In Vitro Assembly of the T=13 Procapsid of Bacteriophage T5 with Its Scaffolding Domain

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The Siphoviridae coliphage T5 differs from other members of this family by the size of its genome (121 kbp) and by its large icosahedral capsid (90 nm), which is organized with T=13 geometry. T5 does not encode a separate scaffolding protein, but its head protein, pb8, contains a 159-residue aminoterminal scaffolding domain (Δ domain) that is the mature capsid. We have deciphered the early events of T5 shell assembly starting from purified pb8 with its Δ domain (pb8Δ). The self assembly of pb8Δ is regulated by salt conditions and leads to structures with distinct morphologies. Expanded tubes are formed in the presence of NaCl, whereas Ca2+ promotes the association of pb8Δ into contracted tubes and procapsids. Procapsids display an angular organization and 20-nm-long internal radial structures identified as the Δ domain. The T5 head maturation protease phb11 specifically cleaves the Δ domain of contracted and expanded tubes. Ca2+ is not required for proteolytic activity but for the organization of the Δ domain. Taken together, these data indicate that pb8Δ carries all of the information in its primary sequence to assemble in vitro without the requirement of the portal and accessory proteins. Furthermore, Ca2+ plays a key role in introducing the conformational diversity that permits the formation of a stable procapsid. Phage T5 is the first example of a viral capsid consisting of quasi-equivalent hexamers and pentamers whose assembly can be carried out in vitro, starting from the major head protein with its scaffolding domain, and whose endpoint is an icosahedral T=13 particle.

The self assembly of the icosahedral capsid of double-stranded DNA (dsDNA) viruses is a highly regulated unidirectional process that proceeds by steps, starting with the association of the full-length capsid protein subunits into a precursor capsid form. Several maturation steps follow (proteolytic cleavage, conformational reorganization of capsid subunits, and stabilization processes) that take place before, during, or after the packaging of the viral genome (34). Finally, the mature capsid has to withstand the high internal pressure imposed by the densely packed genome as well as environmental stresses after exit from the bacterial host. Capsid assembly thus relies on highly regulated protein-protein interactions that transition from the dynamic association and maturation phases to the stable and robust endpoint.

The architectural principles that govern icosahedral capsids comprising several hundred identical protein subunits were formulated by Caspar and Klug (2), who introduced the concept of quasi-equivalence. Accordingly, capsid proteins have enough flexibility in shape and binding interfaces to assemble into hexamers (hexons) or pentamers (pentons) and to form hexon-penton and hexon-hexon contacts that are nearly equivalent to each other. In addition, assembly is tightly controlled to yield a single size in most cases, with the precise geometry of the capsid being described by the lattice triangulation number T. Viruses have developed various strategies to control the proper assembly of their capsid. The T4 bacteriophage (26) and human adenoviruses (14), for example, use distinct proteins to form hexons and pentons, while others, including herpesviruses and phage λ, use the same protein. Capsid assembly may rely on scaffolding proteins that subsequently are expelled from the capsid during maturation and DNA encapsidation (6, 15). If the scaffolding protein is mutated or absent, incorrect structures are formed, which can be open tubes (T4) or smaller particles (P22, φ29, herpesvirus) (1, 3, 10, 27, 28, 35). Capsid assembly also may rely only on the major head protein, as shown in the case of the lambdoid coliphage HK97 (T=7). Its major head protein, gp5, contains a mature domain and a 102-residue amino-terminal Δ domain that is cleaved after initial assembly by a viral protease and thus is considered to play the role of the scaffolding protein in other phages (5, 7). Furthermore, in vitro and in vivo studies have shown that preformed pentamers and hexamers of uncleaved gp5 accurately assemble into T=7 icosahedral shells without the requirement of the portal protein (5, 7, 9, 36). Gp5 thus carries by itself all the information needed to assemble into procapsids.

The Siphoviridae coliphage T5 differs from other members of this family, like phage λ or HK97, by the large size of its dsDNA genome, 121,750 bp (GenBank accession numbers AY587007, AY692264, and AY543070). Similarly to HK97, the T5 genome does not encode a scaffolding protein. The structure of the T5 icosahedral mature capsid was solved by cryoelectron microscopy (cryo-EM) and image reconstruction revealing the first capsid of a wild-type single-layered virus to have the triangulation number T=13 (11) (Fig. 1). The 90-nm-diameter capsid shell consists of the major head protein pb8 (39), whose subunits form 11 pentons located at the vertices and 120 hexons located at the faces. The hexons are decorated with portal and accessory proteins. Furthermore, Ca2+ plays a key role in introducing the conformational diversity that permits the formation of a stable procapsid.
by an accessory protein, pb10 (11, 32). The 12th vertex is occupied by the connector, which comprises a dodecamer ring of the portal protein pb7. pb8 is a 50-kDa protein with two domains in its full-length form (called pb8p). pb8p undergoes proteolytic cleavage during morphogenesis, leaving a 32-kDa mature protein (pb8m) in the viral particle (38). N-terminal sequencing indicated that the severed region of pb8m corresponds to the 159 aminoterminal residues (11). This N-terminal extension, called the H9004 domain by analogy with the HK97 major head protein, is predicted to have a high H9251-helical content mostly organized as a coiled-coil formation, and it may function as a scaffold domain (11). The mechanism by which the H9004 domain is cleaved is unknown but likely involves pb11, a head maturation protease identified on the genome and located upstream of the gene encoding pb8 (GenBank accession number AY692264). A schematic of the T5 head assembly pathway is presented in Fig. 1. Compared to that of HK97, the T5 capsid exhibits a higher level of complexity. Several constraints must be fulfilled to assemble its T13 shell. A unique protein must associate into pentons occupying the vertices and into two kinds of hexons, one interacting both with pentons and hexons and the other exclusively with hexons. This raises the question of whether a T13 procapsid can be assembled in vitro with the unique contribution of the major head protein carrying both structural and scaffold functions. To address these questions, we have expressed and purified pb8 and searched for physicochemical conditions allowing its assembly. We demonstrate that the precursor form of pb8 with its H9004 domain (pb8p) assembles in vitro into diverse structures, including expanded and contracted tubes, and that the conformational flexibility introduced in pb8p by Ca2+ allows it to assemble into procapsids with internal radial structures corresponding to the H9004 domain.

MATERIALS AND METHODS

Cloning procedures. Five overexpression vectors were constructed to produce different forms of pb8 either alone or with the adjacent protease pb11, as represented schematically in Fig. 1C. The corresponding genes were cloned into the pCDF-2 Ek/LIC vector according to the ligation-independent cloning strategy from Novagen into the pET-28b plasmid as indicated. The target genes were amplified from the DNA of phage T5st0 (GenBank accession number AY692264) using the appropriate PCR primers and Phusion high-fidelity DNA polymerase (Finnzymes). The PCR products were purified and inserted into the pCDF-2 Ek/LIC plasmid. Details of the purification procedures are given in Materials and Methods. pCDF refers to the pCDF-2 Ek/LIC plasmid.

FIG. 1. Bacteriophage T5 head assembly and characteristics of pb8 constructs. (A) Schematic of the head assembly pathway. The precursor form of the major head protein, pb8p, includes a scaffolding Δ domain (residues 1 to 159; represented as a white triangle) and a mature domain, pb8m (residues 160 to 458; represented as a gray rectangle). Pb8p is coassembled with the dodecameric portal complex (pb7) to form prohead I. The head maturation protease (pb11) cleaves the Δ domain, 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. In the present work, prohead I (or procapsid I) refers to the portalless assembly, and prohead II (or procapsid II) refers to the purified particles isolated from a T5stAmN5 mutant. (B) The T5 head structure calculated from cryo-EM data displays icosahedral T13 symmetry, as shown previously (11). Vertices are indicated by the number 5 in the schematic at the top, and the T13 pattern of hexamer positions is indicated in the schematic by the number 6. Scale bar = 20 nm. (C) Characteristics of pb8 constructs. The schematics at the left represent pb8p with its mature domain (in gray) and scaffolding domain (in white). The position of the His tag is indicated by H6. The fifth construct encompasses both pb8p and the protease pb11 (in black). Details of the purification procedures are given in Materials and Methods. pCDF refers to the pCDF-2 Ek/LIC plasmid.

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reached an absorbance of 0.5 at 600 nm. The overexpression of His₆-pb8p then was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were further grown for 3 h, harvested by centrifugation (6,000 × g, 30 min at room temperature), and stored at −20°C. The frozen cells (40 ml) were suspended in 10 mM Tris, pH 7.6, broken by two passages in a French press (20,000 lb/in²) at room temperature, and centrifuged at 4°C (10,000 × g, 30 min), and the supernatant was recovered. The pb8p protein carrying the N-terminal His₆ tag (termed pb8p*) was purified by loading the supernatant onto a 5-ml HisTrap FF column preequilibrated in 10 mM Tris, pH 7.6, that was connected to an AKTA purifying system (GE Healthcare). The column was extensively washed with the loading buffer, and pb8p* was eluted with a 0 to 1 M imidazole gradient and dialyzed against 10 mM Tris, pH 7.6. To determine whether the His₆ tag modified its biochemical properties, pb8p also was purified with and without the His₆ tag by the precipitation of the French press supernatants with 7.5% polyethylene glycol (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 to 40% glycerol step gradient in the same buffer, and centrifuged at 75,500 × g for 2 h in an SW41 rotor (Beckman). The purity of the proteins was checked by SDS-PAGE, followed by staining with Coomassie blue. The pb8p concentration was determined by measuring the absorbance at 280 nm and using the theoretical extinction coefficient of 31,860 M⁻¹ cm⁻¹.

Size-exclusion chromatography of pb8p*.

Purified pb8p* (1 g/liter) was run through a Superose 6 (HR10/30) size-exclusion column (GE Healthcare) that previously was equilibrated in 10 mM Tris, pH 7.6. Chromatography was performed at room temperature at a flow rate of 0.5 ml/min. Reference proteins used for calibration were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and bovine serum albumin (BSA) (67 kDa). Dextran Blue 2000 and NaCl were used for the determination of the column void volume and total volume, respectively.

Analysis of pb8p structures by native agarose gel electrophoresis.

Samples were mixed with 10% glycerol and 0.005% bromophenol blue (final concentrations), and pb8p assemblies were separated by horizontal electrophoresis in 1% (wt/vol) agarose gels. Runs were carried out in TA buffer (40 mM Tris base, 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).

Negative-stain EM and cryo-EM.

For negative-stain electron microscopy (EM), samples were deposited onto freshly glow-discharged carbon-coated copper electron microscopy grids, washed with water, stained for 30 s with either 1% (wt/vol) uranyl acetate (UAc) or 2% (wt/vol) phosphotungstic acid (PTA), air dried, and observed with either a Philips EM208 microscope operating at 80 kV and equipped with an AMT charge-coupled-device (CCD) camera or an FEI Tecnai T12 microscope with an LaB₆ tip operating at 120 kV and equipped with a Gatan UltraScan 1000 CCD camera. For cryo-EM, drops of samples (4 μl) were applied to holey carbon film-bearing grids, blotted, vitrified, and imaged by a Tecnai T12 microscope with an LaB₆ tip operating at 120 kV and equipped with an AMT charge-coupled-device (CCD) camera or an FEI Tecnai T20 FEG microscope with an LaB₆ tip operating at 200 kV and equipped with a Gatan 626 cryoholder and Gatan UltraScan 4000 CCD camera. Two-dimensional (2D) averages of the capsomere images were calculated from UAc-stained pb8p* using the defla2dpy program included in the EMAN package (24). One hundred particles were used for averaging.

Proteolytic activity of pb11.

E. coli BL21(DE3) cells harboring the pCDF-pb11 plasmid were grown at 37°C in 750 ml LB medium, collected, and broken in a French press cell as described above for pb8p. The supernatant obtained after centrifugation of the broken cells contained the pb8-pb11 extract that was used for the determination of the proteolytic activities. To prepare this extract, 1 ml of the supernatant was mixed with 1 g of pure glycerol and stored at −20°C. Proteolytic activities were determined by mixing 1 μl of this extract with 20 μl of purified pb8p* (0.3 g/liter in 10 mM Tris, pH 7.6). Samples were incubated overnight at room temperature. Proteolytic activity was assessed by SDS-PAGE.

Preparation of procapsid II.

E. coli F cells were grown at 37°C in 750 ml LB medium to an absorbance of 0.25 at 600 nm. They then were infected at a multiplicity of infection of 5 by phage T5stAmN5, an amber mutant derived from T5st(0) that produces procapsids devoid of the scaffolding domain but containing the portal protein (further called procapsid II) (39). After lysis was completed, the suspension was clarified by centrifugation (6,000 × g, 30 min at 4°C), and procapsid II particles were centrifuged with 7.5% PEG and 0.5 M NaCl overnight at 4°C. The pellet was suspended in 4 ml phage buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂), layered on top of a 10 to 40% glycerol step gradient in the same buffer, and centrifuged at 75,500 × g for 2 h in an SW41 rotor. The fractions containing procapsid II particles were identified by the presence of the major head protein pb8 in SDS-PAGE. They were purified by anion-exchange chromatography through a HiTrapQ HP column (GE Healthcare) and eluted with a 0 to 1 M gradient of NaCl. Their purity was controlled by SDS-PAGE.

RESULTS

Sequence analysis of the pb8 gene product.

pb8p consists of an N-terminal region (residues 1 to 159; the Δ domain) and of a domain (residues 160 to 458) corresponding to the mature protein pb8m (Fig. 2A). We attempted to establish a structural model of both domains using the PHREY server that is based on predicted secondary structure matching (20). Our previous cryo-EM study established the consistency between pb8m and gp5, the HK97 major capsid protein (11). Although both proteins share less than 20% amino acid identity, we could model pb8m using the gp5 crystallographic structure (PDB entry 1OHG) as a template (17) (Fig. 2B). This allowed the determination of the location in pb8m of the putative flexible N arm (residues 174 to 189) and E loop (residues 209 to 236) (Fig. 2A). The pb8 Δ domain, which has a high probability of coiled-coil organization (11), was modeled using the coiled-coil domain of tropomyosin (PDB entry 1C1G) as a template. Its maximum length was estimated to be 20 nm, which is significantly longer than the gp5 Δ domain from HK97 (see Fig. SA1 in the supplemental material). As yet, however, not enough information is available to define its quaternary organization.

Purification and characterization of pb8p.

We expressed the genes encoding pb8m and pb8p in E. coli. Figure 1C summarizes the characteristics of the constructs. Pb8m was produced in a partially soluble form that tended to polymerize with time. EM observations showed the presence of some tube-like structures, but the heterogeneity of the preparation prevented their
are compatible with those of capsomeres from the T5 capsid. Scale top-view particles are shown enlarged in the inset. Their dimensions... of pentons/hexons. Band-passed, filtered EM images (Fig. 3C) showed that pb8p* formed an almost-uniform population of particles that accumulate negative stain at their center and with ring-shaped morphology. The 2D averaging of the images indicated a mean diameter of 10 nm, which is compatible with that of capsomeres in the T5 capsid (11). So far, the resolution was not high enough to permit their reconstruction, so that we could not conclude whether these capsomeres, further termed [pb8p]n, were pentons or hexons. Capsomeres most likely did not arise from the dissociation of tubes or procapsids, since the latter structures were never observed in the bacterial lysate producing pb8p.

We further asked whether [pb8p] could assemble into larger structures and procapsids. Since capsid association generally is governed by electrostatic interactions as demonstrated for other viruses (19, 23), we investigated the effect of monovalent (NaCl) and divalent (CaCl2) salts on the assembly of [pb8p]n.

NaCl promotes the association of [pb8p] into expanded tubes. We first followed the migration of [pb8p] in a native agarose gel after incubation for various periods of time (3 and 24 h) at room temperature in 10 mM Tris, pH 7.6, containing 100 mM NaCl (Fig. 4A). In the absence of added NaCl, [pb8p]n migrated as a unique diffuse band at the bottom of the gel. Incubation with 100 mM NaCl for 3 h resulted in the appearance of a faint and slower-migrating band. Its intensity increased with time and its distance of migration decreased, suggesting a continuous polymerization process that was favored by the salt. The level of polymerization became higher and higher with time, and after 1 week the material stayed in the well. Negative-stain and cryo-EM images of this material showed that it corresponded to thin-shelled and open tubes (Fig. 4B). Although their lengths were variable, their diameter (54 nm) remained constant over time, and they all displayed a 2-nm-thick shell. These tubes originated only from the self association of uncleaved pb8p*, as they migrated in SDS-PAGE at an MM of ~50 kDa (see Fig. 7A). These tubes will be further named expanded tubes.

Ca2+ promotes the association of [pb8p] in contracted tubes and procapsids with internal radial structures consistently with that of the Δ domain. The addition of 40 mM Ca2+ to [pb8p]n in 10 mM Tris, pH 7.6, triggered an increase in turbidity within a few minutes at room temperature. Light-scattering measurements indicated that the kinetics was at least of the second order: a first rapid increase was observed in less than 5 min, followed by a second, slower one that lasted hours (data not shown). EM images of UAc-stained samples
(see Fig. SA2 in the supplemental material) showed that open and closed procapsid-like structures and tubes were formed within the first 15 min. Twelve hours later the number of procapsids had decreased by a factor of two, but the remaining ones were closed. Concomitantly, we observed an elongation of the tubes, suggesting that their formation represented an off-pathway assembly reaction. This distribution remained almost constant over time.

\( [\text{pb8p*}]_n \) samples that were incubated for 24 h with \( \text{Ca}^{2+} \) were further analyzed by native agarose gel electrophoresis (Fig. 4C). The diffuse band characteristic of \( [\text{pb8p*}]_n \) was not detectable any more, in agreement with the fact that no capsomers were visible in EM. Highly polymerized material remained in the well, and a new discrete band appeared. They were tentatively attributed to tubes and procapsid-like structures, respectively (Fig. 4C). A similar electrophoresis pattern was observed with the untagged protein, thus ruling out that the target of \( \text{Ca}^{2+} \) was the His tag (data not shown). A high concentration of \( [\text{pb8p*}]_n \) (10 g/liter, 200 \( \mu \)M) was required to observe the discrete band. Notably, this is also the concentration that is reached in infected cells producing \( \sim 100 \) phages/bacteria. This new band also was observed at a lower \( [\text{pb8p*}]_n \), concentration (40 \( \mu \)M) (Fig. 4C), provided that the samples were incubated with PEG 6000 (4\%), which is known to facilitate protein-protein interactions by increasing the local protein concentration (12).

The \( \text{Ca}^{2+} \)-treated samples were centrifuged, and the pellet and supernatant were observed by EM (Fig. 5 and 6). The pellet contained open tubes with morphology different from that of the expanded tubes observed after NaCl treatment. Their diameter was smaller (44 nm; that of the expanded tubes was 54 nm), and UAc-stained samples displayed an \( \sim 5 \)-nm-thick shell and a thin and unstained line along the central axis (Fig. 5A). This central line, as well as internal structures oriented perpendicularly to the tubes, also were visible on the cryo-EM images (Fig. 5A). Capsomers were visible after PTA staining (Fig. 5B). Those tubes were termed contracted tubes. Interestingly, when these tubes were further incubated with 25 mM EDTA to chelate \( \text{Ca}^{2+} \), they underwent an immediate transition to an expanded morphology characterized by a larger diameter (Fig. 5C) resembling that observed after the incubation of \( [\text{pb8p*}]_n \) with 100 mM NaCl (Fig. 4B).

Samples from the supernatant yielded 70-nm-large procapsid structures (called procapsid I) with a polyhedral appearance (Fig. 6A). Capsomers were visible after PTA staining (Fig. 6B). UAc-stained procapsid I particles displayed a 5-nm-thick external shell and a highly stained central region (Fig. 6C). Cryo-EM images revealed 20-nm-long internal radial structures oriented perpendicularly to the shell (Fig. 6D). These cryo-EM images were compared to those of procapsid II (Fig. 6E). Both procapsids exhibited an \( \sim 5 \)-nm-thick shell and a diameter of 70 nm. Furthermore, a preliminary density map of negative-stain EM images indicated that procapsid II exhibits icosahedral geometry with triangulation number \( T=13 \) (see Fig. SA3 in the supplemental material). Given the morphological similarities of procapsids I and II and the known \( T=13 \) geometry of the phage capsid (11), it is likely that procapsid I also displays \( T=13 \) geometry. Importantly, procapsid II, contrarily to procapsid I, did not display any internal structure. Since procapsid II has undergone the proteolytic cleavage of the scaffolding domain, the internal structure seen in procapsid I most likely corresponds to scaffolding domains. The incubation of procapsid I with 25 mM EDTA resulted in a collapse of the capsid structure into capsomers that were identifiable by negative-stain EM (data not shown).

**Effect of the pb11 head protease on the different \( [\text{pb8p*}]_n \) assemblies.** The experiments described above highlight the critical role of \( \text{Ca}^{2+} \) in triggering conformational changes in \( [\text{pb8p*}]_n \). Since proteolytic cleavage is the next step in capsid maturation, we further assessed the effect of the capsid pro-
FIG. 5. Ca\(^{2+}\) promotes the formation of contracted tubes that expand after EDTA treatment. [pb8p\(^{+}\)]\(_{n}\) was incubated for 24 h in 10 mM Tris, pH 7.6, 40 mM CaCl\(_2\) and centrifuged at 16,000 \(\times\) g for 10 min. EM images were taken from the material recovered in the pellet. This material corresponds to the fraction remaining in the agarose well shown in Fig. 4C. (A) On the left are UAc-stained tubes displaying different regions of density. In the middle is an enlarged view of a contracted tube with considerable internal density within the tube walls (white arrows). On the right is a cryo-EM image of the same sample with the tube walls indicated by black arrows (contrast is reversed compared to that of negative-stain EM), and strong central density is connected to the walls by perpendicular arms. The slightly smaller diameter of tubes in negative-stain EM likely is due to shrinkage by dehydration. (B) Capsomeres are visible on the tubes when imaged with PTA staining. Two examples are indicated with black arrows, one of which is also marked with a dashed black circle. (C) After further treatment with 25 mM EDTA, contracted tubes appear expanded, as visualized by PTA staining. A portion of the tube shown in panel B has been superimposed to aid the comparison of the sizes. Scale bars are 100 nm for the images on the left in A and B (black scale bar) and 25 nm for enlarged views (white scale bars).

FIG. 6. Ca\(^{2+}\) promotes the formation of procapsid I displaying inner radial structures corresponding to the \(\Delta\) domain. EM images of [pb8p\(^{+}\)]\(_{n}\), incubated for 24 h in 10 mM Tris, pH 7.6, 40 mM CaCl\(_2\) and centrifuged at 16,000 \(\times\) g for 10 min. The sample corresponds to the supernatant and to the faint band seen in the agarose gel (Fig. 4C). (A) UAc-stained procapsid I. Scale bar = 100 nm. Enlarged views of PTA-stained (B) and UAc-stained (C) particles. Procapsid I displays an angular organization and a thick external shell, and capsomeres are distinguishable on the UAc-stained image (dashed circles on the duplicate image at right in panel B). (D) By cryo-EM, procapsid I particles reveal the presence of 20-nm-long radial structures that are internal and perpendicular to the shell (black arrow). (E) In comparison, a cryo-EM image of a purified procapsid II does not display internal structure. Scale bar = 25 nm (B to E).

Ca\(^{2+}\) on [pb8p\(^{+}\)]\(_{n}\). Attempts to purify pb11 expressed alone failed due to its high toxicity toward \(E.\) coli cells. However, we could isolate a bacterial lysate from \(E.\) coli cells co-expressing pb8p-pb11 that exhibited specific proteolytic activity against pb8p (see Fig. SA4 in the supplemental material). The overnight incubation of capsomeres with this lysate resulted in the proteolysis of the 50-kDa band and the appearance in SDS-PAGE of a unique band at an MM of \(\sim 27\) kDa, lower than expected for the mature protein (31 kDa) (Fig. 7A). On the other hand, when the lysate was added to [pb8p\(^{+}\)]\(_{n}\), together with 40 mM Ca\(^{2+}\), a major band was found at the MM expected for pb8m (Fig. 7A), and no other bands appeared below that. Comparable patterns of proteolysis were observed with untagged [pb8p\(^{+}\)]\(_{n}\) (data not shown). The N-terminal sequencing of the 31-kDa band revealed a cleavage at position 159 and, thus, that the \(\Delta\) domain was fully proteolyzed. In contrast, the 27-kDa band resulted from cleavage at residue 237, showing that both the \(\Delta\) domain and the amino acids encompassing the modeled N arm and E loop (Fig. 2) were removed. The cleaved \(\Delta\) domain was not detectable in SDS-PAGE, probably because the polypeptide underwent further proteolytic cleavage. The overnight proteolysis of NaCl-induced expanded tubes resulted in the appearance of up to five bands in SDS-PAGE corresponding to cleavage sites located above the pb8m band (Fig. 7A). Their location was not modified by the addition of 40 mM Ca\(^{2+}\), thus indicating that Ca\(^{2+}\) had not affected the specificity of the protease activity. On the whole, these experiments suggest that the protease senses different pb8p conformations. This proposal is in agreement with previous studies on phage HK97 that suggest that the correct cleavage of gp5 depends primarily on the configuration of the polypeptide chain around the cleavage site (8).

Samples were further examined by EM. [pb8p\(^{+}\)]\(_{n}\) first was incubated for 24 h with 40 mM Ca\(^{2+}\) and then submitted to overnight proteolysis by pb11 (Fig. 7B). As expected, the PTA-stained nonproteolyzed samples mostly consisted of contracted tubes with a few procapsids, but once they were proteolyzed and lost their \(\Delta\) domain, the contracted tubes displayed a morphology resembling that of expanded tubes, thus demonstrating that the \(\Delta\) domain is required to maintain the contracted structure. In contrast, procapsids displayed the same ultrastructure before and after treatment with the protease. The most likely interpretation is that the cleavage site is located within the capsid and is not accessible to the external protease. Finally, the proteolyzed capsomeres were no longer detectable by EM, suggesting that they were dissociated.
FIG. 7. Effect of the head protease pb11 on the different \( [pb8p^{*}]_n \) assemblies. (A) At the left is the proteolytic cleavage of \( [pb8p^{*}]_n \) by pb11 as characterized by SDS-PAGE. Samples (0.3 g/liter in 10 mM Tris, pH 7.6, alone or containing 40 mM Ca\(^{2+}\)) were incubated overnight at room temperature with a cytoplasmic extract of E. coli BL21 expressing pb11 (see the supplemental material). Mass markers are indicated on the left. The lysate concentration is low enough so that it is not detectable on the gel. At the right is the proteolysis under conditions similar to those for preformed NaCl-induced expanded tubes. The migration of mature pb8 (pb8\(_m\)) is indicated by an arrow. The lysate concentration is low enough so that it is not detectable on the gel. At the right is the proteolysis under conditions similar to those for preformed NaCl-induced expanded tubes. The migration of mature pb8 (pb8\(_m\)) is indicated by an arrow. (B) EM images of PTA-stained contracted tubes and procapsids formed when incubated for 24 h with 40 mM Ca\(^{2+}\) and then submitted to an overnight proteolysis by pb11. Contracted tubes are converted into expanded tubes, whereas procapsids appear unchanged. The scale bar is 25 nm.

DISCUSSION

The icosahedral capsid of phage T5 exhibits a high level of complexity. To assemble into a T=13 shell, its major capsid protein, pb8, must associate into pentons and hexons that must sense different environments so that hexons can interact with both pentons and neighboring hexons while avoiding the formation of nonproductive hexamer sheets or tubes. Furthermore, since pb8 carries its own scaffolding domain (\( \Delta \) domain), its assembly into shells of a specific geometry must rely on subtle local conformational changes. In this work, we found that rather simple physicochemical conditions permit the self-assembly of pb8 into different structures, including a T=13 procapsid.

Neither mature pb8 (pb8\(_m\)) nor its precursor form (pb8p) spontaneously associates into procapsids during the overexpression/purification process. However, when purified pb8 proteins alone or carrying an N-terminal His\(_6\) tag are incubated at low ionic strength (10 mM Tris, pH 7.6), they form an almost-uniform population of ring-shaped structures whose morphology and diameter (10 nm) correspond to those of capsomeres of purified T5 capsids. However, we failed to determine whether these capsomeres-like structures, called \( [pb8p]_n \), originate from hexons, pentons, or both. In the presence of monovalent ions (100 mM NaCl), \( [pb8p]_n \) capsomeres autoassociate into open tubes of various lengths but have an almost-constant diameter (54 nm) and a thin shell. These tubes are qualified as expanded, considering their diameter and their thin shell as well as by comparison to the morphology of tubes (polyheads) made of gp23, the major head protein of phage T4 (1, 21). Very few angular or closed structures are observed, suggesting that the tubes are formed by hexons exclusive of pentons, similarly to the tubes observed in other viral shell assemblies, such as SPO1 (29) and T4 (1, 21). It should be noted that although these structures may not be relevant to the in vivo assembly situation, tubes made from the uncleaved form of pb8 have been observed in vivo upon the treatment of T5-infected E. coli cells with canavanine (38).

Divalent cations (up to 40 mM Ca\(^{2+}\)) cause significant changes of the \( [pb8p]_n \) conformation that result in the formation of contracted tubes and procapsid I particles. The morpholgy of the contracted tubes is significantly different from that of expanded tubes: their shell is thicker, and capsomeres are clearly visible. Procapsid I particles also have a thick shell and display an angular structure. This is a strong indication that Ca\(^{2+}\) has introduced enough flexibility into the capsomeres, permitting their association into hexons and pentons, or into their interfaces to allow curvature consistent with that of a closed icosahedral shell. Alternatively, Ca\(^{2+}\) might somehow allow pentons to form so that hexons and pentons would be in equilibrium with each other, thus permitting the formation of procapsids.

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The high concentration of protein (10 g/liter, 200 \( \mu \)M) and the long time (24 h) that is required to generate procapsids is consistent with the usual conditions for in vitro assembly (30, 36). Further, in silico models show that capsid assembly is characterized by a slow nucleation phase and needs a nucleus for stabilization (13, 31, 33, 37) and further assembly (30). The propensity of pb8p to form tubes and the low yield of procapsid formation suggest that hexons are more stable than pentons, as shown for HK97 (36). It should be stressed that the conditions that permit the formation of procapsids in vitro are far from mimicking those encountered in bacteria, where Ca\(^{2+}\) is extruded from the cytosol by an active transport mechanism (25).

The structures of contracted tubes and procapsid I are differently affected by the further addition of 25 mM EDTA to chelate Ca\(^{2+}\): tubes immediately recover an expanded morphology, whereas procapsids collapse into capsomeres. Since procapsids are formed of both pentons and hexons whereas the tubes contain only hexons, it may be that Ca\(^{2+}\) is required to sustain the interface between pentons and hexons but not between hexons.

Importantly, internal radial structures oriented perpendicularly to the shell are visible inside procapsid I and contracted tubes. Their length (20 nm) is compatible with that of the \( \Delta \) domain structure predicted in silico. These structures are not visible in expanded tubes, although the protein still carries its \( \Delta \) domain. The \( \Delta \) domain thus may adopt at least two conformations: a disordered one in expanded tubes and a more ordered/rigid one triggered by Ca\(^{2+}\) in contracted tubes and in procapsid I. A similar disordering of the HK97 \( \Delta \) domain of hexons but not pentons has been observed in heated prohead I, which also returns to the starting state on cooling (4).

The difference in the proteolytic cleavage of capsomeres, tubes, and procapsids further highlights the accessibility of the pb8 domains to the head protease. Pb11 uniquely cleaves the \( \Delta \)
domain of expanded and contracted tubes, whereas it also cleaves the predicted N arm and E loop located downstream of the \( \text{H9004} \) domain of pb8 in capsomeres. This indicates that the building of hexon-hexon interactions in tubes results in major conformational changes in pb8 that prevent the N arm and E loop from being susceptible to the protease.

Putative \( \text{Ca}^{2+} \) binding sites in the \( \text{H9004} \) domain were searched for with the PHYRE server (20) and by the calculation of the electrostatic potential on the basis of the structural model using the PME implementation of the VMD software (18). Figure SA1 (in the supplemental material) shows that the \( \text{H9004} \) domain is mainly electronegative, with two particularly strong electronegative sites that could be candidates for calcium binding.

Major molecular reorganizations take place in pb8p besides those affecting the \( \Delta \) domain. Indeed, the thickness of the shell of contracted and expanded tubes is different (5 and 2 nm, respectively). The pb8m atomic structure is not known, but it likely shares a common fold with gp5 from HK97 (11). The structures of gp5 have been solved before (16) and after the expansion (17) of the procapsids. Gp5 is somewhat flexible, undergoing twisting and bending during this process, and it is likely that similar molecular reorganizations take place in pb8, as schematically represented in Fig. 8. These reorganizations are clearly regulated by \( \text{Ca}^{2+} \), which may play the role of molecular switch, as observed in other viral capsid assembly mechanisms (19, 22). The mechanism by which \( \text{Ca}^{2+} \) controls the assembly of thick-shelled structures (procapsid I and contracted tubes) could be that its binding to the \( \Delta \) domain helps to pull apart the mature domain of pb8p. As a consequence, the capsomeres could shift from a planar conformation to a bent and twisted one, thereby generating the conformational diversity required for the assembly of pentamers and hexamers into a \( T_{13} \) lattice.

The pb8 domains contribute differently to the interactions between subunits. The C-terminal domain is likely to be important for correct folding, since a C-terminal His tag renders pb8p insoluble. The \( \text{H9004} \) domain is required, together with \( \text{Ca}^{2+} \), to permit the conformational changes that lead to the formation of contracted tubes and procapsids, although it may not be necessary for the self association of pb8, since recombinant pb8m tends to polymerize into tubes, albeit at a low yield. Finally, the N arm and E loop are critical for intercapsomeric association, since their removal by proteolysis results in the inhibition of capsomere association. A summary of the different assembly states of pb8p is presented in Fig. 8.

An important feature of the \textit{in vitro} assembly process is that procapsid I is of a unique size (70 nm in diameter). Furthermore, this size perfectly matches that of procapsid II purified from a T5 amber mutant that is devoid of the scaffolding domain. We thus propose that the elongated size of the scaffolding domain and the fact that it occupies almost all of the internal shell volume is a steric factor that contributes to regulate the minimum size of the capsid (at least \textit{in vitro}).

This study has revealed that the T5 uncleaved major head protein carries all the information in its primary sequence to assemble \textit{in vitro} into a \( T_{13} \) lattice without the requirement of the portal protein, the protease, or accessory proteins. The
role that these proteins play in the regulation of the assembly, expansion, and stabilization of the capsid presently is under investigation.

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