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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 ß-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 83 kDa 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., 2015; Vodkin and Leppis, 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 Jacquez, 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 ß-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-Nolina et al., 2016; Bondos and Bicknell 2003; Chaudhuri et al., 2014; Shrödel and de Marco, 2005). The low pH lability of soluble rPA83 and PA63 has been well documented, despite the fact that conversion of

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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 ß-sheet content. A structural similarity has been drawn between the closed ß-barrel, as found in the heptameric VCC pore, and an open-ended fibrillar ß-sheet (Harris and Palmer, 2010). Indeed, conversion of ß-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 Fleming, 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): 8 g/L Sodium chloride, 0.072 g/L Disodium hydrogen phosphate, 0.01 g/L Potassium dihydrogen phosphate and 0.2 g/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 1 ml 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%–100% over 30 min 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 100 x 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 24 h 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 s, 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 100 kV, without using low electron dose. Digital images (8 bit) were recorded at direct magnifications up to x 130,000, using an Optronics 1824 × 1824 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 45–50 °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. 2a,b). Whilst there is considerable similarity to the