Biochimica et Biophysica Acta, 558 (1979) 307—313

BBA 78580

RING FORMATION OF PERFRINGOLYSIN O AS REVEALED BY NEGATIVE STAIN ELECTRON MICROSCOPY *

KEN'ICHIRO MITSUI [a], TAKASHI SEKIYA [b], SHOJI OKAMURA [a], YOSHINORI NOZAWA [b] and JUN'ICHI HASE [a]

[a] *Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani-2630, Toyama and* [b] *Department of Biochemistry, School of Medicine, Gifu University, Tsukasamachi-40, Gifu (Japan)*

(Received February 26th, 1979)

Key words: Perfringens O; Cholesterol; Histone; Negative stain electron microscopy; Hemolysin; (Clostridium perfringens)

Summary

Perfringolysin O revealed ring- and arc-shaped structures in the absence of cholesterol by negative staining electron microscopy, while before activation with cysteine it showed indistinct arcs and irregularly curved sticks but no rings. These structures were observed only at high concentrations (more than 17 000 hemolytic units per ml) and seemed to be particle associates with 20—28 particles (about 4 nm per particle) linked in a circle. The toxin produced an inactive and high molecular weight complex in the presence of phosphotungstic acid, which was isolated by Sephadex gel filtration. These findings suggest that the rings are the toxin-phosphotungstic acid complexes produced during specimen preparation on a grid in vacuo. The toxin lost the properties necessary for ring formation though moderate modification with glutaraldehyde, showing spindle- and egg-shaped particles of about 4 nm in minor and 5 nm in major axis by negative staining. These facts suggest that the aldehyde modifies the binding sites for phosphotungstic acid, which probably are the basic groups of the toxin molecules. In the presence of cholesterol, even at a low concentration, the toxin revealed rings and arcs by negative staining and also by carbon shadowing electron microscopy, although the toxin itself did not show any characteristic structure without phosphotungstic acid. These observations suggest that the rings are the toxin-cholesterol complexes themselves. The toxin-phosphotungstic acid complexes seemed to have a structure of a single layer of particle associates, while that of the toxin-cholesterol complexes may consist of

* This is a series on *Clostridium perfringens* Exotoxins (No. VII).

double or triple layers of the associates because its border was thicker and more distinct.

Introduction

Perfringolysin O (θ-toxin of *Clostridium perfringens*) is one of the oxygen-label cytolysins such as streptolysin O, cereolysin and tetanolysin [1—3]. Smyth et al. [4] were the first to find many ring- and arc-shaped structures in negatively stained horse erythrocyte membranes treated with this toxin at a high concentration. We have [5,6] observed similar structures which consisted of a distinct border of 5.5 nm in width around a dark center of 18—29 nm in diameter. Using freeze-fracturing electron microscopy [6], we observed round protrusions and 'cavities', which were assumed to correspond to the rings, in fracture faces of membranes treated with perfringolysin O.

Smyth et al. [4] reported that this toxin occasionally produced the rings alone in the absence of cholesterol. Recently, Cowell et al. [7] reported that cereolysin produced the rings alone at concentrations higher than 6 μg of protein/ml (27 000 hemolytic units) and suggested that the rings are associates of the toxin itself and that sterols enhance its association. We also observed [6] the rings of perfringolysin O at 170 000 hemolytic units per ml in the absence of sterols. In this report, conditions necessary for the ring formation of perfringolysin O were examined.

Materials and Methods

Perfringolysin O

Perfringolysin O was purified from the culture filtrate of *C. perfringens* type A PB6K N5-L9 [8,9]. The toxin preparation used in this report had a specific activity of 134 000 hemolytic units per mg of protein and its concentration was 3.8 mg of protein/ml. For the experiment of the phosphotungstic acid-treatment, a further purified preparation (333 000 hemolytic units per mg, 1.2 mg/ml) was used. These preparations were completely freed from all the other activities found in the culture filtrate [8].

Phosphotungstic acid treatment

After full activation by preincubation with cysteine, 0.7 ml of the toxin solution (280 000 hemolytic units, 840 μg) was added to 1 ml of an 8% (w/v) hosphotungstic acid solution in 70 mM phosphate buffer containing 78 mM NaCl (pH 6.6—6.9) and was left for 40 min at room temperature (about 30° C). The reaction mixture was applied to a Sephadex G-100 column (2.5 × 48 cm), equilibrated with a 2% phosphotungstic acid solution in the phosphate buffer containing NaCl (pH 6.7) and was eluted with the same buffer.

Glutaraldehyde treatment

0.2 ml of the toxin solution (510 000 hemolytic units per ml) was added to 0.4 ml of a 2% (v/v) glutaraldehyde solution in the phosphate buffer containing NaCl (pH 6.7) and was left for 20 or 60 min at room temperature. Treatment

for 20 min decreased the activity to 6%, but after 60 min the activity was completely lost.

Electron microscopy and chemicals

Electron microscopy was carried out using a JEM 100-U and a JEM 7A electron microscope [10]. Histone from calf thymus was kindly given by Dr. N. Sugano, Toyama Medical and Pharmaceutical University, Japan. All the other chemicals were of reagent grade and obtained from commercial sources.

Results

A large number of ring- and arc-shaped structures with distinct borders of 4—7 nm in width and with internal diameters of 20—30 nm were visible in negatively stained specimens of fully activated perfringolysin O at a high concentration (170 000 hemolytic units per ml) in the absence of cholesterol (Fig. 1). They appeared to consist of 20—28 particles linked in a circle (Fig. 2a, b). The ring formation was highly dependent on the toxin concentration; a few rings were visible even at 17 000 hemolytic units per ml but invisible at lower than 17 000 hemolytic units per ml. Before activation the toxin, which retained 2% of the full activity, produced indistinct arcs and irregularly curved sticks of 30—35 nm in length which consisted of the particles jointed together, but did not produce rings (Fig. 3).

These findings suggest a possibility that phosphotungstic acid used in negative staining plays an important role in the association of the toxin. When the fully activated toxin was incubated in a phosphotungstic acid solution and was analysed by Sephadex colum chromatigraphy, the main peak of the protein elution curve was found in the void volume and contaminated proteins were found at smaller molecular regions (Fig. 4a). The peak of the hemolytic curve induced by the residual active toxin (0.03% of the added activity) was

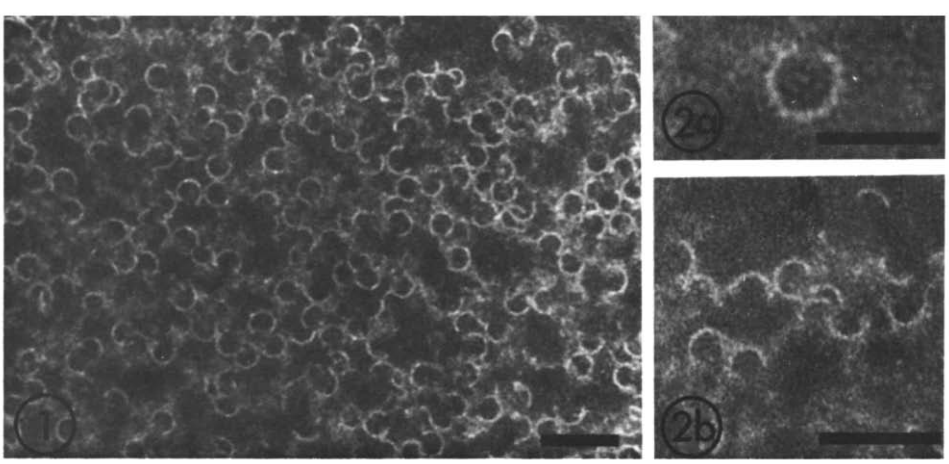
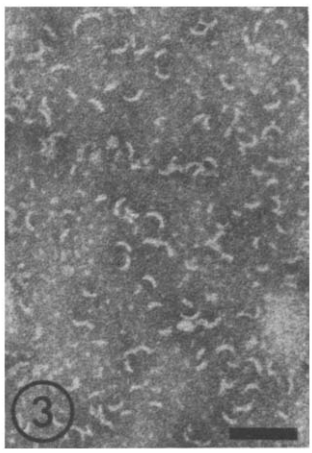


Fig. 1. Negatively stained specimen of the active perfringolysin O alone. Toxin at 1.2 mg/ml (170 000 hemolytic units per ml). Bar = 100 nm.

Fig. 2. Magnified features of ring structure. Bar = 50 nm (a) and 100 nm (b).



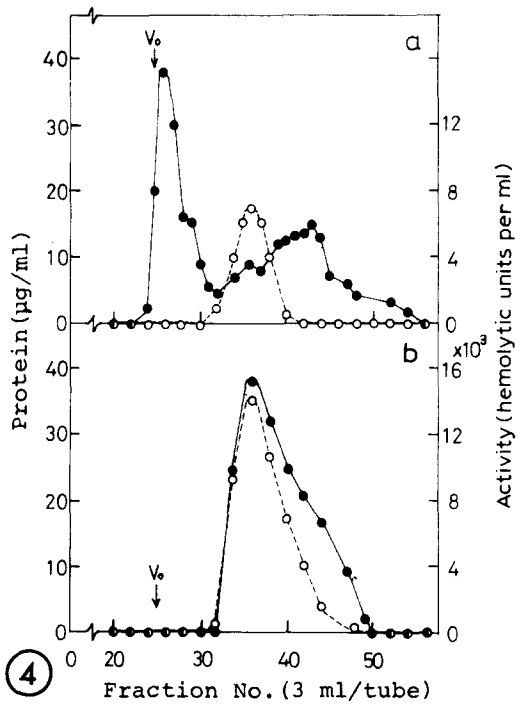


Fig. 3. Negatively stained specimen of perfringolysin O alone before activation by cysteine. Toxin at 1.2 mg/ml. Bar = 100 nm.

Fig. 4. Gel filtration with Sephadex G-100 of perfringolysin O after (a) and before (b) treatment with phosphotungstic acid. o- - - - -o, the hemolytic activity (hemolytic units) assayed by the method of Roth and Pillemer [16] using sheep erythrocyte suspensions; •———•, protein concentration determined by the method of Lowry et al. [17] using bovine serum albumin as standard.

found in the same volume as that of the toxin before treatment (Fig. 4b). The high molecular complexes may be produced by binding of phosphotungstic acid to basic groups of the toxin, because the reagent is used for precipitation of basic amino acids. After treatment with glutaraldehyde for 20 min, the toxin showed a large number of spindle- and egg-shaped particles of about 4 nm in minor and 5 nm in major axis (Fig. 5a). But toxin inactivated completely by the treatment for 60 min showed differently sized globes of 20—90 nm in diameter and spindle-shaped particles (Fig. 5b).

The toxin produces the inactive complexes with a trace of cholesterol. All the negatively stained specimens of the toxin-cholesterol complexes prepared at different toxin concentrations, even at a low concentration such as 170 hemolytic units per ml, revealed the distinct rings and arcs of 5—6 nm in width and of 20—30 nm in internal diameter (Fig. 6a, b and c). These rings showed the distinct borders but not the particle-associates arranged on a plane such as those of the toxin alone. In order to exclude the effect of phosphotungstic acid on the ring formation, the toxin-cholesterol complexes were examined by carbon-shadowing electron microscopy (Fig. 7). Rings and arcs of about 7 nm in width and of about 30 nm in external diameter were visible, and their borders were seen to be quite thick. On the other hand, even at a high con-

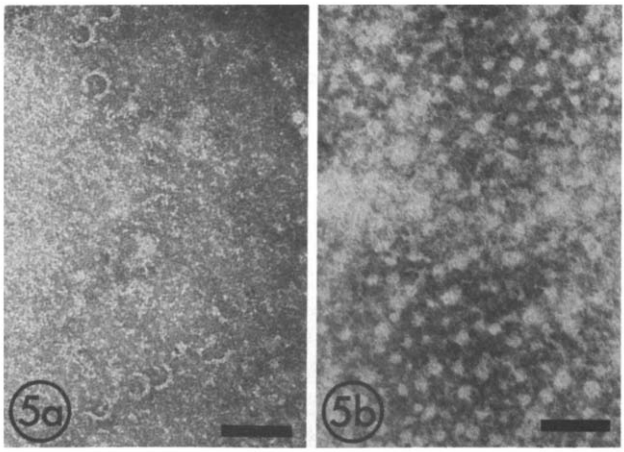


Fig. 5. Negatively stained specimens of perfringolysin O treated with glutaraldehyde for 20 min (a) or 60 min (b) at room temperature. Bar = 100 nm.

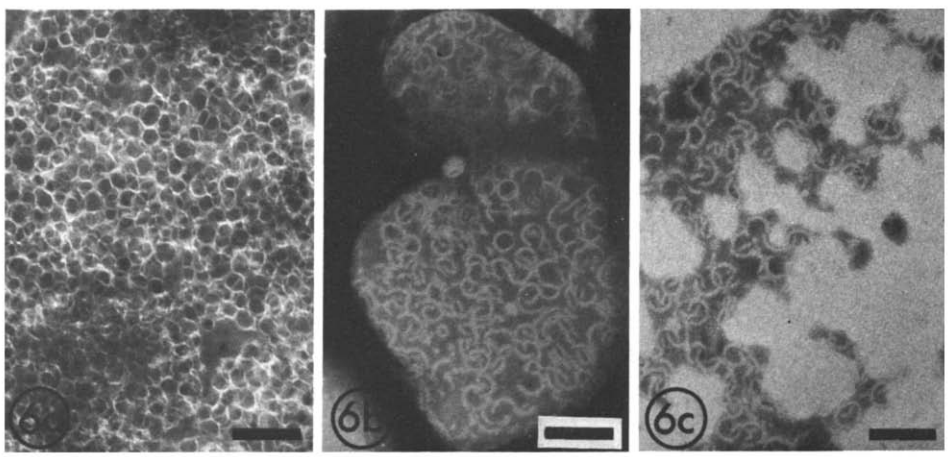


Fig. 6. Perfringolysin O-treated cholesterol dispersion. (a), toxin at 120 μg/ml; (b), at 12 μg/ml, and c, at 1.2 μg/ml. The toxin was preincubated with $1 \cdot 10^{-5}$ M cholesterol dispersion containing 1% EtOH for 60 min at 37°C. Bar = 100 nm.

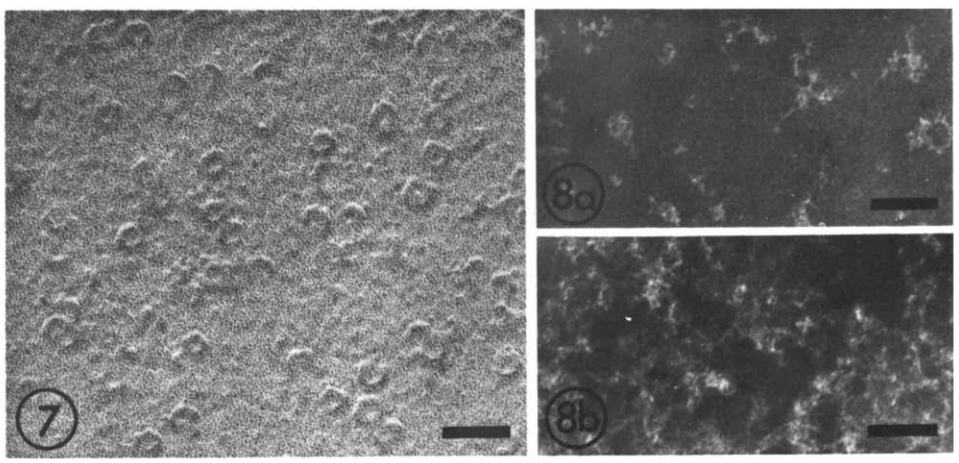


Fig. 7. Carbon-shadowed specimen of toxin-cholesterol complexes prepared by the method described in the legend of Fig. 6a. Bar = 100 nm.

Fig. 8. Negatively stained specimens of histones treated without (a) or with (b) $1 \cdot 10^{-5}$ M cholesterol dispersion containing 1% EtOH for 60 min at 37°C. Bar = 100 nm.

centration such as 170 000 hemolytic units per ml, the toxin alone, either before or after activation, did not show any characteristic structures in the absence of phosphotungstic acid.

Effect of addition of cholesterol on structures of histones (H-1, -2A, -3 and -4) [12] was examined by negative staining (Fig. 8). Cholesterol enhanced aggregation of histones, forming networks.

Discussion

Since perfringolysin O is not known to associate alone and does not reveal any characteristic structure in the absence of cholesterol or phosphotungstic acid by electron microscopy, rings and arcs should be associates of the toxin-cholesterol or -acid complexes. The rings of toxin revealed by negative staining are apparently particle associates linked in a circle, in which a particle may be a monomer of the toxin-phosphotungstic acid complex. The acid binds to basic amino acids and also to proteins, decreasing their solubilities. Glutaraldehyde is used for modification and bridging between molecules of proteins, probably by reactions with lysine, histidine, cysteine and tyrosine residues [11]. The toxin moderately modified with glutaraldehyde revealed spindle- and egg-shaped particles by negative staining, suggesting that it loses properties necessary for ring formation even in the presence of phosphotungstic acid. Thus, the aldehyde and acid probably bind to the same basic groups of the toxin. In Fig. 2, the particles are seen to associate together and inevitably grow from a short arc to a ring. These observations suggest that when hydrophilicity is lowered and mobility in the solution decreases, monomers of the toxin-acid complex inevitably assemble together to form rings and arcs, probably by hydrophobic bonds.

In order to form a ring on a plane, we propose that the toxin molecule is egg-shaped with a hydrophilic site including basic groups at its round end and hydrophobic sites at its middle sites, as shown in a hypothetitical model of the ring (Fig. 9). The rings are formed during specimen preparation on a grid in vacuo, and the higher the concentration of the complex monomer and the longer the reaction time with the acid, the more associates are formed.

The toxin produces the inactive complexes with high molecular weights in the presence of cholesterol. The rings observed in negatively stained specimens of biological and artificial membranes [5,6] containing sterols should be associates of toxin-cholesterol or toxin-cholesterol-phosphotungstic acid complexes. However, the ring formation of toxin-cholesterol complexes was hardly dependent on the toxin concentration, suggesting that shapes of the complexes are ring- and arc-like before binding with phosphotungstic acid. Actually, the complexes revealed rings, having more distinct and thick borders, by carbon shadowing electron microscopy in the absence of phosphotungstic acid. The toxin may bind with cholesterol not only at its active sites but also at its hydrophobic sites. Binding of cholesterol may increase hydrophobicity and enhance association of the complex monomer together to make rings and arcs. As proposed in Fig. 9, the ring structures of toxin-acid complexes seemed to be single layers of the particle associates arranged on a plane, while those of toxin-cholesterol complexes may be double or triple layers of the associates.

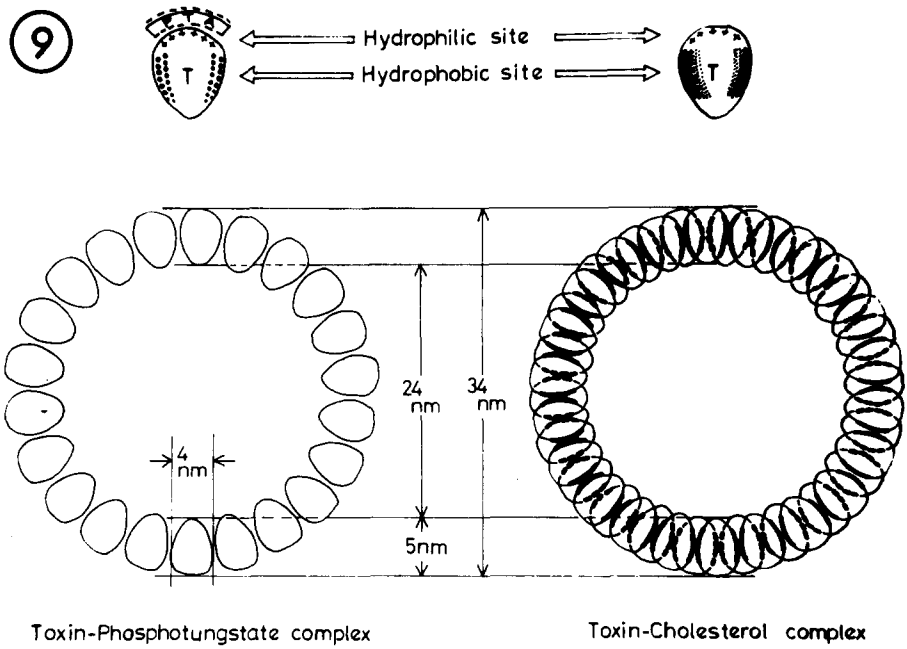


Fig. 9. Proposed models of toxin-phosphotungstic acid and -cholesterol complexes.

Ring formation of streptococcal α-toxin has been observed by negative staining and enhanced by addition of cholesterol, although cholesterol does not affect on its activity [13—15]. Results in this report show that shapes of proteins revealed by negative staining occasionally show their complex with phosphotungstic acid, and that sterols enhance their association or aggregation (see Fig. 8) by binding. Dissociation and composition of the toxin-cholesterol complexes will be represented elsewhere in the near future.

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