Subunit organisation and symmetry of pore-forming, oligomeric pneumolysin

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Abstract **We present a detailed analysis of the oligomeric subunit organisation of pneumolysin by the use of negative stain electron microscopy and image processing to produce a projection density map. Analysis of the rotational symmetry has revealed a large and variable subunit number, between 40–50. The projected subunit density by rotational averaging shows at least two distinct subunit domains at different radial positions. Side views of the rings reveal further details concerning the dimensions of the oligomer in the membrane. On the basis of these observations and our previous knowledge of the monomer domain structure we propose that the 4-domain subunits are packed in a square planar arrangement to form the pneumolysin oligomer.**

Key words: Pneumolysin; Pore-forming toxin; Electron microscopy; Image processing

1. Introduction

Pneumolysin is an important virulence factor of the bacterium *Streptococcus pneumoniae*, which is a major pathogen of man causing pneumonia, meningitis and otitis media [1]. Pneumolysin belongs to a family of protein toxins, all produced by Gram positive bacteria and generally referred to as the thiol-activated toxins. These toxins have been shown to have primary structural similarities and share a number of biological properties [2] including the formation of oligomeric structures in cholesterol-containing membranes.

It has been suggested [3] that cholesterol acts as a membrane receptor for these toxins and facilitates the concentration and resulting oligomerisation of toxin monomers in the membrane. The resulting structures have been reported for many of the thiol-activated toxins [4–8]. However the detailed topology of these oligomers is still unclear. The subunit structure of perfringolysin O was studied by electron microscopy [9] and image processing which showed an oligomeric structure containing an average of 50 subunits, each of which spans the ring and consists of a periodic repeat of 2.4 nm on the outer rim of the ring. A similar analysis was reported for streptolysin O [10] in which the authors proposed an oligomeric structure consisting of two concentric rings of subunits.

We have previously proposed [11] an oligomeric model based on the dimensions of pneumolysin monomers and oligomers, obtained by negative stain and metal-shadow electron microscopy. This model consists of a single ring of subunits which contains ~30 subunits. The incorporation of pneumolysin oligomers into liposomes rather than sheep erythrocytes and the use of image processing have enabled a more refined analysis of the pneumolysin oligomeric structures. Here we present (i) a symmetry-averaged projected density map of a pneumolysin oligomer and (ii) side views of the oligomers. On the basis of these images we propose a compact 4-domain subunit model for the oligomer.

2. Materials and methods

2.1. Pneumolysin expression and purification

Wildtype pneumolysin protein was expressed in *Escherichia coli* JM101 and purified as described previously [12]. Sample purity was checked by SDS PAGE analysis and haemolytic activity assayed as described previously [12].

2.2. Liposome preparation and incorporation of pneumolysin

Liposomes were made by dissolving a mixture of phosphatidylcholine (10 μmol), cholesterol (10 μmol) and dicetyl phosphate (1 μmol) in chloroform/methanol (1:1, v/v) and drying them under nitrogen. The lipids were resuspended in phosphate buffered saline (PBS): 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.137 M NaCl, 2.5 mM KCl, pH 7.3 and dispersed by sonicating in a sonic water bath for 90 s. The suspensions were then filtered through a 0.45 μm filter (Millipore). Equal volumes of pneumolysin (typically ~1.0 mg/ml) and liposomes were incubated for 30 min prior to electron microscopy.

2.3. Negative stain electron microscopy

For negative staining, samples of pneumolysin were vapour fixed for 1 min over glutaraldehyde solution, prior to rinsing with distilled water and contrasting with either 0.8% (w/v) sodium phosphotungstate pH 6.8 or unbuffered 2% (w/v) uranyl acetate. All specimens were examined and photographed in a Jeol 100CX transmission electron microscope fitted with a low dose unit. The magnification was calibrated using a diffraction grating replica.

2.4. Image processing

Images of the oligomers were digitised at 6.5Å/pixel using a Sentinel CCD camera (EEV, Essex) interfaced to a Synergy PC frame store (Synoptics Ltd, Cambridge). Analysis of the 2D rotational symmetry of the oligomer images was done on a PC with SEMPER software [13]. It was possible to centre the rings manually because they are large and well defined. Rotational symmetry was determined by calculating the rotational autocorrelation function for each ring. The appropriate symmetry was applied to yield symmetry-averaged end views.

3. Results

3.1. Face view symmetry

Pneumolysin incorporates into cholesterol-containing liposomes to form ring-shaped oligomeric structures (Fig. 1), which have the same dimensions as those formed in erythrocyte

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Fig. 1. Electron micrograph of negatively stained pneumolysin oligomers incorporated into a liposome. Magnification ×170,000.

membranes [11,14]. Although the majority of these structures are incorporated into liposomes, many are also found attached directly to the grid. These have the same dimensions as membrane-incorporated oligomers. Intact rings were used for image processing and not the arc-shaped structures which are also present. Analysis of the rotational symmetry of the oligomers, by rotational autocorrelation (Fig. 2), reveals a large and variable subunit number of between 40–50. The outer diameter of the structures varies according to the number of subunits present in a particular ring (from ~35–45 nm) and the ring width was 6.5 (± 0.5) nm. Rotational averaging of the negatively stained rings was used to produce projected density maps of pneumolysin oligomers. In Fig. 3 the density map of a 41-fold ring is shown with density contours superimposed. The structure appears as two concentric rings of protein density, separated by a gap of lower protein density (darker staining). One subunit is outlined in Fig. 3. On symmetry grounds (in order that all subunits make equivalent interactions with their neighbours) the projected subunit density must consist of an inner and an outer radial domain. The region of low protein density appears to contain a bridge between the inner and outer radial domains. The inner domains are more compact in projection.

3.2. Side view subunit structure

The incubation of high concentrations (~10 mg/ml) of pneumolysin in the presence of liposomes for long periods (up to 8 weeks) caused the oligomeric structures described above to form stacks (see Fig. 4). The oligomers appear to stack into cylinders which lie on their sides on the grid, providing an excellent opportunity to view the side projection of the oligomer. The resulting side views of the stacks are slightly wider in overall diameter than the face views, as would be expected in a negatively stained preparation, due to the dehydration-induced flattening of the structure onto the grid. The diameter of the stacks is ~45 nm and the axial translation between individual oligomers in the stack is ~10 nm. The inner and outer radial domains can be seen in the side projections and their combined width is ~8 nm. The inner domain, which lines the pore, has a sharper profile. The arrangement of stacked oligomers appears helical in some areas with diagonal regions of protein density, although horizontally separated layers are also seen (indicated in Fig. 4).

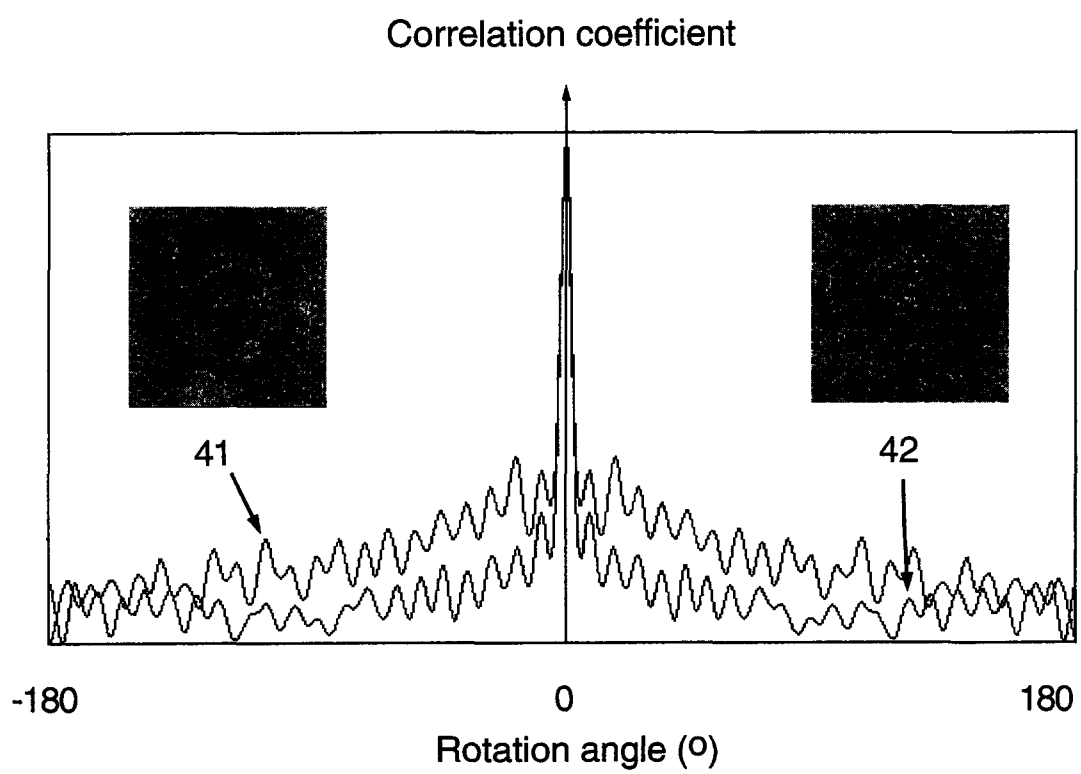

Fig. 2. Negative stain images and their corresponding rotational self-correlation's, showing 41 and 42 peaks, respectively.

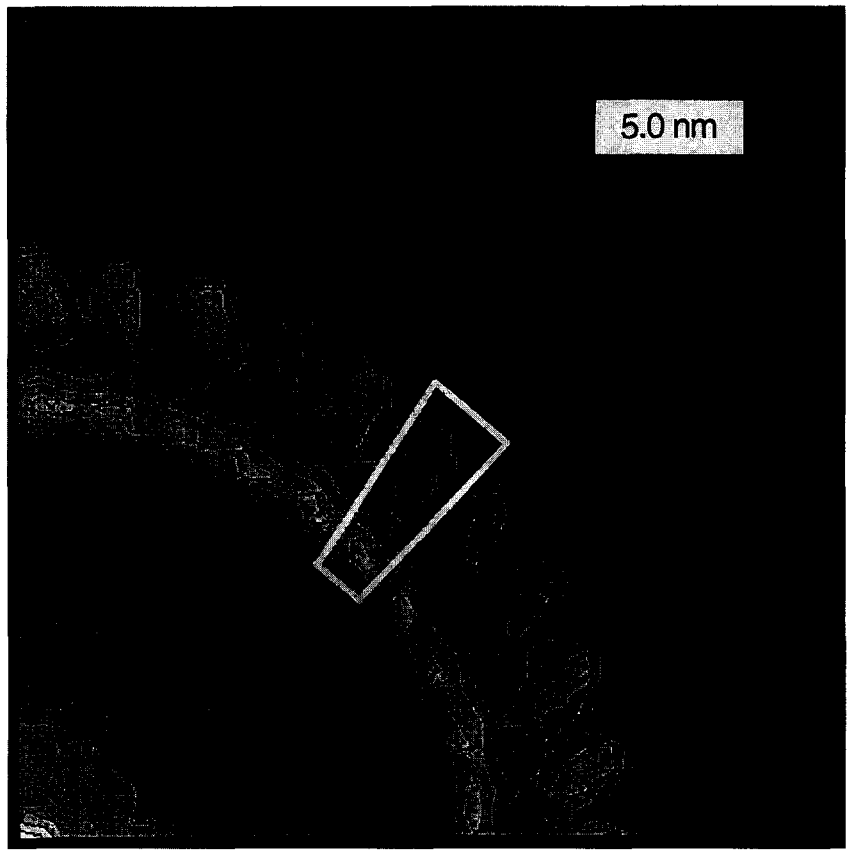

Fig. 3. One quadrant of the 41-fold ring shown in Fig. 2, after rotational averaging. The projected density map shows the subunit structure of the pneumolysin oligomer, with density contours superposed on the light, stain-excluding protein regions. The repeating subunit motif (shown boxed) contains a compact, inner domain, tenuously connected to an elongated outer domain, extending radially outwards.

4. Discussion

We have previously reported [11,14] that water-soluble monomeric pneumolysin incorporates into erythrocyte membranes to form large oligomeric ring and arc structures, as reported for many other of the membrane-damaging thiol-activated toxins [4–8]. The precise mechanism of oligomer formation is currently unclear and consequently a detailed knowledge of the subunit structure of the oligomer may provide further clues. We have extended our preliminary studies (in which we used erythrocyte membranes for oligomer incorporation) by introducing a system of cholesterol-containing liposomes. This system is free of contaminating proteins, which increases the sensitivity and reproducibility of our experiments. We have shown that pneumolysin will incorporate into liposomes to produce oligomers (Fig. 1) equivalent in appearance and size distribution to those found in cell membranes. Negative stain electron microscopy and rotational averaging (Fig. 2) were used to get projected density maps of the individual rings (Fig. 3).

The rotationally averaged image clearly shows two domains of subunit density, with the more elongated domain extending radially outwards from the ring. The compact inner domains appear to make the ring-forming contacts and line the pore. Rotational correlation analysis reveals a large and variable subunit number of between 40–50. Previous analysis of pneumolysin monomers [11] by electron microscopy has shown that pneumolysin has a four domain structure and this information was included in our previous working model for the oligomeric structure. Our previous model estimated a subunit number of ~30 and proposed a more extended conformation for the subunit domains.

The side-view images of the oligomers have enabled us to refine our previous model. Images of stacked pneumolysin oligomers (Fig. 4) provide an estimate of the height (based on their axial translation in the stacks) of the oligomers (~10 nm). This value is in good agreement with our previous estimate (~9 nm) obtained using metal-shadowing methods [11]. As far as we know these stacks have not been detected with other thiol-activated toxins. A slightly larger diameter was measured for the oligomers in the stacks, due to the flattening of the structure on the grid during the negative stain process. Unfortunately the stacks were not regular enough to produce diffraction spots (unpublished results) and so further analysis of the data was not possible. This observation was not surprising bearing in mind the variable subunit number (40–50). In fact there is some evidence of a helical arrangement in the stacks (see Fig. 4) although this appears irregular.

The subunit size and organisation observed for the pneumolysin oligomer is very similar to that determined for perfringolysin O [9] and streptolysin O [10]. Previous studies have shown that the amino acid sequence of pneumolysin has approximately 48% homology with that of perfringolysin O. They have been shown to possess similar biological properties and the ring and arc structures at low resolution appear to have similar

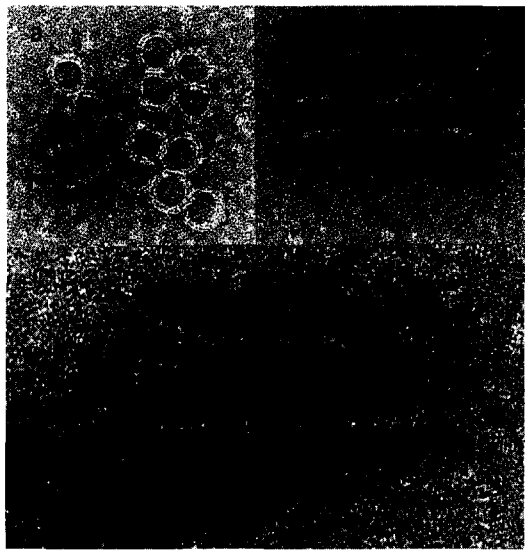

Fig. 4. A composite of electron micrographs showing negatively stained stacks of pneumolysin oligomers. The axial translation of an individual oligomer in the stack is indicated in the higher magnification image (c). Arrows show horizontal and diagonal regions of protein density, the latter suggesting a helical arrangement. Magnifications: (a),(b) ×145,000 and (c) ×330,000.

dimensions. It is likely that the family of thiol-activated toxins will have a conserved 3D structure.

On the basis of the images of pneumolysin oligomers presented here, together with our previous knowledge of the monomer domain structure [11] we propose that each subunit has four domains packed in a square planar arrangement, although the domain connectivity is not yet clear. We now intend to use cryo-EM and image processing to obtain more accurate information on the pneumolysin oligomers. We hope this will eventually enable a 3D reconstruction of the oligomeric structure, which in turn will help define the precise mechanism of membrane damage by these toxins.

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