

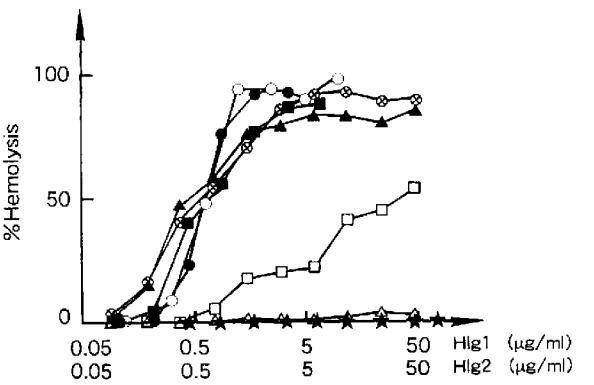

Fig. 2. γ-Hemolysin-induced lysis of human erythrocytes in the presence of polyethylene glycols with different hydrodynamic diameters. Hemolytic activity of γ-hemolysin (Hlg1, 0.05–95 μg/ml and Hlg2, 0.05–95 μg/ml) towards human erythrocytes (0.5%) was assayed at 25°C in the absence of nonelectrolyte (○) or in the presence of ethylene glycol (●), polyethylene glycol 200 (■), 400 (▲), 600 (⊗), 1000 (□), 1500 (△) or 2000 (★) as described in Section 2.

To estimate the functional diameter of the membrane pore formed by γ-hemolysin, we assayed toxin-induced hemolysis in the presence of polyethylene glycols with different hydrodynamic diameters (Fig. 2). Since intracellular hemoglobin gives an osmotic pressure of approximately 33 mosM [16], we added the nonelectrolytes to the extracellular space at a concentration equivalent to 40 mosM. As shown in Fig. 2, ethylene glycol and polyethylene glycols 200–600 did not suppress the toxin-induced hemolysis. In contrast, the toxin-induced hemolysis was inhibited by polyethylene glycol 1000 (with a hydrodynamic diameter of 1.8 nm [13,14]) and polyethylene glycol 1500 (with a hydrodynamic diameter of 2.1 nm [13,14])

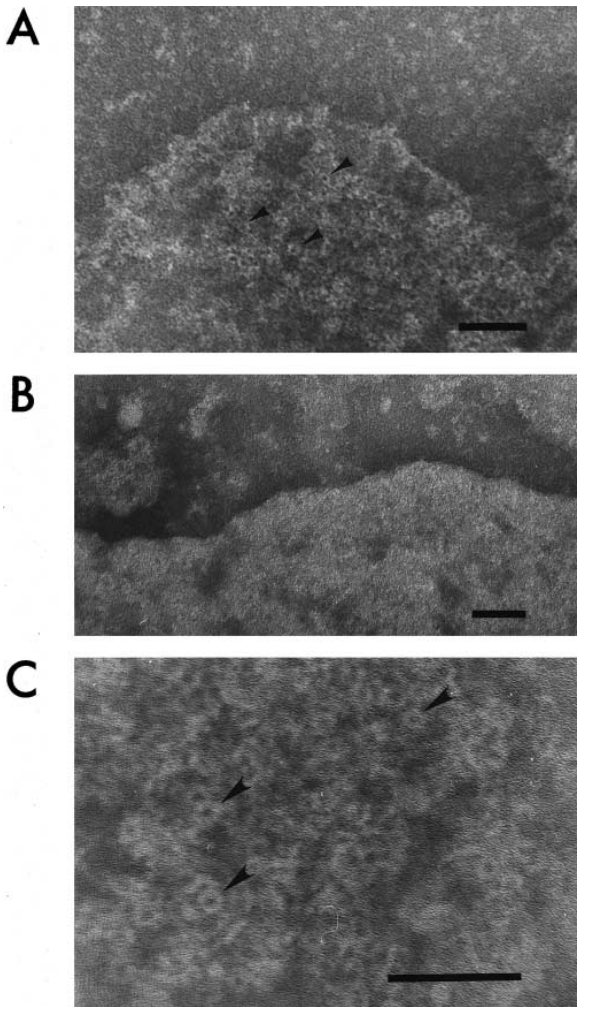

Fig. 3. Ring-shaped structures observed on the toxin-treated human erythrocytes (A) and ring-shaped toxin complexes isolated from the toxin-treated cells (C). Human erythrocytes were lysed by the treatment with γ-hemolysin (5 μg/ml of Hlg1 and 5 μg/ml of Hlg2) (A) or hypotonic 5 mM phosphate buffer, pH 7.4 (B). The complex-containing fraction was obtained from the second sucrose density gradient ultracentrifugation (the same fraction as in Fig. 4B) (C). The lysed erythrocytes and the isolated toxin complexes were stained negatively with 1% sodium phosphotungstic acid, and subjected to electron microscopy. Arrow heads indicate typical ring-shaped structures. The bar represents 50 nm.

in accordance with their size. No more hemolysis was detected in the presence of polyethylene glycols with an average molecular weight of ≥2000 (Fig. 2). Since polyethylene glycol 2000 has a hydrodynamic diameter of 2.4 nm [13,14], these data suggested that γ-hemolysin forms a hydrophilic pore with a functional diameter of 2.1–2.4 nm in the cell membrane of human erythrocytes.

3.2. *Assembly of γ-hemolysin into a ring-shaped structure on the cell surface*

To elucidate the molecular architecture of the membrane pore formed by γ-hemolysin, we performed morphological and chemical studies on the cell-bound toxin. For morphological analysis, toxin-treated erythrocytes were stained negatively with 1% sodium phosphotungstic acid and subjected to electron microscopy. As shown in Fig. 3A, the image of the toxin-treated erythrocytes showed the ring-shaped structures on their surface, whose outer and inner diameter were approximately 7 and 3 nm, respectively. In contrast, osmotically lysed erythrocytes did not carry such a ring-shaped structure on their cell surface (Fig. 3B). Neither Hlg1 nor Hlg2 formed the ring-shaped structure on the cells in the absence of their counterpart (data not shown). These data suggested that γ-hemolysin assembled into the ring-shaped structure with a inner diameter of approximately 3 nm on the cell surface, forming the hydrophilic pore with a functional diameter of 2.1–2.4 nm in the cell membrane.

Our previous studies of the cell-bound γ-hemolysin by using Western immunoblot analysis suggested that γ-hemolysin formed large-molecular-sized (150–240 kDa) complexes on the cell surface [9,10]. However, the molecular architecture of the toxin complexes remained to be elucidated. Based on the observation that the toxin complexes were stable in the presence 2% SDS at the temperatures below 40°C but dissociated to give monomers of Hlg1 and Hlg2 at 100°C [9,10], we attempted to isolate the ring-shaped structure formed by γ-hemolysin from erythrocyte membrane: Cell-bound toxin was solubilized with 2% SDS at 20°C, and the solubilized toxin was fractionated by the two steps of sucrose linear density gradient ultracentrifugation. Fractions obtained from the first ultracentrifugation using a 10–40% (w/w) sucrose linear gradient were subjected to SDS-PAGE on a gradient gel of 3–14% acrylamide, followed by Western immunoblotting using specific antisera raised against Hlg1 and Hlg2. As shown in Fig. 4A, the solubilized toxin was fractionated into two portions (fraction I and II), which gave the immunostained bands corresponding to the toxin complexes of ≥195 kDa and the toxin monomers, respectively. Since the fraction I contained a substantial amount of the ≥195 kDa complexes (Fig. 4A) but few membrane protein (data not shown), it was further fractionated by the ultracentrifugation using a 10–30% (w/w) sucrose linear gradient and analyzed by Western immunoblot (data not shown). The peak fraction containing the ≥195 kDa complexes was treated with 2% SDS at 20 or 100°C for 5 min and subjected to SDS-PAGE, followed by staining with Coomassie brilliant blue R-250 (Fig. 4B). The toxin complexes of ≥195 kDa were dissociated to monomeric Hlg1 and Hlg2 in a molar ratio of 1.0:0.97 when heated at 100°C in the presence of 2% SDS (Fig. 4B and C). A similar value was obtained for the molar ration of Hlg1 to Hlg2 when only the 195 kDa band was cut out from the gel and analyzed by SDS-PAGE (data not shown). Although SDS-PAGE of the peak fraction revealed two faint bands of approximately 100 kDa and 220 kDa (Fig. 4B, lane 1), no increase occurred in the intensity of these bands upon the treatment of the same fraction with 2% SDS at 100°C (Fig. 4B, lane 2). Thus, it is likely that no membrane protein was included in the toxin complexes that were formed on the surface of human erythrocytes. Electron microscopy of the same fraction showed the presence of the ring-shaped structures, whose dimensions coincided with those of the ring-shaped structure observed on the toxin-treated cells (Fig. 3C). Substantial portion of the ring-shaped structures formed clusters in the fraction (Fig. 3C), suggesting that the toxin bands of ≥195 kDa in the fraction (Fig. 4B) may correspond to parts of the clusters of the ring-shaped toxin complexes. We also observed that neither Hlg1 nor Hlg2 formed the ring-shaped structures as well as the large-molecular sized complexes of ≥195 kDa in the absence of their counterpart (data not shown).

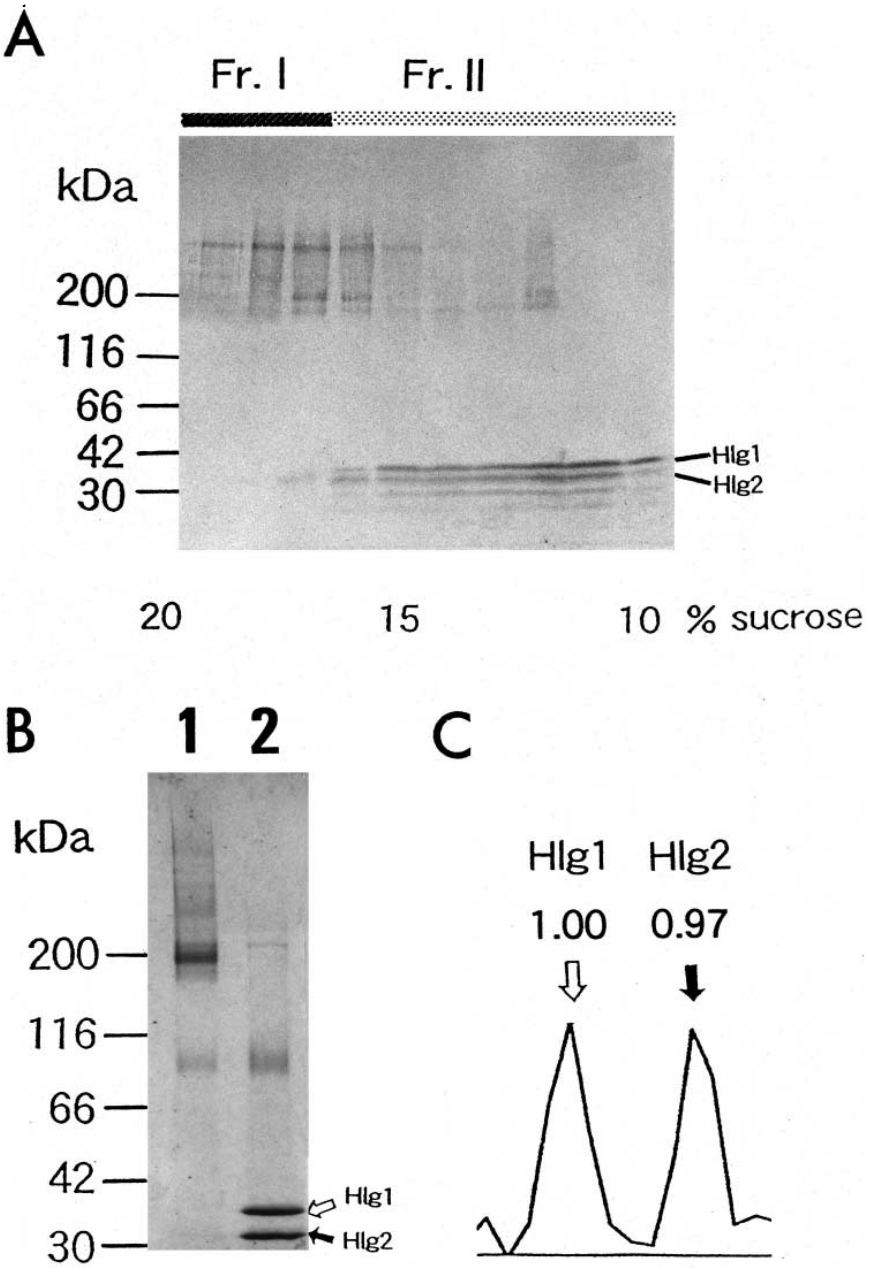

Fig. 4. Separation of the solubilized toxin complex(es) by sucrose density gradient ultracentrifugation. (A) Western immunoblot analysis of the toxin complexes in the fractions obtained from the 1st sucrose density gradient ultracentrifugation. Cell-bound γ-hemolysin was solubilized with 2% SDS at 20°C, fractionated by a 10–40% (w/w) sucrose linear density gradient ultracentrifugation, and subjected to Western immunoblot as described in Section 2. (B) SDS-PAGE of the toxin complex-containing fraction from the 2nd sucrose density gradient ultracentrifugation. The fraction I obtained from the first sucrose gradient ultracentrifugation was further fractionated by a subsequent 10–30% (w/w) sucrose linear density gradient ultracentrifugation. The peak fraction for the ≥195 kDa complexes was treated with 2% SDS at 20 (lane 1) or 100°C (lane 2) for 5 min, and subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250. (C) Densitometry was performed for the stained bands of Hlg1 and Hlg2 in lane 2 of (B).

Based on the data described above, we concluded that γ-hemolysin assembles into a ring-shaped complex composed of Hlg1 and Hlg2 in a molar ratio of 1 : 1, which may form a membrane pore with a functional diameter of 2.1–2.4 nm in the cell membrane. A single ring-shaped Hlg1-Hlg2 complex may be a hexamer structure consisting of three molecules of each of Hlg1 and Hlg2.

Acknowledgements: We are very grateful to Mr. Tsuruji Sato for his excellent technical assistance in electron microscopy. This work was supported in part by the grants from The Ministry of Education, Science, Sports and Culture of Japan (08307004 to Y.K.; 08670298 and 08219203 to T.T.) and from Takeda Science Foundation (to Y.K.).

References

[1] Smith, M.L. and Price, S.A. (1938) J. Pathol. Bacteriol. 47, 379–393.
[2] Guyonnet, F. and Plommet, M. (1970) Ann. Inst. Pasteur 118, 19–33.

[2a] Tomita, T. and Kamio, Y. (1997) Biosci. Biotech. Biochem. 61, 565–572.
[3] Kamio, Y., Rahman, A., Nariya, N., Ozawa, T. and Izaki, K. (1993) FEBS Lett. 321, 15–18.
[4] Rahman, A., Izaki, K. and Kamio, Y. (1993) Biosci. Biotech. Biochem. 57, 1234–1236.
[5] Supersac, G., Prevost, G. and Piemont, Y. (1993) Infect. Immun. 61, 580–587.
[6] Coony, J., Kienle, Z., Foster, T.J. and O'Toole, P.W. (1993) Infect. Immun. 61, 768–771.
[7] Rahman, A., Izaki, K., Kato, I. and Kamio, Y. (1991) Biochem. Biophys. Res. Commun. 181, 138–144.
[8] Rahman, A., Nariya, H., Izaki, K., Kato, I. and Kamio, Y. (1992) Biochem. Biophys. Res. Commun. 184, 640–646.
[9] Ozawa, T., Kaneko, J. and Kamio, Y. (1995) Biosci. Biotech. Biochem. 59, 1181–1183.
[10] Kaneko, J., Tomita, T. and Kamio, Y. (1997) Biosci. Biotech. Biochem. 61, 846–851.
[11] Nariya, H., Asami, I., Ozawa, T., Beppu, Y., Izaki, K. and Kamio, Y. (1993) Biosci. Biotech. Biochem. 57, 2198–2199.
[12] Tomita, T., Watanabe, M. and Yasuda, T. (1992) J. Biol. Chem. 267, 13391–13397.
[13] Scherrer, R. and Gerhardt, P. (1971) J. Bacteriol. 107, 718–735.
[14] Sabirov, R.Z., Krasilnikov, O.V., Ternovsky, V.I. and Merziliak, P.G. (1993) Gen. Physiol. Biophys. 12, 95–111.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Freedman, J.C. and Hoffman, J.F. (1979) J. Gen. Physiol. 74, 157–185.