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Recombinant Anthrax Protective Antigen: Observation of Aggregation Phenomena by TEM reveals specific effects of sterols

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Abstract

Negatively stained transmission electron microscope images are presented that depict the aggregation of recombinant anthrax protective antigen (rPA83 monomer and the PA63 prepore oligomer) under varying *in vitro* biochemical conditions. Heat treatment (50°C) of rPA83 produced clumped fibrils, but following heating the PA63 prepore formed disordered aggregates. Freeze-thaw treatment of the PA63 prepore generated linear flexuous aggregates of the heptameric oligomers. Aqueous suspensions of cholesterol microcrystals were shown to bind small rPA83 aggregates at the edges of the planar bilayers. With PA63 a more discrete binding of the prepores to the crystalline cholesterol bilayer edges occurs. Sodium deoxycholate (NaDOC) treatment of rPA83 produced *quasi* helical fibrillar aggregate, similar but not identical to that produced by heat treatment. Remarkably, NaDOC treatment of the PA63 prepores induced transformation into pores, with a characteristic extended β -barrel. The PA63 pores aggregated as dimers, that aggregated further as angular chains and closed structures in higher NaDOC concentrations. The significance of the sterol interaction is discussed in relation to its likely importance for PA action *in vivo*.

1. Introduction

Recombinant anthrax protective antigen (rPA) from *Bacillus anthracis* has been available as an 83kDa protein for many years, produced in *B. subtilis*, *E. coli*, yeast and plant cells (Miller et al. 1998, Gupta et al. 1999, Hepler et al., 2006; Palais et al., 2014; Vodkin et al., 1983). Anthrax PA has been studied extensively at the structural level and in connection with the development of anthrax vaccines (Bento et al., 2015; Jiang et al., 2015; Katayama et al., 2008; Merkel et al., 2013; Nguyen, 2004; Ren et al., 2004).

In vivo, the proposed sequence of anthrax toxin action is complex. Anthrax toxin receptor 2-mediated cell surface binding of the PA83 to the host cell (Sun and Jaquez, 2016) is followed by proteolytic / proprotein convertase cleavage to yield the PA63 and PA20 cleavage products. Oligomerization of the PA63 prepore on cholesterol-rich lipid rafts (Abrami et al., 2003) is followed by binding of the enzymatic edema and lethal factors, which contribute the toxic activity. This is followed by endocytosis, and in the acidic endosome environment the lethal and oedema factors are released and the PA63 pre-pore undergoes partial unfolding to generate the membrane-penetrating β -barrel of the anthrax pore. This in turn facilitates entry of unfolded lethal and edema factors into the cytoplasm via the pore channel (Finkelstein, 2009; Gao and Schulten, 2006; Lacy et al., 2004; Qa'dan et al., 2005, Jennings-Antipov et al., 2011). Considerable structural similarity exists between the anthrax PA63 pore, the *Staphylococcus aureus* α -toxin pore and *Vibrio cholerae* cytolysin (VCC) pore, although the length of their membrane-penetrating beta barrel and modes of action are different (Jiang et al., 2015).

The rPA83 monomer can be cleaved *in vitro* by the protease furin or trypsin. After purification rPA63 then spontaneously associates in solution as a ring-like heptamer and octamer (Milne et al., 1994; Petosa et al., 1997; Kintzer et al., 2009, 2010), generally considered to be equivalent to the anthrax PA63 prepore. Instability of rPA83 in terms of its molecular lability with spontaneous aggregation and loss of activity initially presented technical difficulties for recombinant biotechnology during purification and storage, as is often the case with other biotherapeutic products (Amador-Molina et al., 2016; Bondos and Bicknell 2003; Chaudhuri et al., 2013; Schrödel and de Marco, 2005). The low pH lability

of soluble rPA83 and PA63 has been well documented, despite the fact that conversion of the endosome membrane-bound PA63 prepore to pore conversion occurs at low pH (Gupta et al., 2003; Qa'dan et al 2005).

The factors involved in protein aggregation have been reviewed in depth by Wang et al. (2010) who included freeze-thawing and increased temperature among other conditions. Molecular aggregation can be considered to be either random clumping or a more organized amyloid-like fibrillar aggregation, with generation of intermolecular β -sheet content. A structural similarity has been drawn between the closed β -barrel, as found in the heptameric VCC pore, and an open-ended fibrillar β -sheet (Harris and Palmer, 2010). Indeed, conversion of β -barrel structures into amyloid-like fibrils has been reported for VCC in the presence of sodium deoxycholate (Harris and Palmer, 2010), for unfolded bacterial outer membrane protein OmpA (Danoff and Flemming, 2015) and the bacterial AAA⁺ chaperone RavA under acidic conditions and elevated temperature (Chan et al., 2016).

The prolonged storage of *B. Anthracis* PA at 37°C PA revealed thermal lability (Radha et al. 1996), with partial protection offered by magnesium sulphate, trehalose and sodium citrate. The thermal unfolding of rPA83 and PA63 studied by circular dichroism revealed a single unfolding transition near 50°C, whilst acidic transitions were also observed (Chalton et al., 2007). Further biophysical characterisation of the thermal unfolding of rPA83 was performed by Ganesan et al. (2012) with emphasis upon the study of the aggregated state of the protein. Thioflavin T binding with increased fluorescence from the aggregated protein provided evidence for the presence of β -sheets. The morphology of thermally induced rPA83 aggregates was studied using environmental scanning electron microscopy (ESEM) by Belton and Miller (2013); their data revealed large tangled clusters of protein, at a relatively low resolution. rPA83 and PA63 binding to aluminium hydroxide adjuvant (Alhydrogel[®]) has been studied by Soliakov et al. (2012) and Harris et al. (2012), respectively; the latter publication provided a strong indication that transmission electron microscopy (TEM) offers considerably greater resolution for the detailed study of protein aggregates than does ESEM. Accordingly, we have utilized this approach to reveal the

aggregate structure of rPA83 and PA63, produced under thermal and freeze-thaw conditions, together with cholesterol and deoxycholate treatment, by TEM of negatively stained specimens.

2. Materials and Methods

Recombinant protective antigen (rPA83) was provided by Avecia Biologics, Billingham, (UK). Buffer formulation (pH 7.4): 8g/L Sodium chloride, 0.072g/L Disodium hydrogen phosphate, 0.01 g/L Potassium dihydrogen phosphate and 0.2g/L Potassium chloride in water for injection, Tween 20 0.04% v/v (+/-0.02%); protein concentration ~1.5 mg/ml. Cholesterol, phospholipids, sodium deoxycholate and other routine laboratory reagents were purchased from Sigma Aldrich (UK).

2.1. Preparation of PA63 heptamer

4 mg of rPA83 (Avecia) was incubated with 10 U of Furin (Biolabs) overnight at ambient temperature in 20 mM Tris, 5 mM CaCl₂, 150 mM NaCl pH 8 buffer to produce nicked PA (PA63-PA20). The sample was dialysed against 5 L of 20 mM Tris, pH 8.4 buffer using Spectra/Por porous membrane with molecular cut off point of 6-8 kDa at ambient temperature overnight and then loaded onto HiTrap Q HP 1ml anion exchange column (GE Healthcare) connected to an AKTA FPLC system (GE Healthcare). The protein was eluted from the column by gradient elution of 0 % to 100 % over 30 minutes using 20 mM Tris, 0.5 M NaCl, pH 8.4 buffer. Three peaks were eluted and collected as individual fractions. These corresponded to PA83, PA63 and PA20, determined by SDS PAGE (data not shown). PA63 heptamers/pre-pores formed by spontaneous oligomerisation.

2.2. Treatment of PA solutions

Heat treatment of rPA83 and PA63 (0.2 mg/ml) was performed at 50°C, using 100 µl aliquots for a 5 h period. Cholesterol microcrystal (stacked rigid bilayers) solutions were prepared by rapid injection of 100 mg/ml ethanolic solutions of the lipid into a 100x excess

of deionized water, generally termed the *stranding* procedure (Harris, 1988). Anthrax rPA83 and PA63 (0.2 mg/ml) was added to the cholesterol microcrystal solutions and incubated for 24h at ambient temperature. Treatment of rPA83 and PA63 (0.2 mg/ml) with sodium deoxycholate was performed for 24 h at ambient temperature, using 1.0 mM to 50 mM concentrations of the surfactant at pH 7.

2.3. Preparation of negatively stained EM specimens

20 µl aliquots of the different PA solutions were taken directly and following treatment, and negatively stained specimens were prepared on glow-discharge treated continuous carbon support films, using the Parafilm droplet procedure (Harris, 1997). PA samples were adsorbed to the carbon film for 30 sec, washed rapidly up to four times with distilled water droplets (the number of wash steps depended upon the salt or buffer concentration in the sample), negatively stained with 2% w/v uranyl acetate solution (pH 4.5) or 5% w/v ammonium molybdate, 0.1% w/v trehalose (pH 7.0), and finally air-dried at room temperature.

2.4. Transmission electron microscopy

TEM studies were performed at ambient temperature using a Philips CM100, at 100kV, without using low electron dose. Digital images (8 bit) were recorded at direct magnifications up to x 130,000, using an Optronics 1824x1824 pixel CCD camera with an AMT40 version 5.42 image capture engine, supplied by Deben UK. Image compilation for publication was performed in Adobe Photoshop.

3. Results

3.1. Anthrax rPA83 aggregation

Negatively stained specimens of untreated anthrax rPA83 monomer solution do not reveal any definable protein structures (Fig. 1a), although the occasional small aggregate is present. This could be due to the overcrowding of the carbon surface with protein or, more likely, the relatively featureless nature of the protein molecule under these conditions. Using the previously defined temperature of 45 to 50°C for thermally-induced rPA83 aggregation (Ganesan et al, 2012; Belton and Miller 2013) we selected 50°C for 5 h, without agitation. After this incubation visible aggregation was detectable by eye in the microcentrifuge tubes. Negatively stained specimens prepared from aliquots taken from the heated rPA83 suspension (after thorough mixing) revealed a range of small to very large aggregates (Fig. 1b), in accord with the ESEM data of Belton and Miller (2013). The thicker regions of the rPA83 aggregates tend to retain too much negative stain, but around the edges the more thinly spread regions showed detail of the fibrous nature of the aggregate (Fig. 1c,d). The coarse helical nature of the rPA83 aggregate is shown more clearly in Fig. 1d and at higher magnification in Fig. 1e and f.

Interaction of rPA83 with 33 mM sodium deoxycholate (NaDOC), induced the formation of short lengths of coarse helical aggregate (Fig. 2 a,b). Whilst there is considerable similarity to the fibrous aggregate formed by heating rPA83, this surfactant-induced aggregate possesses a hollow stain-filled core around which the protein spirals. Also, contrary to the heat treatment, no large rPA83 aggregates were detected.

Addition of rPA83 to aqueous microcrystal suspensions of cholesterol, with incubation at room temperature (22°C) for 24 h induced the formation of small fibrous aggregates (Fig. 2c) together with the marked binding of a layer of small aggregates onto the edges of the planar microcrystals (Fig. 2d). The absence of rPA83 aggregates on the cholesterol bilayer planar surfaces is clearly evident (Fig. 2c,d).

3.2. Anthrax PA63 aggregation

The anthrax PA63 heptamer/prepore is soluble and stable at neutral pH (Fig. 3a,b), although previous studies have shown it to aggregate at lower pH (e.g. pH 5) (Kintzer et al., 2012; Kohler and Collier, 1991). The ring-like heptamer has two distinct surfaces (Lacy et al., 2004), one of which possesses the cell surface receptor binding site, leading *in vivo* (in the acidic endosome) to unfolding and membrane penetration by the extended 14-stranded β -barrel. We have found that freeze-storage and repeated freeze-thawing of PA63 leads to the production of chain-like stacks of the prepore. Freeze-thaw is known to destabilize some proteins, in this instance creating an inter-prepore linkage (Fig. 3c). Within the PA63 prepore stacks, all the oligomers are orientated on-edge, alongside the single face-on ring-like images (Fig. 3c), whereas when PA63 prepores are incorporated into the stacks few ring-like images remain (Fig. 3d). Attempts to dissociate the PA63 prepore stacks with the non-ionic surfactant octylglucoside did not release the individual prepores, but the clarity of the prepores within the stacks was reduced (Fig. 3e), in all probability due to binding of surfactant.

Heat treatment of the PA63 prepores (50°C for 5 h) produced random aggregates (Fig. 3f), with no indication of fibrous material or conversion to PA63 pores.

Addition of PA63 prepores to an aqueous suspension of cholesterol microcrystals reveals binding to the bilayer edges of the microcrystals. In Fig. 4 a,b the cholesterol microcrystals are orientated flat and surrounded by relatively shallow negative stain, showing little detail of the bound PA63 prepores at the edges of the microcrystals. However, occasionally the thin cholesterol microcrystals are positioned on-edge in deeper negative stain, the bound PA63 prepores are shown to be aligned with the stacked cholesterol bilayers (Fig. 4c,d).

As previous studies using the cholesterol dependent *Vibrio cholerae* cytolysin (VCC) revealed conversion to a fibrillar state by sodium deoxycholate (Harris and Palmer, 2010), this surfactant was added to anthrax PA63 prepores. Under these conditions low concentrations of NaDOC (1.0 to 10 mM) resulted in conversion of prepores to pores,

together with the formation of random aggregates (data not shown). However, at higher NaDOC concentrations (20 to 30mM) ordered aggregates were produced. At 20mM NaDOC precisely aligned-defined PA63 pore dimers were formed, with the β -barrels extending out from the opposite sides (Fig 5, a,b). The binding of micellar and molecular NaDOC is undoubtedly responsible for the conversion to PA63 pores and for their aggregation as dimers. At 25 to 50 mM NaDOC the aggregation of the PA63 pores progresses further, with the formation of angular chains, with the extended β -barrels linked by NaDOC micelles (Fig 5, c,d). At the higher NaDOC concentrations there is a loss of clarity of the individual PA63 pores within the aggregates, due to the increased amount of bound surfactant (data not shown).

4. Discussion

Protein science uses the term aggregation with some degree of precision to refer to ordered structures rather than disordered/random precipitates. Indeed, in the field of amyloidogenesis aggregation generally refers to fibrillogenesis and the creation of a range of polymorphic fibrils (*eg* Harris, 2012; Milton and Harris, 2014). In most cases fibril formation can be analysed structurally in terms of the presence of rigid fibrils containing inter molecular β -sheets, supported by increased thioflavine-T fluorescence. Thioflavine-T is weakly fluorescent in aqueous conditions, but readily intercalates within ordered cross- β sheet channels to produce a strong fluorescence when excited at 425 nm (Krebs et al., 2005). This property of Thioflavine-T is widely exploited to differentiate between amyloid fibrils and disordered amorphous aggregates. In some cases the amyloid fibrils appear to be more disordered, but exhibiting helicity (Tyco, 2014). With thermally-treated rPA83 the structural indication of amyloid-type aggregation (Fig.1) is supported by thioflavin fluorescence (Ganesen et al., 2012).

Sodium deoxycholate (NaDOC) is a widely-used surfactant for the solubilisation of membrane proteins and is also known to interact with water-soluble proteins (Ren et al., 2015). Bhakdi et al. (1981) showed that the *Staphylococcal* α -toxin monomer formed oligomers through contact with deoxycholate. Aqueous NaDOC has a relatively small micellar size, each micelle containing approximately 10 molecules (Matsuoka and Moroi, 2002). Both molecular and micellar NaDOC have the potential to interact with protein molecules. With rPA83 we found that NaDOC potentiates the transformation to fibrillar helical aggregates at ambient temperature, similar but not identical to those produced by heat treatment. These helical structures formed by the uncleaved full protein do not resemble any obvious intermediate in the pore formation process but curiously do appear to show long range order. Clearly the presence of the PA20 domain 1 inhibits formation of the normal heptameric pore structure and the destabilised rPA83 adopts an alternative complex. Nevertheless these are not random aggregates and may be driven by the same partial unfolding of the beta barrel forming domain 2 which drives PA63 (prepore) spontaneous oligomerisation.

Cholesterol microcrystal suspensions potentiate the aggregation of rPA83, which then clusters in the vicinity of the microcrystals. Although *in vivo* PA83 binds to a cell surface receptor (Sun and Jacquez, 2016), it has been shown here, under *in vitro* conditions, to bind to cholesterol microcrystals (Fig. 4). That the binding occurs predominantly at the cholesterol bilayer edges indicates that a sterol ring interaction is involved rather than the 3 β hydroxyl that is more accessible on the planar microcrystal surface.

In solution, the PA63 prepore is a stable ~ 440kDa ring-like oligomer, with two well defined faces, one of which unfolds to generate the β -barrel of the PA63 pore (Lacy et al., 2004). Freeze-thawing leads to an ordered face-to-face aggregation of the PA63 prepore, without any extension of the β -barrel. Whilst surfactant dissociation of such a protein aggregate might be predicted, this was not achieved with octyl glucoside (octyl β -D-glucopyranoside). Unlike rPA83, heat treatment of PA63 yields random disordered

aggregates, with no indication of transformation to pores. Similar to rPA83, PA63 prepores bind to the edges of cholesterol microcrystals, without transformation to pores. A comparable situation has been shown for the *Vibrio cholerae* cytolysin VCC prepore (Harris et al., 2002).

Contrary to octyl glucoside, we have shown that the sterol surfactant sodium deoxycholate induces the PA63 prepore to pore conversion. Others have also achieved PA63 pore formation using 1 M urea in the presence of the *E. coli* chaperone GroEL (Katayama et al., 2008) and on PerfectPro Ni-NTA Agarose by brief pH 5.5 treatment followed by binding to lipid nanodiscs and vesicles (Katayama et al., 2010). PA63 pore formation directly on polylysine treated carbon support films was also achieved by Jiang et al. (2015) using a low pH treatment in the presence of the non-ionic surfactant IGEPAL[®] CA-630 (equivalent to Nonidet P40). Although our emphasis here has been on PA63 aggregation, our direct treatment of PA63 prepores in solution with NaDOC at neutral pH could provide suitable protein samples for the further structural analysis of the PA63 pore by cryo-electron microscopy and X-ray crystallography. The controlled formation of PA63 pore dimers in the presence of NaDOC correlates with the further aggregation of the dimers to form angular chains and closed structures (Fig. 5b), incorporating molecular and/or micellar NaDOC.

This study has revealed a common and unexpected theme in the behaviour of rPA83 and PA63 in their response to sterol containing molecules. In both the case of the NaDOC and cholesterol crystals both proteins show ordered associations. While the interaction with the crystalline phase has clear long range order, the effect of the micellar detergent is comparatively less clear but none the less shows a degree of specificity. In the case of rPA83 the filaments produced by the two treatments appear similar and presumably result from ordering of rPA83 monomers around some specific interaction with the sterols. In the case of PA63 the effects are even clearer, with the heptameric rings forming regular arrangements on the edges of the cholesterol crystals and transforming from prepores into

pores in the presence of NaDOC. It is worth noting that treatment with a non-sterol detergent octyl glucoside does not dissociate the stacks formed by freeze thawing and does not produce the elongated pore form. There is no previous evidence for a direct molecular interaction between anthrax PA and cholesterol, as has been shown the cholesterol dependent cytolytins and VCC (Farrand et al., 2010; Harris et al., 1998; Harris et al., 2002; Harris et al., 2011). However it is known that the anthrax toxin receptor ATR migrates to cholesterol-sphingomyelin rich membrane domains after binding to PA63 and that the *in vivo* activity of the toxin is inhibited by reducing in the levels of cholesterol or sphingomyelin in cultured cells (Abrami et al., 2003). Whilst these effects are likely to be due to ATR activity the fact that PA63 forms its pores in cholesterol rich membranes may explain why it also shows sterol specific effects *in vitro*. Once in the acidic endosome the PA63 ring becomes more stable and this helps explain the effects of low pH on the structure of all the forms of PA.

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Legends

Fig. 1. Heat-treated recombinant anthrax PA83: (a) Untreated PA83 solution (thawed from -80°C), revealing only a dispersion of thinly spread protein with no visible structural features, and only the occasional small aggregate. (b) A low magnification image of rPA83 following treatment at 50°C for 5h. This image correlates well with the ESEM image of heat-aggregated rPA83 in Belton and Meller (2013). At higher electron optical magnification the rPA83 aggregate is shown to be a tangle of thread-like strands (c,d). When spread at the edges of the aggregates greater detail of the threads is revealed (d). The individual rPA83 filaments (~21 nm diameter) possess a quasi helical feature (e, f). All samples were negatively stained with 2% w/v uranyl acetate. The scale markers indicate 100nm (a,c,d,e,f), 500nm (b).

Fig. 2. Anthrax rPA83 following interaction with sodium deoxycholate and cholesterol microcrystals: (a,b) rPA83 treated with 33mM sodium deoxycholate for 24 h at room temperature (22°C). Quasi helical protein filaments (~25 nm diameter) are produced, similar to those produced by heat-treatment of rPA83(Fig. 1 ef), but showing a more pronounced stain-filled central channel. No large aggregates were produced. (c,d) Cholesterol microcrystals with added rPA83 for 24h at room temperature (22°C). The rPA83 has undergone filamentous aggregation on and around the cholesterol microcrystals, but without the production of large aggregates (c). Small aggregates also cluster predominantly at the bilayer edges of the cholesterol microcrystals (d). All samples were negatively stained with 2% w/v uranyl acetate. The scale markers indicate 100 nm.

Fig. 3. PA63 heptamers (prepores), untreated and following freeze-thawing, octylglucoside and heat treatment: PA63 prepores adsorbed to a carbon support film and negatively stained with uranyl acetate (pH 4.5) (a), and ammonium molybdate (pH 7.0) (b). In (a) all the PA63 prepores are adsorbed end-on, displaying the ring-like structure of the oligomer. With ammonium molybdate some release of the adsorbed PA63 from the carbon film has occurred, generating more confused image, with the PA63 oligomers in varying orientations (b). This phenomenon has been observed for other proteins negatively stained with ammonium molybdate. Freeze-storage (-18°C) of the PA63 prepore solution generates flexible stack-like aggregates, with free prepores (c) and with repeated freeze-thaw almost total incorporation into the stacks (d). Attempts to dissociate the PA63 stacks with octylglucoside were unsuccessful (e), but the increased diameter and loss of clarity of the individual PA63 prepores within the stacks is indicative of surfactant binding. Heat treatment (50°C) for 5 hours produced disorganized aggregates, with loss of clarity of the individual PA63 prepores and no indication of conversion to PA63 pores (Cf Fig. 5). All samples were negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 4. Anthrax PA63 interaction with cholesterol microcrystals: Stacked planar cholesterol microcrystals in shallow negative stain bind PA63 prepores at the bilayer edges (a,b). When the cholesterol crystals are positioned on-edge in deeper negative stain the linear PA63 binding is shown to be imposed by the accessible bilayer edges (c,d). All samples negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 5. Anthrax PA63 following treatment with sodium deoxycholate: At a sodium deoxycholate concentration of 20 mM at neutral pH induces the formation of paired PA63 heptamers that have undergone structural transformation into PA63 pores, as shown in the survey image (a). At higher magnification the extended β -barrels of the paired PA63 pores are indicated (arrows, b). When the sodium deoxycholate concentration is increased, further aggregation of the PA63 pores occurs, leading to extended chains and angular structures (c). The extended β -barrels of the paired PA63 pores (arrows, d) are linked, in all probability by micellar or molecular deoxycholate. All samples negatively stained with 2% w/v uranyl acetate. The scale bars indicate 100 nm.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5