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Correct assembly of the bacteriophage T5 procapsid requires both the maturation protease and the portal complex

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Abstract

The 90 nm diameter capsid of coliphage T5 is organized with T=13 icosahedral geometry and encloses a dsDNA genome that measures 121 kbp. Its assembly follows a path similar to that of phage HK97 but yielding a larger structure that includes 775 subunits of the major head protein, 12 subunits of the portal protein and 120 subunits of the decoration protein. As for phage HK97, T5 encodes the scaffold function as an N-terminal extension (σ -domain) to the major head protein that is cleaved by the maturation protease after assembly of the initial prohead I form and prior to DNA packaging and capsid expansion. Although the major head protein alone is sufficient to assemble capsid-like particles, the yield is poor and includes many deformed structures. Here we explore the role of both the portal and protease in capsid assembly by generating constructs that include the major head protein and a combination of protease (wild type or an inactive mutant) and portal proteins, and overexpressing them in *E. coli*. Our results show that the inactive protease mutant acts to trigger assembly of the major head protein, probably through binding to the σ -domain, while the portal protein regulates assembly into the correct T=13 geometry. A cryo-EM reconstruction of prohead I including inactivated protease reveals density projecting from the prohead interior surface toward its center that is compatible with the σ -domain, and additional internal density that we assign as the inactivated protease. These results reveal complexity in T5 beyond that of the HK97 system.

Graphical abstract

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Keywords

Capsid assembly; connector; portal; scaffolding; tailed bacteriophage; maturation protease

Introduction

Capsids of the tailed dsDNA bacteriophages assemble in a tightly regulated process involving hundreds of copies of a major capsid protein organized in a regular repeating pattern of hexameric and pentameric capsomers on an icosahedrally symmetric lattice. During this process, the capsid proteins avoid being trapped into non-productive planar or helical networks of hexamers or into shells of the incorrect size or shape. Despite the vast array of icosahedral capsid morphologies observed, including prolate icosahedral geometries, every capsid of the tailed dsDNA bacteriophage family detailed to date, including phage T5, appears to exploit a common fold – that first described for phage HK97¹². The HK97 fold is also likely used in the eukaryotic herpesvirus family³ and has recently been observed in an archaeal virus⁴ and in non-viral structures⁵. The principles guiding assembly of these structurally related subunits into different but specific icosahedral geometries with high fidelity are the subject of our on-going work.

Proteins essential for tailed dsDNA phage capsid assembly and function, in addition to the major capsid protein, include a scaffolding protein, a portal and sometimes a maturation protease. The scaffolding protein plays a critical role in regulating correct head assembly⁶⁷⁸ and its absence leads to aberrant structures, as has been demonstrated in bacteriophages P22, phi29 and T4⁹¹⁰¹¹¹². Incorporation of the portal complex appears to be another function of the scaffolding protein¹³¹⁴¹⁵. The portal forms a dodecameric ring complex located at one icosahedral vertex and through which the dsDNA enters and exits the capsid, and is also the binding site for head completion proteins that seal the portal gate after DNA packaging termination¹⁶. This specialized vertex forms the connector that binds the tail¹⁴. Some evidence suggests that the portal may also act to nucleate capsid assembly^{1517; 18} but its role in regulating assembly is less clear. Some phages, such as T4 and phi29, require the portal for generating the correct prolate shape¹¹¹⁰¹⁹ while others such as P22²⁰, SPP1²¹

and HK97²² can make correctly sized proheads in the absence of the portal. Virally encoded proteases are present in most but not all phages. When present they appear to regulate capsid maturation including scaffold digestion and auto-inactivation, but structural roles during assembly have only recently been suggested, as for example in phage HK97^{23,24}.

Unlike many other phages, the T5 assembly pathway does not involve a separately encoded scaffolding protein that would typically be encoded immediately upstream of the major capsid protein gene. Instead, as for HK97, phage T5 employs a scaffolding domain that is fused to the N-terminus of the major capsid protein gene and is termed the γ -domain^{25,26}. The T5 γ -domain of the 50 kDa full-length precursor major head protein (pb8p) comprises the N-terminal 159 amino acids and it is strongly predicted to be mostly α -helix arranged in a coiled-coil²⁶. Interestingly, the larger T5 capsid (90nm diameter) correlates with the longer T5 γ -domain, compared to the HK97 capsid (60nm) and its γ -domain (102 residues), suggesting that the γ -domain may have a size-regulating role for the capsid, although as yet there is no experimental evidence to support this.

Bacteriophage T5 has a genome of 121 kbp and an icosahedral capsid that has a triangulation number $T=13$ ^{26,27}. As a consequence, 775 copies of the major head protein (120 hexons and 11 pentons of pb8) and one portal complex (12 copies of portal protein, pb7) compose the shell, and each hexon binds a single copy of the decoration protein pb10 according to the assembly scheme indicated in Fig. 1 (the function of the decoration protein is still unclear). The full-length pb8p together with both the portal complex and the maturation protease (pb11) form prohead I. All 775 copies of the 50 kDa pb8p are cleaved to the 32 kDa mature major head protein (pb8) by release of the γ -domain, yielding the metastable prohead II. It should also be noted that the first 10 amino-acids of the portal protein are also cleaved¹⁶. Assembly proceeds with packaging of the viral dsDNA into the prohead II capsid through the portal as the capsid undergoes an expansion transition to the mature, angular form. A single copy of the decoration protein binds to the center of each hexon of the expanded capsid to give the final head particle. Despite the capsid geometries differing significantly between phage HK97 ($T=7$) and phage T5 ($T=13$), the apparent similarity of their assembly pathways has led us previously to investigate whether the T5 major head protein alone has all the information to make a capsid of the right size and shape starting from purified protein²⁸, as for the major head protein of HK97²². While we obtained capsids of about the right size, visualization by cryo-electron microscopy (cryo-EM) revealed that the particles were irregularly shaped (Supplemental Fig. S1) suggesting that other capsid proteins are critical for regulating assembly. Thus, we investigate here the roles of the portal pb7 and the maturation protease pb11 by attempting to assemble T5 proheads *in vitro* and *in vivo* in the presence of wild type or inactivated protease, or the portal, or both together.

Results

Overexpression of the structural proteins

Purified T5 major capsid protein, pb8p, is able to assemble into prohead I-sized particles (called pHI-8p) in the presence of calcium²⁸. However, cryo-EM reveals that their shape is somewhat irregular (Supplementary Fig. S1) and image reconstruction of these particles

yields a density map limited to 30 Å resolution, despite including more than 1000 images (see below). We surmise that other capsid proteins may play a critical role in controlling the fidelity of assembly, and the most likely candidates are the maturation protease, pb11, and the portal protein, pb7, because they are present during the earliest stages of assembly. We constructed a total of 6 plasmid-based expression vectors to explore the effects of co-assembling one or both proteins with the pb8p head protein, as detailed in Fig. 2a and Table 1. Two of the constructs, 11-8 and 7-10-11-8, exist in two versions, one with the wild-type protease and the other with an inactivated protease (described below). A second version of each of the six vectors was made with a hexa-histidine tag as indicated in Table 1 to aid in purification. Also the gene coding for the decoration protein is present in the expression vectors containing all of the portal, protease and major head proteins so as to maintain a wild-type-like context of the structural capsid genes that may include any potential regulating domain of the translation. The structural region is arranged in the order 7 – 10 – 11 – 8, encoding portal (pb7), decoration protein (pb10), protease (pb11) and major capsid protein (pb8), respectively, although we do not consider that the decoration protein plays a role in the early steps of assembly since it is not found in prohead II²⁷ and presumably is also absent in the earlier prohead I.

To study the role of the protease during the early steps of prohead assembly, we sought to eliminate its proteolytic activity by mutation of one of the residues critical for that function, and thereby accumulate prohead I-like particles. Multiple sequence alignment of the T5 maturation protease with those of other phages revealed highly conserved residues potentially involved in the proteolytic activity. Previous studies on this family of bacteriophage proteases have suggested that the catalytic site involves a structurally organized triad of a histidine, a serine and a glutamate residue, as indicated in Fig. 2B²⁹. Point mutations of each of these residues all result in inactivation of the protease (Supplementary Fig. S2) and we have chosen one, H76A (named mutpb11), to use in this study.

We first analyzed the soluble fraction after overexpression of each vector by SDS-PAGE. In particular, the soluble fractions after overexpression of the constructs 11-8 and 7-10-11-8 with wild-type pb11, and mut11-8 and 7-10-mut11-8 with inactivated pb11, all without his-tags, are shown in Fig. 2C. The equivalent constructs with His-tags gave the same results. Overexpression of the wild-type protease in the plasmids 11-8 and 7-10-11-8 causes a large decrease of bacterial growth upon induction likely due to its high toxicity²⁸. In addition, on SDS-PAGE the 50 kDa band corresponding to the unprocessed major capsid protein pb8p is not visible, likely due to being proteolysed, while instead a faint band at the size expected for the mature 32 kDa form of the major head protein (pb8m) appeared, especially in the case of the 7-10-11-8 expression. N-terminal sequencing of this 32 kDa band confirms that it corresponds to pb8m, indicating that the protease is cleaving the N-terminal α -domain as it does *in vivo*³⁰. Nonetheless, the high toxicity of the maturation protease is likely reducing the yield of protein expression as shown by the small amount of pb8m produced. When the protease is rendered inactive by mutation (constructs mut11-8 and 7-10-mut11-8), the precursor major head protein, the mutated protease, the portal (for 7-10-mut11-8) and the decoration protein (for 7-10-mut11-8) were clearly detectable in the soluble fractions. We

note that the T7 promoter in the plasmid yields particularly high overexpression of the proteins mutpb11 and pb7 in the constructs mut11-8 and 7-10-mut11-8, respectively.

***In vitro* assembly reveals a structural role for the maturation protease**

With the purified pb8p already in hand, we expressed and purified pb7 portal and mutpb11 protease to study their influence on pb8p assembly *in vitro*. We purified the his-tagged versions of these two proteins by nickel-affinity chromatography (Fig. 2D, top-left). The purified portal protein required an ionic strength $[\text{NaCl}] > 500 \text{ mM}$ in order to remain soluble, and by negative-stain EM the sample appeared as the expected ring-shape structures, 15 nm in diameter and with a ~4 nm hole in the middle (Fig. 2D). In contrast, the inactive protease was highly soluble.

To analyze whether the mutated protease or the portal protein play a role in the regulation of capsid assembly *in vitro*, we mixed them individually and together with the major head protein and assayed for the formation of protein assemblies by native agarose gel and negative-stain EM (Fig. 3). The concentrations of the pb8p, pb11 and pb7 proteins in the assembly reactions were 10, 2, and 0.2 μM , respectively, approximating the expected stoichiometries of the subunits in the capsid considering about one maturation protease per hexamer and 12 portal proteins per capsid (775:~120:12, respectively). Since the portal protein requires high salt for solubility, but high salt concentration triggers pb8p to assemble into tubes²⁸, all experiments were done at an intermediate level of 100 mM NaCl buffered at pH 8.0. We also tested the effect of adding 20 mM Ca^{2+} as used previously for assembling purified pb8p into prohead I-like particles (irregular particles), although in the absence of NaCl²⁸.

Without Ca^{2+} and with 100 mM NaCl (Fig. 3A, lanes 1 to 7), purified pb8p gives a diffuse band that suggests it is composed of capsomers (lane 1)²⁸. Also, a slow migrating species – too slow to be prohead I – formed a faint band that likely corresponds to a high order assembly such as tubes or monster capsids. The purified inactive maturation protease formed a discrete but very diffuse band (lane 2). The purified portal did not produce any bands in the gel (lane 3), suggesting that it is aggregated or very heterogeneous in this condition. The mutated protease (mutpb11) affects migration of pb8p (lane 4): the intensity of the band corresponding to mutpb11 decreases while the intensity of the slow migrating band of pb8p increases. This suggests that pb11 interacts with pb8p and triggers its association, and EM analysis of this sample shows tubes with inner density regularly spaced that likely corresponds to pb11 (Fig. 3B). Under the same conditions (lanes 5 to 7), the portal did not show any effect on the assembly of pb8p, mutpb11, or pb8p in the presence of mutpb11.

The presence of 100 mM NaCl abolished formation of most of the PHI-8p capsids induced by Ca^{2+} (Fig. 3A lane 8 and Fig. 3B) that we have seen previously in the absence of NaCl²⁸, although a few PHI-8p particles were present (Fig. 3B, inset). In contrast, the presence of pb11 protease changed the migration pattern dramatically (lane 9) where both the capsomer band and diffuse pb11 band (as seen in lane 4) are replaced by a long smear together with a discrete band (arrowhead on Fig. 3A) positioned where prohead I runs. EM visualization of this sample (Fig. 3B) confirms the presence of prohead I-like particles as well as tubes that have a different morphology from those induced by NaCl: the mutpb11

prohead-I particles (called PHI-mut11) exhibit a white core at their center that was not seen in particles of pI-8p (²⁸ and Fig. 3B), and the tubes also exhibit white density along their axis. We attribute this extra density to mutpb11 and conclude that while NaCl inhibits the formation of PHI-8p particles, purified mutpb11 facilitates the formation of prohead and other assembled species. Addition of the pb7 portal protein in the presence of Ca²⁺ had no significant effect on the banding pattern (lanes 10 and 11).

If our assignment of the extra-density in the cores of mutpb11 particles (both prohead-like and tubes) is correct, then mutpb11 appears to bind the γ -domain of pb8p where it may trigger assembly of pb8p into capsids or tubes. To confirm that pb11 promotes assembly of pb8p, we monitored the light scattering (OD at 400 nm) from purified pb8p (10 μ M) in the presence of various concentrations of pb11, as indicated in Fig. 4A. After 3 hours of incubation, samples were centrifuged at 14000 rpm followed immediately by light scattering measurements in order to remove any tubes that would alter the prohead I scattering signal. We found that the intensity of scattering correlated with the concentration of pb11, suggesting that pb11 is indeed promoting pb8p assembly into proheads. After 24 hours, the samples were also centrifuged and the supernatants were subjected to SDS-PAGE analysis (Fig. 4B), which revealed that pb8p levels in the supernatant diminish as pb11 concentration in the reaction increases. Imaging of the supernatant by cryoEM at 3h and 24h (Fig. 4C and D, respectively) showed prohead I as well as tubes, also consistent with pb11 promoting pb8p assembly. At the later time-point, the pb11-induced proheads were fewer and the tubes longer, suggesting that the prohead I-like particles produced this way are unstable and convert to tubes. Close inspection of the prohead I particles revealed that they are not regular, much as was observed with particles assembled from purified pb8p (Supplementary Fig. S1); they also appeared to be slightly larger and often had an unclosed side that may be the precursor for tube formation through accretion of hexamers. Unfortunately, this heterogeneity did not permit a capsid reconstruction to be calculated from the pb11-induced prohead I particles.

The portal protein regulates the correct assembly of proheads *in vivo*

The presence or absence of the purified pb7 portal protein, *in vitro*, did not significantly affect assembly of proheads. However, adding the portal protein to assembly reactions requires high salt for portal solubility, which also induces the major head protein to polymerize into tubes. Instead, we used our 7-10-11-8, 7-10-mut11-8, 11-8 and mut11-8 plasmids, with and without his-tags, to study the role of the portal in the assembly of the overexpressed proteins *in vivo*. Glycerol gradient, agarose gel, SDS-PAGE and EM were used to analyze the particles obtained.

The protein patterns of the crude extract obtained from plasmids 11-8, 7-10-11-8, mut11-8 and 7-10-mut11-8 are shown in Fig. 2C. For 11-8 and 7-10-11-8, putative proheads were concentrated by PEG/NaCl precipitation, then purified by glycerol gradient sedimentation and anion exchange chromatography. The purification process was followed by SDS-PAGE to check for the presence of a 32 kDa band corresponding to mature pb8. The eluate containing pb8 was imaged by negative-stain EM for both the 11-8 and 7-10-11-8 samples (Fig. 5A). Co-expression of the major head protein with the protease (11-8) gives high

molecular weight particles that are open and thin-shelled but neither tubular nor spherical in appearance. In contrast, expression of the major head protein with the wild type protease, the portal and the decoration protein (7-10-11-8) yields round prohead-like particles. In addition, the pb7 portal protein was detected by Western blot in the purified fraction of 7-10-11-8 (Fig. 5A, inset), indicating that pb7 is present in these particles.

We further investigated the assembly of pb8 in the presence of portal and inactivated protease after co-expression *in vivo*. The soluble fractions obtained after over-expression of the constructs 7, 8p, mut11-8, and 7-10-mut11-8 were PEG/NaCl precipitated and then analyzed by glycerol gradient, native agarose gel and SDS-PAGE (Fig. 5B). For this experiment, all glycerol gradients included 500 mM NaCl to maintain solubility of the portal. Comparison of the glycerol gradient light scattering patterns revealed that neither 7 nor 8p showed significant light scattering, while mut11-8 gave intense scattering that increased from the top to the bottom of the gradient suggesting that particles of different sizes are made. In contrast, 7-10-mut11-8 exhibited a discrete band consistent with uniformly sized particles, perhaps proheads. The band was still observed even when the sample was held at room temperature for 24h before centrifugation, demonstrating its stability. All the gradients were subsequently analyzed on both native agarose gel and SDS-PAGE, and the analysis corresponding to 7-10-mut11-8 is shown in Fig. 5C. For 7-10-mut11-8, the SDS gel shows that the major head protein was present in fractions 1 and 2 near the top of the gradient and in fraction 6, which corresponds to the discrete band seen by light scattering. The native agarose gel failed to show any discrete band that corresponds to proheads in fraction 6. Instead, the fractions at the top and fraction 6 all showed a similar wide band that runs in a position we identified as capsomers in Fig. 3A (lane 1), suggesting the assembled prohead particles in fraction 6 dissociate into capsomers under the conditions we use for native agarose gel electrophoresis. Also, as revealed by Western blot (Fig. 5C, bottom), the portal protein largely co-sedimented with the major head protein, suggesting that they associate with each other.

Because we suspected that the particles in glycerol fraction 6 might correspond to prohead I, we carried out an extensive analysis of all such fractions obtained by different constructs (as indicated on Fig. 6) by both negative-stain and cryo-EM, where the latter technique is expected to preserve fragile structures. Negative stain images of the glycerol gradient fraction 6 for 8p and mut11-8 are shown in Fig. 6A. For sample 8p, EM showed only a few round particles of varying size that we attribute to membrane vesicles rather than protein assemblies. For mut11-8 we observed tubes of various lengths likely made mainly of pb8p. Interestingly, those tubes have internal white density resembling that seen in the tubes made *in vitro* with pb8p and mutpb11 (Fig. 3B).

Fraction 6 for 7-10-mut11-8 observed by cryo-EM (Fig. 6B left) reveals prohead I-like particles (named PHI-7mut118) with dense interiors. More importantly, these particles appeared regular in shape and thus amenable to image reconstruction. However, the particles proved too unstable to concentrate and their fragility has been demonstrated already by their dissociation on native agarose gels. Nonetheless, we collected images of 150 prohead I-like particles and calculated a 3D reconstruction to 25 Å resolution (see below). As a control, cryo-EM of 7-10-11-8 purified proheads (Fig. 6B right) showed particles with empty

interiors that we identify as prohead II as well as expanded prohead and some irregular structures.

We also visualized glycerol gradient fraction 6 for each of the alternate pb7-his-tagged versions of these two constructs, namely his7-10-mut11-8 and his7-10-11-8 (Fig. 6C), by cryo-EM. Interestingly, both samples exhibited irregular particles and neither yielded any icosahedral capsids resembling those seen in the untagged samples. We infer that the N-terminal domain of the pb7 portal protein plays a crucial role in controlling the correct assembly of the prohead.

Prohead I-like structures of the 7-10-mut11-8 (PHI-7mut118) and purified pb8p (PHI-8p) constructs

Due to the heterogeneity evident in the cryo-EM data, the prohead I-like particles obtained from purified pb8p in presence of Ca^{2+} yielded a 30 Å resolution structure despite the inclusion of about 1000 particles, whereas the smaller set of 156 particles of the more uniform PHI-7mut118 sample was resolved to 25 Å (Fig. 7 and Supplementary Fig. S3). The diameter of both prohead structures is 67 nm, and the surface geometry has the expected triangulation number of $T=13$. The most distinctive feature of these structures is the array of internal protrusions from the capsid interior surface that are directed towards the center of the capsid cavity. These are consistent with the appearance of the prohead I images by cryoEM (Fig. 6), and also of tubes constructed from the full-length pb8p (Supplementary Fig. S2, ²⁸) The length of the protrusions is consistent with a model of the N-terminal domain that is arranged entirely as an extended coiled-coil (Fig. 7B). The exterior capsid surface reveals that both kinds of hexamer (H1 and H2) have a skewed conformation in both particles that is reminiscent of the phage HK97 prohead surface ³¹. Three main differences can be observed at this modest resolution: (i) the PHI-7mut118 particle is more angular than PHI-8p; (ii) PHI-7mut118 exhibits an additional internal structure extending between radii 60–125 Å that is not seen in PHI-8p and (iii) the internal protrusions are organized differently in that PHI-7mut118 has no visible protrusion under the penton while PHI-8p has a strong density in this location (Fig. 7C). We expect that the remarkable prohead structure and the morphological differences between these mutants offer important insights into the basis of capsid assembly regulation that will be explored in future work.

Discussion

Our results show that the phage T5 capsid requires three viral proteins for efficient and correct assembly – the major head protein, the protease and the portal. In addition to the primary functions ascribed to the protease (proteolysis) and portal (gating DNA entry and exit), both proteins are also important for capsid morphogenesis – the protease as a trigger for assembly of the capsid protein, and the portal for governing correct capsid geometry. These additional functions have not been reported for the phage HK97 capsid and may be a consequence of requirements for assembling a larger capsid from almost twice as many major head protein subunits (775) as HK97 (415). Given the well-developed structural basis provided by the simpler and more tractable HK97 system, our studies on T5 are revealing how additional complexity is integrated into this framework to expand capsid size and

genetic complement. Fig. 8 summarizes the results obtained in this paper and illustrates the complexity of T5 capsid assembly.

The maturation protease promotes capsid assembly

An important but poorly understood step of the capsid assembly pathway is incorporation of the soluble protease into the assembling procapsid despite its high toxicity for *E. coli* especially when overexpressed²⁸. Since the protease targets the α -domain, it likely binds this region and indeed this interaction has been demonstrated recently in the HK97 system^{32; 33}. We show here that the T5 protease does interact with the major capsid protein both *in vitro* – from purified components – and *in vivo* – by plasmid co-expression. Further, we visualize by EM the presence of density in the center of capsids and tubes that we ascribe to the T5 protease and which is not observed in absence of the protease protein or gene. Beside its proteolytic activity, the HK97 maturation protease has also been implicated in procapsid stability^{23,24}. Our work takes this a step further in demonstrating that the inactivated T5 maturation protease promotes capsid assembly in a concentration-dependent way. Unlike the HK97 protease, that of T5 is highly toxic to the host (²⁸ and Fig. 2C). We may suppose that this role in assembly serves to rapidly sequester newly-formed protease inside the capsid, protecting the host as well as corralling the protease to where its proteolytic function is subsequently employed. Another possibility could be that the protease is inactive when associated with the major head protein's α -domain, preventing toxicity to the host as well as premature cleavage of the α -domain while also promoting capsid assembly. On completion of procapsid assembly, the protease is rendered active whereupon it not only processes the accessible α -domains but also itself so that it is no longer lethal to the host on expulsion from the capsid as DNA is packaged.

If we assume that T5 capsids assemble from capsomers, as is thought to be the case for HK97, then there are two general ways to accomplish this – hierarchically or by accretion. For the first, capsomers could assemble into larger structures that correspond to a discrete portion of the completed capsid, and an appropriate number of these assemblies would join together to make the complete capsid. Alternatively, the capsomers could add individually to the growing edge of the shell until it is complete. By either scenario new capsomers must add to a growing structure, and as we suggest above the protease may promote this process by interacting with capsomers through their α -domains. However, we also observed that the particles formed by the major head protein and the maturation protease include both irregular shells and helical tubes, indicating that the protease promotes assembly but does not regulate geometry.

The portal regulates capsid shape

Our *in vivo* data (overexpression of plasmid in *E. coli*) show that the construct that includes the portal together with the decoration protein/inactivated protease/major head protein leads to the formation of proheads that are more regular in shape than without the portal. Similarly, with the active, wild-type protease, the overexpression yields closed particles of two sizes that resemble prohead II and expanded head, suggesting that our overexpression system mimics the capsid assembly that takes place during bacteriophage infection.

In contrast, purified portal did not appear to affect the capsid assembly process *in vitro* even though it adopts a ring conformation resembling that of the native portal complex. This is somewhat unexpected as portals are considered likely to nucleate assembly to ensure exactly one portal is incorporated per capsid¹⁵. Possible explanations for this absence of affect include: (i) capsid assembly *in vivo* may depend on location, such as is the case for phage T4 that assembles on a membrane¹⁹; (ii) purified portal may not be in the correct 12-mer conformation for interacting with the major head protein; (iii) an essential viral or host chaperone is absent from our *in vitro* system; or (iv) the N-terminal histidine tag abolishes interaction with the major head protein. While investigating this point is beyond the scope of our current work, we note that both the his7-10-mut11-8 and his7-10-11-8 constructions with His-tagged portal do not yield regular prohead particles suggesting that it may be the N-terminal His-tag that interferes with any nucleation function of the portal protein.

Prohead I structure

The T5 prohead I samples obtained in this work and previously²⁸ display internal density in both negatively-stained and cryo-EM images that likely corresponds to the γ -domain. We have now calculated low-resolution cryo-EM reconstructions of two prohead I-like particles: those obtained previously by Ca^{2+} treatment of purified pb8p (pI-8p), and from our new *in vivo* overexpression of the construction 7-10-mut11-8 (PI-7mut118). These structures represent the largest unproteolysed proheads for which a structure has been determined to date. Like the smaller procapsid of HK97, the hexamers of these two structures exhibit a skewed conformation that is “cured” to local 6-fold symmetry after capsid expansion. Also like HK97²⁴, the T5 prohead I density maps exhibit striking projections from the capsid interior surface that are directed toward the capsid center, which are compatible in length with the expected size of the T5 γ -domain.

Despite important morphological similarities between the pI-8p and PI-7mut118 particles, differences are clearly visible even at our modest resolution: the pI-8p particle is less angular, has a different pattern of projections under the capsomers, and lacks additional inner density. Interpreting this innermost pI-7mut118 density between radii from 60-125 Å is rather speculative given the resolutions achieved for the density maps. However, since we observed in our *in vitro* experiments that inclusion of mut-pb11 is correlated with the presence of internal density in PI-mutpb11, we suppose that the inner density near the center of the PI-7mut118 is likely due to mut-pb11. Unfortunately the relative instability of the PI-7mut118 prevents its extensive purification to estimate the pb11/capsid stoichiometry. The number of protease subunits incorporated into the HK97 prohead I is estimated to be between 60³⁴ and 120²⁴ (roughly one or two per hexamer) and assuming a similar ratio for T5 we would expect about 200 copies of pb11 in the T5 prohead I. If we further consider that the T5 maturation protease has a similar structure to that of herpes simplex virus (PDB id: 1AT3), which has an average diameter of about 20 Å, then we could fill the internal density observed in the reconstruction – the spherical volume between the radii 125 Å and 60 Å is about $8 \times 10^5 \text{ Å}^3$ – with 200 spheres of 20 Å diameter each. This internal density is thus compatible with extended γ -domains that bind about 200 copies of the maturation protease at their N-terminal end.

Despite the structural similarities between the capsids and capsid subunits of bacteriophages T5 and HK97, the assembly pathway of T5 exhibits differences that may be crucial for yielding a larger and more complex capsid. The major capsid protein of HK97 is sufficient by itself for generating capsids of the correct T=7 geometry²⁵²²³¹, but for T5 we find that the protease and portal proteins have significant roles for ensuring formation of regular T=13 particles. We have previously determined that the primary sequence of the T5 major head protein encodes the information governing capsid size although the end-point of assembly is more often tubular or irregular than icosahedral²⁸. We now understand that the fidelity of assembly is greatly improved by inclusion of the portal, but the specific mechanism that targets T=13 lattice geometry over other capsid sizes is not yet known. In the phage P22 system, for example, the scaffold protein regulates capsid size: absence of the scaffold protein yields a mixture of large, irregular particles as well as empty procapsids with T=4 and T=7 geometry³⁵¹². We suspect that the α -domain of the T5 major head protein may serve this role as one of its functions simply through steric constraints – the α -domains are too long to fit inside a capsid smaller than T=13.

In summary our data reveal that larger capsids require more regulatory elements in order to be correctly shaped, even when the primary sequence of the major head protein specifies the correct capsid geometry.

Materials and Methods

Cloning procedures

Twelve expression vectors were constructed as shown on table 1. The corresponding genes were cloned into the pCDF-2 Ek/LIC vector according to the ligation-independent cloning strategy from Novagen or into the pET-28b plasmid as indicated. The target genes were amplified from the DNA of phage T5 *st0* (GenBank accession number: AY692264) using the appropriate PCR primers and the Phusion High-Fidelity DNA Polymerase (Finnzymes). The PCR products were purified and inserted into the pCDF-2 Ek/LIC vector following the Novagen User Protocol TB163 or by digestion/ligation for pET-28b vector. Plasmids were amplified and purified after transformation into *XL1Blue Escherichia coli* cells (NovaBlue GigaSingles competent cells). All constructions were checked by DNA sequencing. For the 11-8, 7-10-11-8, mut11-8, 7-10-mut11-8, the his-tag was removed from the equivalent His-tagged construction using the strategy of Adareth and coworkers³⁶. Briefly, 4 primers were used, and a first round of PCR amplify the DNA sequences before and after the part of the sequence that code for the His-tag. The part that codes for the His-tag is not amplified. Two of the primers that have been used are phosphorylated in order to ligate by the T4 DNA ligase. This new ligated product can then be amplified by a new round of PCR that will give the cDNA without the His-tag ready to be cloned into the plasmid. For 7-10-mut11-8 and 7-10-11-8, we used the following oligos (from 5' to 3'): primer #1, GGCAGCTTCCACAGCAATGGC; primer #2 (phosphorylated)CTTGTCGTCGTCATCCACGTGA; primer #3 (phosphorylated) ATGGGTTTTAAAGCTGGATTA; primer #4 GTGCTTATATCCATGAATGGA. For mut11-8 and 11-8, we used: primer #1, GGCAGCTTCCACAGCAATGGC; primer #2

(phosphorylated) CTTGTCGTCGTCATCCACGTGA; primer #3 (phosphorylated) ATGACACAAGCTGCTATTGACT; primer #4 GCAACCCAAGTAGCTTTACCAG.

Expression and purification of the proteins and the particles

E. coli BL21(DE3) cells harboring the expression plasmids were grown at 37° C in 750 mL Luria-Bertani (LB) Broth medium supplemented with 50 µg/mL streptomycin until the culture reached an absorbance of 0.5 at 600 nm. Expression of the proteins was then induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the cells were further grown for 3 h, harvested by centrifugation (6000 g, 30 min at room temperature) and stored at -20°C. The frozen cells (40 mL) were suspended in 20 mM Tris, pH 8.0, broken by two passages in a French press (20000 psi) at 4°C, centrifuged at 4°C (10000 g, 30 min) and the supernatant was recovered.

His-tagged proteins were purified by loading the supernatant onto a 5 mL HisTrap™ FF column pre-equilibrated in 20 mM Tris, pH 8.0 connected to an AKTA purifying system (GE Healthcare). The column was extensively washed with the loading buffer and the protein eluted with a 0–1 M imidazole gradient. The purified His-pb8p and the inactive His-pb11 were dialyzed against 20 mM Tris, pH 8.0. The purified pb7 protein aggregates in low salt and adjusting the eluate at 500 mM NaCl solubilizes the protein, suggesting that the low-salt aggregation/polymerization of His-pb7 is reversible. The purity of the proteins was checked by SDS-PAGE followed by staining with Coomassie blue. Protein concentrations were determined by measuring the absorbance at 280 nm and using the theoretical extinction coefficients of 31860, 32110 and 27180 M⁻¹cm⁻¹ for pb8p, pb7 and pb11, respectively.

If the particles/protein had no His-tag, the supernatant was precipitated by treatment with 7.5% PEG and 0.5 M NaCl overnight at 4°C. The pellets were suspended in 10 mM Tris, pH 7.6, layered on top of a 10–40% glycerol step gradient and centrifuged at 75500 g, 2 h, in a SW41 rotor (Beckman). The light-scattering band was extracted and analyzed on a native agarose gel, SDS-PAGE and by electron microscopy (negative stain and cryo-EM). For the constructions 11-8 and 7-10-11-8, an additional step of purification by anion exchange chromatography through a HiTrapQ HP column (GE Healthcare) was done. SDS-PAGE was used to follow the purification and to check the purity.

In vitro assembly reaction

Purified pb8p, pb7 and pb11 were used at concentrations indicated in the Figures in a tris 10mM pH 8.0, NaCl 100 mM buffer with or without 20 mM calcium at room temperature. Samples were then analyzed by native agarose gel: samples were mixed with 10% glycerol and 0.005% bromophenol blue (final concentrations) and separated by horizontal electrophoresis in 1% (w/v) agarose gels. Runs were carried out in TA buffer (Tris-base 40mM, 20 mM acetic acid pH 8.3) in a 6 cm long gel and at 4 V/cm for 90 min. Gels were stained overnight with the Bio-Safe Coomassie blue G-250 solution (Bio-Rad). The impact of pb11 concentration on pb8p assembly was measured by monitoring turbidity at 400 nm (nanodrop ND-1000).

Anti-pb7 antibody and Western blot

Rabbit polyclonal antiserum raised against purified portal was produced according to a standard procedure³⁷. Those antibodies were then used to check for the presence of pb7 in the purified particles made by the plasmid 7-10-11-8 by western blot. After SDS-PAGE, the proteins were transferred on a PVDF (polyvinylidene fluoride) membrane that was then incubated in TBST-milk buffer (10mM tris pH 8.0, 100mM NaCl, 0.5 % tween 20, 4% non-fat milk) for an hour, incubated one more hour with the anti-serum (dilution 1/100) at room temperature, washed in TBST-milk and incubated with the horseradish peroxidase-conjugated anti-rabbit antibody (dilution 1/10000) for 1 hour. The Membrane was washed with TBS (no tween, no milk) and the peroxidase activity was detected by the Amersham ECL system (GE healthcare life sciences).

Negative-stain electron microscopy (EM)

Samples were deposited onto freshly glow-discharged carbon-coated copper EM grids, washed with water, stained for 30 seconds with either 1% (w/v) uranyl acetate (UAc) or 2% (w/v) phosphotungstic acid (PTA), air dried and observed in an FEI Tecnai T12 microscope (FEI, Hillsboro OR) with an LaB₆ tip operating at 120 kV and equipped with a Gatan UltraScan 1000 CCD camera (Gatan, Pleasanton CA). 2D-averages of the portal images were calculated from the UAc-stained pb7 sample using the refine2d.py program included in EMAN package³⁸. 100 particles were used for averaging.

Cryo-EM and Image reconstruction of prohead I

3 µl of purified capsid sample were pipetted onto a Quantifoil R2/1 holey carbon EM grid (Quantifoil Micro Tools GmbH, Jena, Germany), blotted, and plunge frozen into a 50:50 mix of liquid ethane-propane³⁹ using an FEI Vitrobot Mark III. The frozen grid was mounted on a Gatan 626 cryoholder and images were collected on an FEI Tecnai TF20 microscope operating at 200 kV with a Gatan UltraScan 4000 CCD camera. Particles were selected using x3d software⁴⁰ and defocus/astigmatism values were estimated manually using BSoft⁴¹. The icosahedrally averaged density map of each capsid was calculated using AUTO3DEM⁴². Final resolutions were estimated by the FSC = 0.5 criterion. The density maps were then analyzed using UCSF Chimera⁴³.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

kbp	kilo-base pairs
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dsDNA	double-stranded DNA
cryo-EM	cryo-electron microscopy

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Highlights

- The major capsid protein of T5 alone does not form regular prohead I
- The maturation protease triggers assembly of the capsid protein
- The portal helps to regulate the shape of the capsid
- T5 capsid assembly is more complex than HK97, requiring more proteins

Figure 1. Scheme of T5 assembly

(A) Gene organization of the capsid proteins. The precursor form of the major head protein, pb8p, includes a scaffolding α -domain (residues 1 to 159; represented as a white triangle) and a mature domain, pb8m (residues 160 to 458; represented as a black trapezoid). (B) Pb8p is co-assembled with the dodecameric portal complex (pb7) to form prohead I. The head maturation protease (pb11) cleaves the α -domain, and the 10 N-terminal residues of pb7, yielding prohead II. *In vivo*, DNA encapsidation through the portal triggers capsid expansion and the binding of the decoration protein (pb10) to the capsid exterior. (C) The T5 prohead II and head structure calculated from cryo-EM data displays icosahedral T=13 symmetry, as shown previously²⁶²⁷. Penton is indicated by P and the two symmetrically unrelated kinds of hexon are indicated by H1 and H2, respectively. Scale bar = 20 nm.

Figure 2. T5 capsid protein constructs used in this work

(A) Schematic description of the constructs: pb7, pb8, 11-8, 7-10-11-8, as well as 11-8 and 7-10-11-8 with mutated protease (mut11-8 and 7-10-mut11-8 respectively). Each of the six has a His-tag variant for a total of 12 different constructions. (B) Multiple alignment of the T5 protease, pb11, with the U35 family of maturation proteases of bacteriophages P12026 (YP_006560244.1), ST64B (NP_700378.1) and HK97 (NP_037700.1) and identification of the putative residues involved in the catalytic domain (black arrowheads). This identification is based on alignment and a computational analysis of phage proteases²⁹ (C) Coomassie-

stained SDS-PAGE analysis of protein in the supernatant of the *E. coli* lysate after expression of the genes present on the plasmid indicated. The low level of protein expression for 11-8 and 7-10-11-8 is due to the high toxicity of the wild-type maturation protease. (D) Purified mutated pb11 protease and pb7 portal analyzed by Coomassie stained-SDS-PAGE (top left) and negatively stained (uranyl acetate) EM imaging of purified pb7. Inset, bottom right: 2D average of the isolated pb7 portals showing that the purified portal exhibits a ring shape of the dimension expected for such a particle. Scale bar = 20 nm.

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Figure 3. *In vitro* analysis of the roles of the pb7 portal and mutated pb11 protease in pb8p capsid assembly

(A) Native agarose gel of the mixture of proteins indicated above each lane. All the experiments were done in 20mM Tris pH 8.0 and 100 mM NaCl. 20 mM of Ca²⁺ was added where indicated. The samples were kept 24h at room temperature before being loaded on the gel. (*) indicates that for lane 2 and 6 the mutpb11 concentration was 10 μM. The arrowhead shows a discreet band that could correspond to prohead I (B) Negatively stained (uranyl acetate) EM images of the samples loaded in lanes (4), (8) and (9) of panel (A). EM of lane (4) shows tubes with a thin shell and an inner white density attributed to mutpb11. EM of lane (8) shows that in presence of NaCl and Ca²⁺, pb8p polymerizes mainly as capsomers and few prohead I-like particles or tubes are found. EM of lane (9) shows that mutpb11 promotes pb8p assembly into prohead I-like particles and tubes. Furthermore a white density is visible inside the capsids and tubes (white arrowheads, figure and insets) that is not seen in the absence of mutpb11.

Figure 4. Concentration effects of mutpb11 on pb8p assembly

(A) Measure of light scattering (400 nm) of pb8p (10 μM) in the presence of various concentrations of mutpb11 (as indicated) after 3 hours at room temperature followed by centrifugation at 14000g to remove tubes. (B) SDS-PAGE of the same samples after 24h at room temperature followed by centrifugation at 14000g. (C) and (D) CryoEM images of pb8p at 10 μM in the presence of 2 μM of pb11 in 20 mM tris pH 8.0, 100 mM NaCl and 20 mM CA^{2+} after 3 hours (C) and 24 h (D). Scale bar = 100 nm.

Figure 5. Analysis of the role of the protease and the portal on pb8p assembly *in vivo*. (A) Negatively stained (phosphotungstic acid) EM images of the purified (PEG/NaCl precipitation, glycerol gradient and anion exchange chromatography) particles produced by overexpression of the constructs 11-8 and 7-10-11-8. Scale bar = 100 nm. Inset, SDS-PAGE, Western blot and anti-pb7 staining of the purified particles, showing that pb7 is present in the particles. The two lanes are for two different concentrations of the sample. (B) Glycerol gradient (10 to 40%) of the supernatant of the bacterial extract obtained after expression of the genes from the indicated constructs. Pb7 and pb8p show slight scattering at the bottom of the tube while mut11-8 exhibits intense scattering all along the tube with a progressive increase from the top the bottom. 7-10-mut11-8 shows a main discrete band in the lower part

of the tube which is also seen if the supernatant is kept 24h at room temperature before being layered on the gradient. (C) Native agarose gel, SDS-PAGE and western blot (probed with anti-pb7) of each fraction of the 7-10-mut11-8 gradient. The major head protein is mostly found at both the beginning of the glycerol gradient and at the level of the discrete band. The portal is found at the same locations suggesting that they interact together. Fraction 6 and the fractions at the top of the gradient exhibit the same band on native agarose gel (similar to the one observed for capsomer) which suggests that the fraction 6 particle is dissociated in the native agarose condition.

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Figure 6. EM-imaging of the fraction 6 of different glycerol gradients

(A) Fraction 6 of pb8p (left) and mut11-8 (right) imaged by negative stain EM (uranyl acetate). Scale bar = 200 nm. (B) Fraction 6 of 7-10-mut11-8, 7-10-11-8, and (C) his7-10-mut11-8 and his7-10-11-8, respectively, imaged by cryoEM. Scale bar = 50 nm.

Figure 7. Structural analysis of the prohead I-like particles

(A) Surface views of reconstructions from cryoEM data viewed along the 2-folds axis. From top to bottom inside view, exterior view and cross section of prohead I from 7-10-mut11-8 construct (left) and prohead I obtained by Ca^{2+} treatment of the purified pb8p (right). The triangle drawn on the center panel defines one facet of the icosahedron. P, H1 and H2 stand for penton, hexon1 and hexon2 respectively. 3 hexons and 1 penton are marked with black and white markers respectively, the proheads are $T=13$. On the cross-section are indicated the positions of the points of view used to see the organization of the α -domain inside the capsid on (C), where the views are limited to the slabs between the yellow lines. (B) Top, Average density of prohead I as a function of radius for PI-7mut118 (dashed line) and PI-8P (continuous line). The black bar indicates the length of the α -domain model. Bottom, model of the T5 α -domain made by threading using the Phyre server ⁴⁴. The colors indicate the putative independent domains. (C) View of the organization of the α -domain under the penton for PHI-7mut118 (top) and PHI-8p (bottom) observed as shown in (A, bottom).

Figure 8. Summary of the results obtained in this work

Correctly shaped prohead I, prohead II and expanded empty head have been obtained only when the portal and the protease (mutated or wild type) were present in the expression system.

Table 1**Plasmids used in this study**

The expressed proteins and the particles obtained from these plasmids are indicated together with a reference to the figures where the plasmids were used.

Plasmid name	vector	expressed proteins	particles obtained	figures
7	<i>pET-28b</i>	pb7	not purified	5B
his7	<i>pCDF-2 Ek/LIC</i>	his-pb7	pure pb7	2C; 3; 7
8	<i>pET-28b</i>	pb8p	tubes	5B
his8	<i>pCDF-2 Ek/LIC</i>	his-pb8p	pure pb8p	3A–B; 4
11-8	<i>pCDF-2 Ek/LIC</i>	pb11; pb8p	irregular structure	5A
his11-8	<i>pCDF-2 Ek/LIC</i>	his-pb11; pb8p	not analyzed	
mut11-8	<i>pCDF-2 Ek/LIC</i>	mutpb11; pb8p	tubes	5B; 6A
hismut11-8	<i>pCDF-2 Ek/LIC</i>	hismutpb11; pb8p	pure pb11	2C; 3; 4
7-10-11-8	<i>pCDF-2 Ek/LIC</i>	pb7; pb10; pb11; pb8p	prohead II, expanded head	5A; 6B
his7-10-11-8	<i>pCDF-2 Ek/LIC</i>	his-pb7; pb10; pb11; pb8p	irregular spherical structure	6C
7-10-mut11-8	<i>pCDF-2 Ek/LIC</i>	pb7; pb10; mutpb11; pb8p	prohead I	5B–C; 6B; 7
his7-10-mut11-8	<i>pCDF-2 Ek/LIC</i>	his-pb7; pb10; mutpb11; pb8p	tubes	6C