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The projection structure of Perfringolysin O (*Clostridium perfringens* θ-toxin)

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The cytolysin Perfringolysin O was applied to lipid layers and the obtained ring-shaped oligomers analyzed by electron microscopy and image processing. The final result shows the periodic repeat of 2.4 nm along the outer rim of the ring. The asymmetric protein unit, corresponding to one monomer, spans the ring from the convex to the concave surface. It shows a clear protein peak close to the outer radius and less density in the middle of the oligomer. The number of monomers in the average ring is 50, and the inner radius of the aggregate is approximately 15 nm.

Perfringolysin O; Electron microscopy; Image processing

1. INTRODUCTION

Perfringolysin O, which was earlier designated as θ-toxin, is secreted by the gas gangrene bacterium *Clostridium perfringens*. Perfringolysin O belongs to a group of at least 15 sulphydryl-activated toxins, including streptolysin O, tetanolysin, cereolysin and listeriolysin [1]. A feature common to these exotoxins is their ability to lyse cholesterol-containing membranes, and it is generally accepted that cholesterol is the membrane receptor for these cytolysins. The nucleotide sequence of the perfringolysin O gene exhibits approximately 60% homology with the streptolysin O gene and 48% homology with the pneumolysin gene [2]. Upon incubation with human erythrocyte plasma membranes perfringolysin O has been shown to aggregate to arcs and rings [3,4], but no substructure within the rings has hitherto been demonstrated. It was thus the starting point of the current work to clarify whether such substructure could be observed with the aid of electron microscopy and image processing in order to show the connection between monomers and the possible existence of an internal pore. The plasma membranes of human platelets have a cholesterol/lipid molar ratio of about 0.5 [5] and is thus a suitable target medium for perfringolysin O. The use of lipid layers, instead of liposomes or intact cells may facilitate a more refined analysis due to a more flat substrate, and it was thus decided to employ the lipid layer preparation technique [6,7] with the lipids extracted from human platelets.

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2. MATERIALS AND METHODS

Perfringolysin O was produced from *Clostridium perfringens* (type A, strain ATCC13124) and purified and tested by the method of Smyth [8]. Briefly the bacterium was cultivated in a pre-reduced proteose peptone medium using a 10 l fermentor. Toxin in the culture supernatant was concentrated by ammonium sulphate precipitation (50–60% saturation) and purified by isoelectric focussing in two steps. The resulting toxin was homogenous on SDS-PAGE with an apparent molecular weight of 62 kDa. The pI was 6.8–6.9 and the specific activity, as determined by hemolysis of sheep erythrocytes, was approximately 55 000 hemolytic units per milligram protein. Human platelet membranes were obtained by a 3-fold centrifugation procedure and the lipids were extracted by dissolving the membranes in a chloroform/methanol (1:1.2) mixture followed by a centrifugation step [9]. For incubation, 13 μl of the toxin solution (1.5 mg/ml) was placed in a cavity in a Teflon plate and 4.5 μl of lipid in chloroform/methanol was added on top. The Teflon plate was kept in a humid atmosphere at 22°C for 45 min whereupon the lipid layer was removed by gently touching the surface with a carbon-coated grid. The specimens were negatively stained with a mixture of 0.8% sodium phosphotungstic acid, pH 7.0, and 0.2% glucose for 1 min.

Electron microscopy was performed under minimal dose conditions on a Philips 420 instrument. Images to be analyzed were selected with the aid of optical diffraction, assuring that the first zero of the contrast transfer function occurred at frequencies beyond 1/1.4 nm^{-1}. The chosen micrographs were digitized in an Eikonix EC 1412 diode array densitometer at a scan spot aperture corresponding to 0.185 nm. The image processing was performed by using a Sun SPARC station 2 computer, employing the EM-program system [10] and the Sun Vision program package. Molecular aggregates that formed closed rings were selected for analysis by making cutouts of 512 pixels squares around each ring. The center of symmetry for the ring was found prior to the transformation of the image into polar coordinates. A small cutout (128 $pixels^2$) was made from the straight structure and a correlation averaging search was performed in two rounds. The polar coordinate image was also projected along the φ-axis to a one-dimensional vector to allow determination of the average radius. A total of 34 rings, from 5 micrographs, were analyzed to give 1 200 individual molecules. The slightly varying radius for the different rings will give a magnification difference along the φ-axis in the polar coordinate image. This magnification difference was corrected for prior to the merging of the data set. The combined result was averaged over the neighbouring unit cells

and back-transformed into real coordinates. The final average is shown in Fig. 2. In order to test the consistency of the result the correlation averaging step was performed with different references. Furthermore, the final data set was split into two halves, a classification step (the odd men out procedure [11]) run on each half and the 2×17 averages scaled together to two independent results, to allow a comparison.

3. RESULTS

Interaction of perfringolysin O with lipid layers results in the formation of arcs and rings such as the ones in Fig. 1. Few single particles were seen, thus suggesting that the protein was almost completely converted to the oligomeric form. No structural difference was evident between the open and closed structures by visual inspection. The average inner radius, as defined by half height of the density gradient, was 15 nm (S.D. 1.3 nm) for the analyzed aggregates. The outer radius was 21 nm (S.D. 1.4 nm) and the width 6 nm (S.D. 0.7 nm). The result of the correlation averaging did not significantly depend on the initial choice of the reference, thus showing that no gradual change along the circumference is present. Furthermore, the two independent averages obtained by splitting the data set exhibited essentially the same features. The total average in Fig. 2 shows an oligomer with a sharply defined inner edge and a less sloping outer gradient. The periodicity of 2.4 nm, close to the outer radius, is clearly resolved, while the periodicity of 2 nm along the inner rim is only faint, thus suggesting a resolution of 2 nm, or slightly better, for the final result. In the middle of the oligomer are some areas with less protein density, separated by stain-deficient bridges. The projected surface area for one monomer is approximately 13 nm^2 and the number of molecules that would fit into the average radius is around 50. In the surroundings of the oligomer some periodic noise is present due to the averaging over neighbouring unit cells, done in the final step. The average density level on the inside of the ring is slightly lower than on the outside.

4. DISCUSSION

Perfringolysin O is one of several thiol-activated cytolysins that are lethal and cardiotoxic to animals. Although the pathophysiology of this phenomenon is not fully understood a common feature of these toxins is their ability to form arcs and rings on the target membrane. It is thus of interest to analyze the substructure within these aggregates by electron microscopy and image processing. It has been shown earlier that monolayer films of phosphatidyl choline mixed with cholesterol is a suitable model for the study of perfringolysin O–membrane interaction [12], in concordance with the preparation method chosen in this work. When averaging over the ring a prerequisite is that the aggregate consists of circularly repeating units oriented in the same direction relative to the membrane plane. That

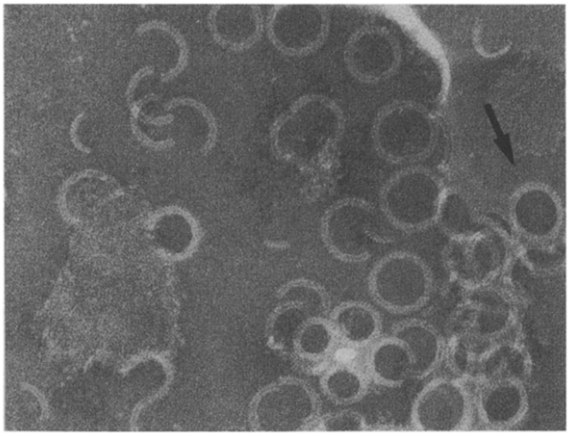

Fig. 1. Electron micrograph showing negatively stained oligomers of perfringolysin O as obtained after incubation with lipid layers. The arrow indicates a ring which exhibits a density difference between the inner and the outer surface. Magnification × 200 000.

this is the case was found during the analysis from the consistent results. This could be expected as it is likely that the protein can interact with the membrane in one preferential direction. Performing the averaging in polar coordinates is a computationally convenient procedure, but it is important to ensure that the original image is sufficiently oversampled so that no information is lost during the transformations between the two coordinate systems.

The final result shows the periodic repeat and substructure within the monomer. Due to the constraints imposed by volumetric considerations it can be deduced that the monomer spans the arc. The border between adjacent monomers is most likely situated in the regions with low protein density. The values of the average inner radius and width of the ring (15 nm and 6 nm) are comparable to the figures given by Mitsui and co-workers [3,4] (13 nm and 5.5 nm). The values obtained for streptolysin O [1] is a 13–16 nm inner radius of curvature and a width of 7.5 nm. The number of monomers in the average ring (50) for perfringolysin O is a factor of two higher than the figure given by Mitsui and colleagues [4] based on visual inspection.

Perfringolysin O is a voltage-dependent pore former with reversible action [13]. In order to clarify whether or not the area in the middle of the oligomer with low protein density constitutes a small pore, a more refined analysis would be necessary. No significant regions of hydrophobic residues are, however, present in perfringolysin O [2], thus suggesting that the membrane-bound toxin may not span the lipid bilayer. A model for the mode of streptolysin O action, proposed by Bhakdi and Tranum-Jensen [14], assumes the convex side of the polymerized toxin to show an apolar surface that anchors the toxin complex to the membrane lipids, and the concave side of the rod to be hydrophilic which accordingly would repel membrane lipids. This model would be consistent with the small density difference seen be-

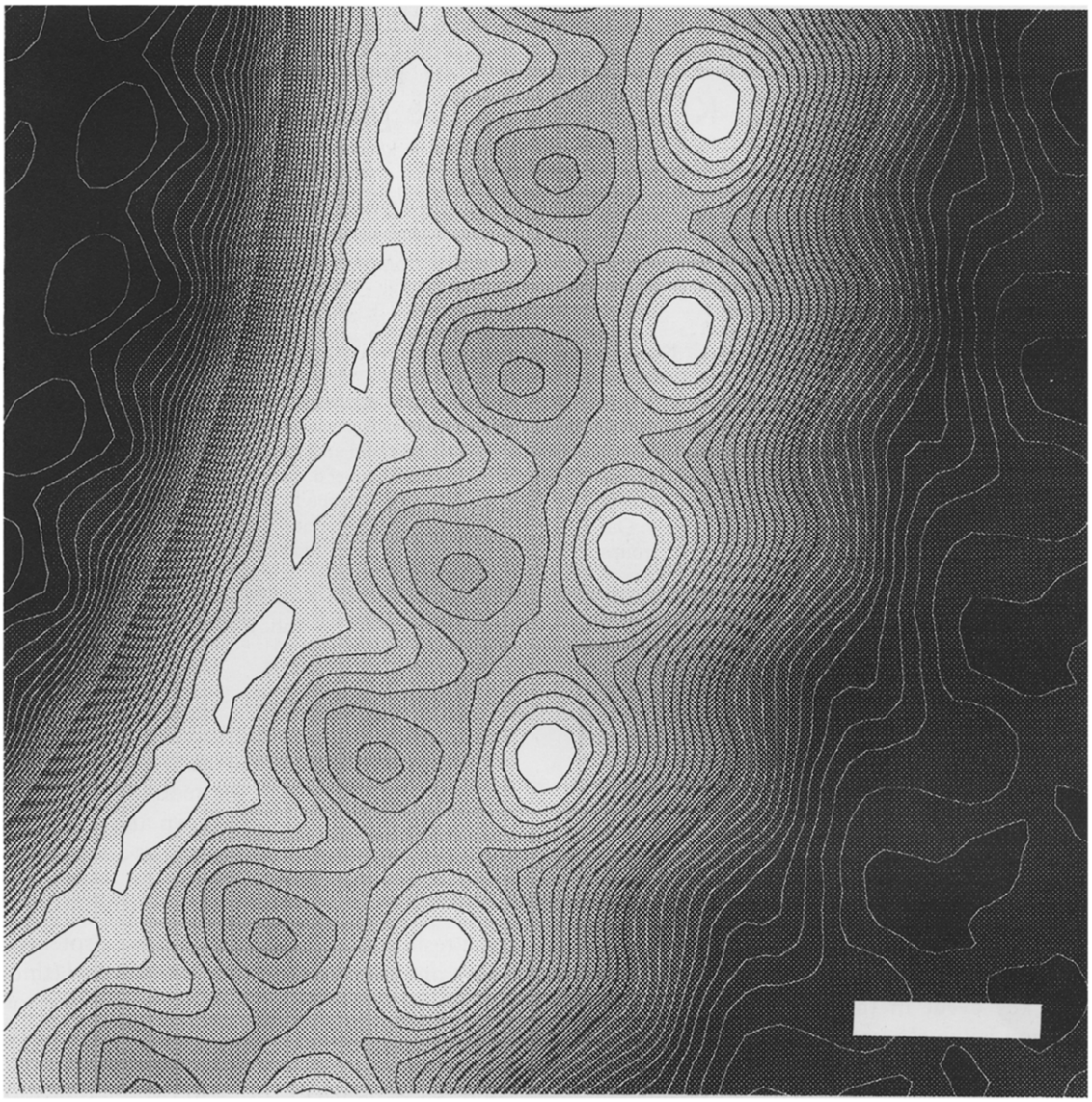

Fig. 2. The final projection density map averaged from 1 200 molecules. Bar = 2 nm.

tween the inner and outer surface of the ring (Fig. 2). By visual inspections of the closed structures (Fig. 1) only a few, such as the one at the head of the arrow, show a marked density difference. As perfringolysin O shows both primary structure homology and visual similarity with, for example streptolysin O [1], the devised averaging procedure can be applied to other thiol-activated cytolysins.

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REFERENCES

[1] Bhakdi, S., Tranum-Jensen, J. and Sziegoleit, A. (1985) Infect. Immun. 47, 52–60.
[2] Tweten, R. (1988) Infect. Immun. 56, 3235–3240.
[3] Mitsui, K., Sekiya, T., Nozawa, Y. and Hase, J. (1979) Biochim. Biophys. Acta 554, 68–75.
[4] Mitsui, K., Sekiya, T., Okamura, S., Nozawa, Y. and Hase, (1979) Biochim. Biophys. Acta 558, 307–313.
[5] Fauvel, J., Chap, H., Roques, V., Levy-Toledano, S. and Douste-Blazy, L. (1986) Biochim. Biophys. Acta 856, 155–164.
[6] Uzgiris, E.E. and Kornberg, R.D. (1983) Nature 301, 125–129.
[7] Olofsson A., Kavéus, U., Thelestam, M. and Hebert, H. (1992) J. Struct. Biol. 108, 238–244.
[8] Smyth, C.J. (1975) J. Gen. Microbiol. 87, 219–238.
[9] Olofsson, A., Kavéus, U., Hacksell, I., Thelestam, M. and Hebert, H. (1990) J. Mol. Biol. 214, 299–306.
[10] Hegerl, R. and Altbauer, A. (1982) Ultramicroscopy 9, 109–115.
[11] Unser, M., Steven, A.C. and Trus, B.L. (1986) Ultramicroscopy 19, 337–348.
[12] Alouf, J.E., Geoffroy, C., Pattus, F. and Verger, R. (1984) Eur. J. Biochem 141, 205–210.
[13] Pasternak, C.A., Bashford, C.L. and Menestrina, G. (1989) Biosci. Rep. 9, 503–507.
[14] Bhakdi, S. and Tranum-Jensen, J. (1988) Prog. Allergy 40, 1–43.