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Cryo-electron microscopy study of bacteriophage T4 displaying anthrax toxin proteins

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Abstract

The bacteriophage T4 capsid contains two accessory surface proteins, the small outer capsid protein (Soc, 870 copies) and the highly antigenic outer capsid protein (Hoc, 155 copies). As these are dispensable for capsid formation, they can be used for displaying proteins and macromolecular complexes on the T4 capsid surface. Anthrax toxin components were attached to the T4 capsid as a fusion protein of the N-terminal domain of the anthrax lethal factor (LFn) with Soc. The LFn-Soc fusion protein was complexed *in vitro* with $Hoc^{-}Soc^{-}$T4 phage. Subsequently, cleaved anthrax protective antigen heptamers $(PA63)_7$ were attached to the exposed LFn domains. A cryo-electron microscopy study of the decorated T4 particles shows the complex of PA63 heptamers with LFn-Soc on the phage surface. Although the cryo-electron microscopy reconstruction is unable to differentiate on its own between different proposed models of the anthrax toxin, the density is consistent with a model that had predicted the orientation and position of three LFn molecules bound to one PA63 heptamer.
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Introduction

Bacteriophage T4 is a large, double-stranded DNA virus that belongs to the *Myoviridae* family and infects *Escherichia coli*. The T4 virion consists of a capsid and a contractile tail terminating in a baseplate to which is attached six long tail fibers (Eiserling and Black, 1994). The T4 head is a prolate icosahedron (Fig. 1A) with a length of 1200 Å and a width of 860 Å (Fokine et al., 2004). The major capsid protein, gp23* (48.7 kDa, 930 copies per capsid), forms a hexagonal lattice with triangulation numbers (Caspar and Klug, 1962) $T_{end}=13$ (*laevo*) in the icosahedral caps and $T_{mid}=20$ in the cylindrical midsection (Fokine et al., 2004). The distance between adjacent gp23* hexameric capsomers is ~140 Å. Eleven vertices of the capsid are occupied by pentamers of the special vertex protein gp24* (47.2 kDa), whereas the 12th vertex, occupied by a gp20 dodecamer, is the portal for DNA entry, tail attachment, and DNA exit (Driedonks et al., 1981). Gp24* and gp23* (Fokine et al., 2005) have a protein fold similar to that of the bacteriophage HK97 capsid protein (Wikoff et al., 2000).

A unique feature of T4 architecture is the presence of two accessory proteins, the highly antigenic outer capsid protein (Hoc, 39.1 kDa) and the small outer capsid protein (Soc, 9.7 kDa), which decorate the outside of the capsid shell (Figs. 1A and 2A) (Fokine et al., 2004; Ishii and Yanagida, 1977; Olson et al., 2001). Both Hoc and Soc are dispensable for capsid formation and able to bind to the capsid during the final stages of capsid maturation. Soc helps to stabilize the capsid against extremes of pH and temperature, whereas Hoc has only a marginal effect on capsid stability (Steven et al., 1992). One Hoc molecule binds at the center of each gp23* hexamer, giving 155 Hoc molecules per T4 capsid. The rod-like Soc molecules form a nearly continuous mesh on the capsid surface that encircles the gp23* hexamers (Figs. 1A and 2A). The Soc molecules bind between gp23* hexamers, but not between gp23* hexamers and

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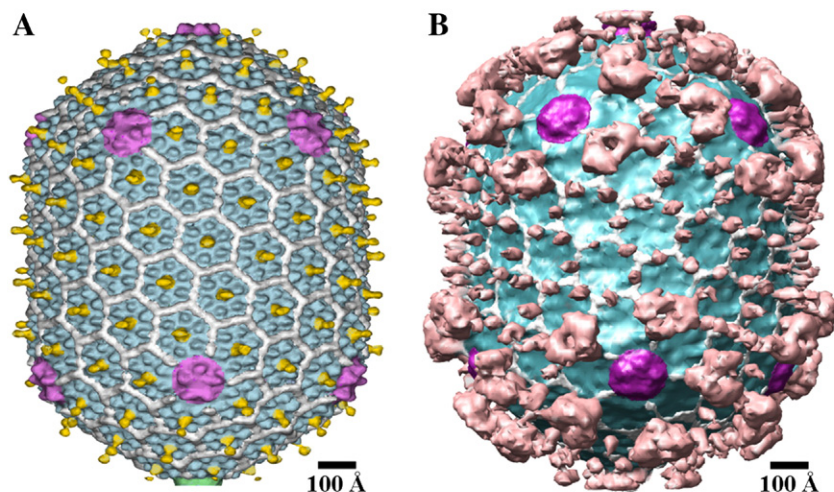

Fig. 1. Cryo-EM reconstructions. (A) The wild-type T4 capsid at 22 Å resolution (figure from Fokine et al., 2004). (B) The T4 capsid with attached $(PA63)_7$ and LFn-Soc molecules at 35 Å resolution. The gp23* hexamers are shown in blue, the gp24* pentamers in magenta, the Hoc molecules in yellow, and the Soc molecules in white. Protrusions corresponding to the anthrax toxin proteins are colored in pink.

gp24* pentamers or between gp23* hexamers and the gp20 dodecameric portal protein. The total number of Soc molecules on the T4 capsid is 870.

Bacillus anthracis, the causative agent of anthrax, secretes three proteins, namely protective antigen (PA), lethal factor (LF), and edema factor (EF), that assemble into toxic complexes on mammalian cell surfaces. The assembly of these complexes starts with the binding of PA to a cellular receptor (Bradley et al., 2001), followed by cleavage of PA by furin (a membrane-bound protease) causing removal of a 20 kDa N-terminal PA fragment. The remaining receptor-bound 63 kDa fragment of PA (PA63) oligomerizes to form a ring-shaped heptamer (Milne et al., 1994). The PA63 heptamer, $(PA63)_7$, then interacts with the N-terminal domain of LF (LFn) and/or EF (EFn) to form the biologically active anthrax toxin complex (Cunningham et al., 2002; Leppla, 2006; Melnyk and Collier, 2006). The assembled toxin can enter the cell via receptor-mediated endocytosis. The intracellular delivery of LF, a Zn-metallo protease, and EF, an adenylate cyclase, dramatically alters the cellular metabolism, eventually causing cell death (Leppla, 2006).

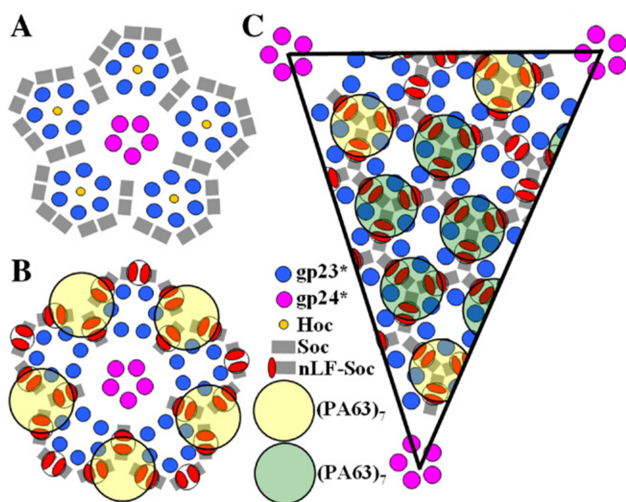

Fig. 2. Schematic representation of the distribution of proteins on the T4 capsid surface. (A) View down a 5-fold axis of a wild-type virus and (B) showing also the attached $(PA63)_7$–$(LFn\text{-}Soc)_3$ complexes. (C) One possible distribution of the $(PA63)_7$–$(LFn\text{-}Soc)_3$ complexes in the equatorial region of the T4 capsid. The yellow rings show the higher occupancy positions of $(PA63)_7$ in the vicinity of the 5-fold vertices, whereas the green rings show a possible distribution of the less occupied sites.

Currently available information on the stoichiometry of the anthrax toxin complex is contradictory. Originally, Singh et al. (1999) showed by non-denaturing gel electrophoresis that each PA63 subunit in the heptamer can bind one LF molecule. Mogridge et al. (2002) studied the stoichiometry of the anthrax toxin by measurements of isotope ratios in complexes assembled from differently labeled toxin subunits. Also, they determined the molecular weights of the complexes using multi-angle laser light scattering. Both approaches yielded the value of three molecules of LF or EF per PA63 heptamer. However, cryo-electron microscopy (cryo-EM) reconstruction of the complex of PA63 heptamer with LF showed only one LF molecule (Ren et al., 2004; Tama et al., 2006). Recent studies of the interactions between PA63 and LF using peptide amide hydrogen/deuterium exchange mass spectrometry and directed mutagenesis (Melnyk et al., 2006) supported the finding that PA63 heptamer can bind a maximum of three molecules of LF or EF (Mogridge et al., 2002). The previously determined crystal structures of PA63 heptamers (Lacy et al., 2004) and LF (Pannifer et al., 2001) were used to construct a model of the $(PA63)_7$–$(LFn)_3$ complex, consistent with mutational studies (Lacy et al., 2005).

In this paper, we report a cryo-EM study of T4 particles associated with PA63 heptamers complexed with LFn-Soc. A fusion protein between LFn (residues 1 to 264) and full-length Soc (80 residues) was constructed, with a hexa-histidine N-terminal tag and a flexible four-residue (SASA) linker. The LFn-Soc molecules were attached *in vitro* to a Hoc^-Soc^-T4 phage mutant. The anthrax PA63 heptamers were then attached to the capsid-exposed LFn domains. This represents probably

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