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Structure and Energetics of Encapsidated DNA in Bacteriophage HK97 Studied by Scanning Calorimetry and Cryo-electron Microscopy

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Abstract

Encapsidation of duplex DNA by bacteriophages represents an extreme case of genome condensation, reaching near-crystalline concentrations of DNA. The HK97 system is well suited to study this phenomenon in view of detailed knowledge of its capsid structure. To characterize the interactions involved, we combined calorimetry with cryo-EM and native gel electrophoresis. We found that, as in other phages, HK97 DNA is organized in coaxially wound nested shells. When scanned in buffer containing 1mM [Mg⁺⁺], filled capsids exhibit a complex thermal profile between 82° and 96°, to which DNA melting and capsid denaturation both contribute. In the absence of (unbound) [Mg⁺⁺], DNA melting shifts to lower temperatures and the two events are resolved. Filled capsids release their DNA at temperatures well below the onset of DNA melting or capsid denaturation. On heating, the internal pressure increases, causing the DNA to exit – probably, via the portal vertex; the capsid, although largely intact, sustains local damage that leads to an earlier onset of thermal denaturation. Filled capsids differ structurally from empty capsids in the curvature of their protein shell, a change attributable to outwards pressure exerted by the DNA. We propose that this transition is sensed by the portal which is embedded in the capsid wall, whereupon the portal's structure and its interactions with terminase, the packaging enzyme, are altered, thus signaling that packaging is at or approaching completion.

Keywords

three-dimensional image reconstruction; DNA condensation; head-full packaging; conformational changes; mechanosensor

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Introduction

The assembly pathways of tailed bacteriophages afford a rich source of insight into the mechanisms that govern the synthesis and activation of macromolecular complexes in general (reviews; 1;2;3). There are separate convergent pathways for the head and tail. Despite considerable diversification in detail, the basic features of head assembly are widely conserved, including: scaffold-assisted assembly of an icosahedral procapsid with a dodecameric channel for DNA (portal) at one vertex; the core fold of the capsid protein; packaging of DNA by the viral terminase, an ATPase-*cum*-DNA translocase-*cum*-endonuclease; and maturation – a sequence of conformational changes that stabilizes the nascent nucleocapsid. These highly distinctive features recur in the assembly of herpesviruses 4, suggesting a common evolutionary origin 5;6;7. In contrast, phage tails are entirely different from herpesvirus membranes, reflecting the respective adaptation of these viruses to infection via the bacterial cell envelope or via the plasma membrane of eukaryotic cells.

Thus, tailed phages first build a receptacle for DNA – the procapsid – then fill it from the replicating concatemer. Packaging is a rapid dynamic process (up to 300 bp/sec; 2 bp packaged per ATP hydrolyzed 8). Many details remain unclear but two broad classes of mechanism are distinguished: site-specific packaging whereby the terminase recognizes a DNA sequence that signals for the terminating cleavage; and a mechanism whereby packaging arrests and the terminating cleavage takes place when a “head-full” condition is detected. In head-full packaging, the capsid is thought to function as a mechanosensor that somehow activates the endonuclease, detaches the terminase, and facilitates downstream events.

Encapsidated DNA of phages (and herpesviruses) is compressed to remarkably high concentrations, comparable to those of crystalline DNA 9. As to its arrangement, a growing body of cryo-EM observations of phages with isometric heads indicates that their DNA is coiled in coaxial shells around the portal axis, with a progressively lower degree of order towards the capsid interior 10;11;12;13;14. Early in packaging, the DNA is less ordered but gradually assumes a highly ordered arrangement, particularly in the outer layers 15. The interplay of physical forces involved in successive stages of packaging has received much recent attention, both theoretical and experimental (e.g. 16;17;18), but a consensus picture has yet to emerge. The principal interactions involved are electrostatic repulsion between segments of duplex DNA and between the DNA and the capsid; the bending of the DNA; and protein-protein interactions in the capsid shell.

Differential scanning calorimetry provides a valuable approach to investigate the energetics of complex macromolecular systems 19. As a sample is heated, thermal transitions are recorded at characteristic temperatures that represent the disruption of particular sets of interactions; moreover, the energy (enthalpy) exchanges associated with each transition may be measured quantitatively. By performing electron microscopy of appropriately heated samples, it is possible to correlate structural and thermodynamic data. In previous work, we applied this approach to the lambdoid *E. coli* phage HK97 which provides an attractive system for assembly studies because its pathway (Fig. 1) – which includes covalent crosslinking of neighboring capsid protein subunits as a stabilization device - and underlying genetics have been well worked out 20;21. Moreover, structural information is available on multiple conformational states of its capsid 22;23;24; in particular, the interior surface is negatively charged and therefore DNA-repelling 22. In this way, we investigated procapsid assembly 25;26 and maturation of the protein shell 27. Here we have extended this approach to focus on DNA-DNA and DNA-protein interactions in filled HK97 heads and complete tailed virions.

Results

Cryo-electron microscopy of tailed and tail-less nucleocapsids

We examined intact HK97 virions and tail-less DNA-filled capsids (“heads”) by cryo-EM (Figs. 2a, b). The latter particles were isolated from non-permissive *E. coli* cells infected with the amber mutant amE1, which is defective in tail assembly. When virions are confined to thin films of buffer, they assume orientations such that the whole particle, including the tail, remains hydrated i.e. with its long axis in the plane of the film: as such, they present side-views of the nucleocapsids, so-defined relative to the portal axis. In cryo-electron micrographs, their DNA presents partially ordered arrays of punctate densities with center-to-center spacings of ~ 25 Å (Figs. 2a, c), each density point representing a near-axial projection of a DNA duplex.

Not being so constrained, tail-less heads present a wider range of views. However, these particles tend to orient such that they present views along a three-fold axis in which two opposing facets are parallel to the air-water interface of the thin film of buffer, and the capsids have hexagonal profiles. In this property, they resemble capsids of herpes simplex virus (28; N.C., unpublished observations) but differ from bacteriophage T7, whose tail-less heads tend to orient with their portal vertex (which is apparently hydrophobic) at the air-water interface, so that they present axial views with near-circular profiles 10. Despite this predilection for three-fold views, some HK97 heads are found in near-axial orientations in which their DNA presents the distinctive “fingerprint” pattern of concentric rings 10— Fig. 2d. Taken together, these observations of side-views and axial views indicate that HK97 DNA is packed in the same “coaxial spool” fashion as has been observed in other isometric phage heads (see Introduction).

Three-dimensional reconstructions in which icosahedral symmetry was imposed were performed for both filled and empty capsids (Fig. 3). The latter particles constituted a small subpopulation in preparations of filled capsids and thus were observed under identical imaging conditions. Both reconstructions depict a thin-walled (~ 18 Å) polyhedral protein shell. These shells are similar to each other and to the crystal structure of Head II 29, when the latter is limited to the same resolution (data not shown). However, on close inspection, it is apparent that the shell of the empty capsid bows out more than that of the filled capsid, whose facets are almost perfectly planar (cf. Figs. 3c and 3f). This difference in curvature may be appreciated with reference to the dashed line in Figures 3c and 3f.

Inside the DNA-filled capsid, the reconstruction shows seven concentric shells spaced at radial intervals of 25 Å, surrounding an inner region that is not empty but does not show the same stratification (Figs. 3B, C). We infer that these shells were generated by applying icosahedral symmetry to a set of projections of a coiled spool with the same spacing (25 Å) between successive layers (see Fig. 9 of reference 2) and that the innermost region ($r < 90$ Å or so) is occupied by less ordered DNA.

Thermal scanning of HK97 heads at different magnesium concentrations

A DSC scan of DNA-filled HK97 heads in our “standard calorimetry buffer” containing 1 mM Mg^{++} (SCB-Mg) is shown in Fig. 4a. (SCB was our initial choice of buffer, to facilitate comparisons with earlier calorimetric studies on other HK97 capsids 25;26;27). A single jagged heat absorption profile extending from $\sim 80^{\circ}C$ to almost $100^{\circ}C$, and peaking at a T_p (peak temperature) of $90^{\circ}C$ is observed. We infer that it has contributions both from the melting of DNA and from thermal transitions of the capsid protein. For comparison, a scan of empty portal-less capsids (Head II) in SCB is reproduced 27 in Fig. 4d; it has a sharp denaturation endotherm with a T_p of 96° .

Aiming to separate the respective contributions of the DNA and protein to the thermal profile, we performed scans at lower ionic strength in the absence of Mg^{++} , which conditions should shift the DNA melt to lower temperature 30. The result of such an experiment is shown in Fig. 4b. It shows two well resolved endotherms with peaks at 85.5° and 93°, respectively. We infer that the upper peak represents denaturation of the capsid. Its position matches that of the upstream shoulder seen on the SCB-Mg scan (Fig. 4a). Consistent with this interpretation, the main event in the Mg-free scan (Fig. 4b) has no such shoulder.

Conversely, we infer that the lower temperature part of the SCB-Mg scan (Fig. 4a), i.e. from ~90°C down, is due almost entirely to melting of the HK97 DNA, as is the major event between 78°C and 90°C in the Mg-free scan (Fig. 4b). To confirm that the latter thermal event represents melting of the DNA, we recorded an ultraviolet melting curve for HK97 DNA in SCB-Mg buffer (Fig. 5). Denaturation took place between 82° and 93°, with a T_m of 88.3°. In a second scan recorded after cooling the Mg-free specimen for two hours, the thermal signature of the DNA - including its fine structure - was reproduced (Fig. 4c). It follows that the melting of the phage DNA is reversible whereas denaturation of the capsid – as with other forms of the HK97 capsid - is irreversible.

The total energy associated with the composite thermal event recorded for HK97 heads in SCB-Mg in Fig. 4a was measured from the area under the curve to be 20.0 mcal. We can attempt to account for this result quantitatively if we apply the previously determined denaturational enthalpy of Head II 27 to these capsids, and we can estimate the energetic contribution from melting the DNA. The details of this calculation are given in the Methods section; according to it, denaturation of the capsid protein contributed 4.9 mcal and melting of the DNA, 15.4 mcal. Thus DNA-filled heads denature with an enthalpy change that is, within experimental error, the sum of the denaturation enthalpies expected for isolated DNA and the capsid, with no detectable contribution from interactions between DNA and the capsid. The enthalpy change associated with DNA melting is approximately three times higher than that associated with capsid denaturation. (For reference, the genome mass is ~ 24 MDa, and the capsid mass is ~ 13 MDa).

In summary, we conclude the following:

- i. In both buffers, the lower-temperature portions of the thermograms are dominated by melting of the genome. As expected, its melting temperature is sensitive to which buffer was used, being 5° lower in Mg-free low-salt buffer.
- ii. The 93° event in the Mg-free scan and the shoulder at this temperature in the scan in SCB-Mg represent thermal denaturation of the capsid. Thus the T_p of this event is unaffected by the choice of buffer, but is ~ 3° lower than that of Head II.

Melting profile of the HK97 genome

The thermal profile of HK97 DNA is complex, exhibiting seven features (peaks and shoulders), with the highest peak at 85.5°C (Figs. 4b, c). Experiments performed using two other buffers exhibited the same fine structure but were offset by a few degrees (data not shown). The observed complexity arises from the melting of domains with differing GC:AT ratios and lengths. (GC-rich regions have a higher T_m than AT-rich regions, having three rather than two H-bonds per base pair). In an analysis of phage λ DNA by high resolution optical melting curves, 32 such sub-transitions were detected 31. Based in part on this work, a computer program (MELTSIM) was written that predicts the melting curve of a given DNA sequence 32. We applied this program to the 40-kilobase sequence 33 of the HK97 genome for comparison with the thermograms. In order to compensate for the thermal response time of the calorimeter, we convolved the calculated curve (Fig. 6a) with Gaussian functions of various widths – e.g. Figs. 6b, 6c. The curve obtained with a sigma of 0.33°C gave an excellent match

to the experimental curves (cf. Figs. 6c and 6d). We attribute the minor offsets in temperature to the fast scan rate (120°/hr), which was approximately ten times faster than the rate required for thermally perturbed λ DNA to equilibrate 31 (the model calculation simulates an idealized, arbitrarily slow, melt), and to differences in ionic conditions.

Cryo-EM of filled capsids heated to 80°C

As described above, cryo-micrographs of filled HK97 capsids exhibit strong signals at spacings of $\sim 25\text{\AA}$, which is the typical center-to-center distance between DNA duplexes in locally ordered bundles. We sought to ascertain how the appearance of the DNA might change in nucleocapsids that had been incubated at 80° in low-Mg buffer, under which conditions the DNA should melt but the capsid should remain intact. The result of such an experiment is shown in Figure 7. The capsids do indeed appear to be intact but they are mostly empty, although a few contain small amounts of filamentous material – presumably residual DNA (e.g. arrowed particle in Fig. 7a). This observation indicates that DNA exits from the capsid at temperatures lower than 80° and melts when free in solution. This scenario readily accounts for the indistinguishable thermal signatures of the DNA when free and when scanned in the form of heads (filled capsids).

Genomes are released from HK97 nucleocapsids heated above 50°C

We further investigated the temperature dependence of genome release by exploiting the property that filled heads and free DNA have different mobilities in native gel electrophoresis. Thus, heads were incubated in Mg-free low-salt buffer at various temperatures in 10° increments, then cooled and treated with DNase, and subjected to electrophoresis (Fig. 8). Release of DNA should register as a decrease in intensity of the filled capsids' band in the gel. (Virions were also included in this experiment to address the possibility that tail addition may affect the stability of the head). The results (Fig. 8) show that there is significant depletion of the filled head band above 50°, and hardly any of these particles remain in the 70° sample. It follows that DNA is released from HK97 heads at temperatures significantly lower than those associated with DNA melting or thermal denaturation of the capsid. Virions appeared to be slightly more sensitive to heating than tail-less heads and the surviving virions exhibit increased electrophoretic mobility, perhaps indicating that tail proteins are damaged by heating at these temperatures.

In order to determine whether a population of heads responds uniformly to heat treatment, we performed cryo-EM on samples that had been incubated at 50°, 70° and 80° for 10 min. This experiment was performed with virions in order to gain insight into the possible mode of egress: if the lesion that leads to loss of DNA were to be gross perturbation of the portal vertex, then the virion would be likely to lose its tail, which should be evident in micrographs of virions whereas such perturbation would be less evident for tail-less heads. We found that that almost all heads in each sample were either full or empty. Already with the 50° sample, almost 20% of virions had lost their DNA. In the 70° and 80° samples, almost all were empty, but a few percent remained packaged (Fig. 9). We also examined a time course, heating samples at 65° for various times between 1 min and 20 min (Fig. 7b). Most were already voided after 2 min incubation, but a few remained full after 20 min. These observations imply that, for any given particle, after a certain threshold temperature – which is no higher than 50°C – is reached, DNA release may initiate stochastically, and then proceeds rapidly. At higher temperatures, this initiation event comes sooner. We observed almost no loss of tails until the 80° sample in the first experiment or the 20-min time point in the 65° incubation, indicating that exit of DNA precedes loss of tail. Interestingly, the tails of heat-voided virions are visibly hollow compared with those of filled virions (cf. Figs. 9c and 9d), indicating that they have axial lumens that are initially occupied by some material – possibly DNA – that is expelled by the heat treatment.

Discussion

In this study, we used calorimetry, cryo-EM, and native gel electrophoresis to probe the interactions that stabilize HK97 nucleocapsids. Thermal scans of capsids record major endotherms that represent thermal denaturations, and minor endotherms that represent thermally induced phase transitions of the capsid. Both kinds of events are distinctive for a given conformation of the capsid's protein lattice. All HK97 capsids studied to date 25;27 remain intact until the onset of the denaturation endotherm. It appears that dissociation of the capsid and denaturation of its subunits are coupled processes: after a certain threshold temperature is passed, any given particle sustains, stochastically, an initial local lesion, with damage spreading out from that site where subunits are no longer fully surrounded by stabilizing inter-subunit interactions. Here our goals were two-fold: to investigate the effect of encapsidated DNA on the thermal stability of the protein shell and *vice versa*.

Pressure of packaged DNA augmented by elevated temperature, not heating *per se*, poses the major physical challenge to retention of DNA in the HK97 nucleocapsid

All five structural states of the maturing HK97 capsid, from Prohead 1 through Head – see Fig. 1 – are quite thermostable, having relatively high T_p 's that range from 82° (Prohead I) to 96° (Head II). Cross-linking, which starts at the third (Expansion Intermediate) stage, confers stability that is crucial in that, in the absence of crosslinking, no infectious phage particles are produced 27. We tentatively assigned this loss of infectivity to mounting pressure exerted by encapsidated DNA that causes the capsids to release their DNA. The imputed breakdown occurs *in vivo* at 37° – much lower than the T_p of any capsid. In the present study, we found that when filled cross-linked HK97 capsids are incubated *in vitro* at elevated temperatures in the absence of Mg^{++} , they release their DNA at 50 - 60° – also much lower than their capsids' T_p of 93°. It appears, therefore, that breakdown and loss of DNA is caused not by thermal denaturation of the capsid, but by an increase in internal pressure caused by heating the packaged DNA.

In this context, we may enquire as to the location of the weak spot(s) where rupture might occur. We envisage two candidates: (1) the portal, being perturbed so that it is no longer able to confine the viral DNA; (2) as cross-linking never goes to 100% completion (RLD – unpublished results), there should be a few local sites that are not cross-linked where a lesion might occur. The cryo-EM data documenting the release of DNA from HK97 virions (Fig. 9) tend to favor the former possibility. Observations of a λ variant that is sensitive to chelating agents and dependent on Mg^{++} for stability 34 support the idea that its capsid is under duress from packaged DNA and Mg^{++} acts as a counter-ion to mitigate this effect.

Not with a bang but a whimper

As DNA packaging proceeds, it slows down as counter-pressure builds up 35. Contributions to the cumulative potential energy stored in a packaging particle derive from electrostatic repulsion between adjacent duplexes, electrostatic repulsion between the DNA and capsid inner surface 22, and bending of the DNA 36. With this consideration in mind, we entertained the possibility that our calorimetric scans of filled HK97 capsids might exhibit an exothermic event, corresponding to liberation of the encapsidated DNA into the surrounding buffer. However, no such event was observed (Figs. 4a, 4b). Accordingly, we suspect that the exit of the DNA from a given nucleocapsid involves a gradual seepage rather than a single explosive release; moreover, this event is not synchronized over the population as a whole.

Differences in the thermal profiles of filled and empty mature capsids

The position of the 93° peak exhibited by filled capsids in Mg-free buffer (Fig. 4b), which represents thermal denaturation of the protein shell, matches that of the shoulder in

thermograms recorded with SCB-Mg (Fig. 4a). Thus, unlike the DNA melt, capsid denaturation is insensitive to Mg^{2+} concentration. This T_p is significantly lower, by 3°C, than the 96° of Head II. We can imagine two possible sources for this discrepancy. First, Head II lacks a portal, unlike the filled heads of the present study. If the portal were to be dislodged at a lower temperature (see above), a vacancy would be created at that vertex from which thermal denaturation could more readily proceed. We see no thermal event that might correspond to denaturation of the portal although it should, in principle, be marginally detectable, as the mass of a portal dodecamer is ~ 5% of that of the gp5* subunits that make up the rest of the capsid shell. Probably, it is submerged in the much larger thermal signal from the melting DNA and denaturing capsid protein.

To conclude, the main calorimetric difference between the protein shells of filled capsids and empty Head II is the 3° reduction in T_p : this shift may result from either (i) an irreversible conformational change in the gp5* lattice, stemming from the capsid's having been packaged; or (ii) a local lesion in the capsid, caused either by outwards pressure of the DNA or from dislodging of the portal, that leads to earlier onset of thermal denaturation of the capsid.

On the thermodynamics of DNA condensation

DNA forms ordered structures within phage heads as shown in Figure 3 for HK97, or it may be condensed by multivalent cations into hexagonal arrays with comparable separation (~ 25 Å) between duplexes. The forces involved in DNA condensation have been reviewed 37. DNA bending, mixing entropy, Coulombic interactions, and hydration forces were each found to make small contributions, resulting in a free energy of condensation of between 0.01 kT and 0.1 kT per base pair. The favorable entropic ($T\Delta S$) and opposing enthalpic (ΔH) contributions appear to be about ten times as large. Direct measurement by the osmotic stress technique 38 gave a value of ΔH between 0.3 and 0.6 kT/bp for condensing DNA into hexagonal arrays with 25 Å inter-axial spacing. It follows that the energies involved in ordering DNA are miniscule compared with the ΔH of 15 kT/bp associated with DNA melting and that electrostatic interactions in phage heads are calorimetrically undetectable on account of the relatively small enthalpy changes associated with these processes 39.

Structural differences between filled and empty mature capsids

The only distinction that we detected between filled and empty capsids was in the more bowed appearance of the empty capsid shell (Fig. 3). This difference is reminiscent of prior observations. In the final stage of maturation, the capsid changes in shape from spherical to icosahedral 40; concomitantly, the final crosslinks, involving the pentameric gp5* subunits, form 41. However, there appear to be additional, approximately isoenergetic, states between these two extremes; for instance, a crystal structure of Head I – a capsid that is nominally mature but unable to crosslink because one of the cross-linking residues is mutated – captured a conformation no longer spherical but not fully icosahedral 41. The empty capsids imaged in this study (Fig. 3b) may represent another such state. As the filled and empty capsids were imaged in the same micrographs, the scaling of the two density maps is consistent. When the maps are overlaid, the pentamers superimpose and, at its center (the icosahedral 3-fold axis), the wall of the empty capsids extends out ~ 8 Å further than the flat wall of the filled capsid. We infer that as DNA packaging advances and internal pressure increases, the capsid is coerced into its fully mature conformational state.

A mechanism for signaling the termination of DNA packaging transmitted via the capsid wall

The internal organization of P22 nucleocapsids has been characterized by “asymmetric” reconstruction of cryo-micrographs 12. These authors observed density, which they interpreted as DNA, encircling the inwards-protruding portion of the portal and proposed a “pressure valve” mechanism whereby this DNA coil might pinch the portal, inducing a conformational

change that halts packaging - a necessary step for P22 which packages by a head-full mechanism 42. The P22 portal has long been thought to play a decisive role in determining when the head is sufficiently filled because certain mutations in its gene were found to lead to over-packaging of DNA (by ~ 4.5%) 43. Similar mutations have been found in the portal protein of phage SPP1 44, which also employs a head-full mechanism. Little is yet known about the details of HK97 DNA packaging, but it belongs to a class of lambdoid phages that cleave their chromosome ends at specific sites 45. Thus, HK97's packaging mechanism is likely to be more like lambda's than P22's. While lambda's terminating cleavage is nominally invoked by the terminase recognizing a specific sequence, there appears to be a headfull-like mechanism superimposed on this mechanism, as it has been observed that when the genome length is shortened below 80% of the normal size, cleavage efficiency drops dramatically 46. Whether this effect is due to some type of head-full sensing is not known, but we can imagine how a head-full sensor might work. Noting the difference in structure between filled and empty HK97 capsids, we posit that the portal senses the accompanying transition in the surrounding hexamers of capsid protein. Thus outwards pressure of the DNA elicits a change in the capsid wall that is transmitted to the portal which responds by undergoing a conformational change; this change alters its interactions with the terminase which is thereby switched into a state that promotes the final cleavage, separating the packaged genome from the concatemer.

We note that small but functionally influential structural differences have also been detected between filled and empty capsids of herpes simplex virus 47. Here, packaging-induced changes in the otherwise mature capsid promote the binding of heterodimeric proteins that appear to signal that the nucleocapsid is ready to exit the nucleus of the infected cell – an event with no counterpart in bacteria, despite the overall similarities in capsid assembly and DNA packaging between herpesviruses and tailed phages. Based on the foregoing discussion, it is plausible that the observed changes may also be coupled to the termination of DNA packaging.

Methods

Preparation of filled capsids

Tail-less DNA-filled Heads were made using an amber mutant, *amE1* in gene H 33;48. *E. coli* strain MM594 (non-suppressor) was grown with vigorous aeration to a density of 4×10^8 cells/ml in 1 L Luria Broth plus 0.4% maltose and infected with 10 phage per cell. Two hours later, chloroform was added to 0.15% (v/v) to lyse the cells. After lysis was complete, all remaining steps were done at 4°C. The lysate was clarified by low speed centrifugation and the capsids were precipitated by adding NaCl (to 0.5 M) and PEG 8000 (to 15% w/v). After overnight storage the precipitated particles were collected by centrifugation and resuspended in phage resuspension buffer (PRB, 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 150mM NaCl, 1 mM dithiothreitol). Residual cell debris was removed by low speed centrifugation and the Heads pelleted in a Beckman 45Ti rotor at 20,000 rpm for 1 hr after filling the tubes with PRB buffer. Supernatants were discarded and the pellets overlaid with 2 ml PRB buffer and left to resuspend overnight. Residual cell debris was removed by low speed centrifugation and the Heads (3 × 3.5 ml) were purified by velocity centrifugation in sucrose gradients. 10 to 40% (w/v) gradients (35.5 ml) in PRB were made using a BioComp Gradient Master and run in a Beckman SW-28 rotor at 28,000 rpm for 45 min. The fastest-sedimenting band contained Heads and was extracted from the gradient tubes and concentrated by centrifugation at 30,000 rpm for 20 min in a Beckman Type 80Ti rotor. The final pellets were resuspended in a small volume of PRB and stored until use, at which time samples were dialyzed into the appropriate buffer.

Phages were grown as described above, except that the amber-suppressing host Ymel was used. After PEG precipitation, virions were purified on equilibrium buoyant density CsCl gradients (16 hr at 38,000 rpm in a Beckman Type 70.1Ti rotor at 16°C), starting with an average density

of 1.5 g/ml. Following centrifugation, phage bands were extracted from the tubes with a needle and dialyzed to remove CsCl. DNA was extracted from phage by three sequential phenol extractions in small tubes rotated on a wheel at 30 rpm for 20 min followed by centrifugation to separate phases. Extracted DNA was dialyzed initially into TE (10 mM Tris HCl pH 8, 1 mM ethylene diamine tetra-acetic acid disodium salt (EDTA)), and later into experimental buffers, as needed.

Native gel electrophoresis of heat-treated samples

Samples were electrophoresed in standard agarose gels using TAMg (40 mM Tris base, 20 mM acetic acid (pH 8.1), and 1 mM magnesium sulfate) buffer, which is the TAE buffer that is routinely used for DNA electrophoresis, but with the EDTA replaced with 1mM MgSO₄ to stabilize encapsidated DNA. Samples were run in 0.8% agarose gels, and visualized using UV light and a yellow filter after staining with 0.5 µg/ml ethidium bromide in gel buffer. Purified virions and heads were dialyzed into SCB buffer (see below) and aliquots were placed in a water bath set at 30°C. The temperature control was raised in 10° increments: at each step, 3 min after equilibration at the higher temperature, samples were removed and placed on ice; the temperature setting was then advanced again. Before gel analysis, half of each sample (5 µl) was mixed with 1 µl of a DNase master mix containing 60 µg/ml DNase I (Sigma D5025), 6 mM MgSO₄ and 60 mM Tris HCl, pH 8, and incubated at 30°C for 30 min.

Cryo-electron microscopy

4µl drops of sample were applied to holey carbon film-bearing grids, blotted, vitrified, and imaged at low electron dose as described 49, using a Philips CM200-FEG electron microscope fitted with a Gatan 626 cryoholder and operating at 120keV. Micrographs were recorded at 50,000x magnification (for heads) or 38,000x (for virions). For heating experiments, a custom-built environmental chamber that mounts over our Reichert KF80 cryo-fixation unit was used 26. This chamber, which is manually accessible for grid preparation operations (blotting and quenching grid), can maintain temperatures of up to 80°C and humidity up to 100%, as set via an electronic control unit. 40µl samples of heads or virions were sealed with parafilm in 0.2ml thin-walled Eppendorf tubes and incubated at the specified temperature (50°, 70° or 80°C) for 10 min in a microprocessor-controlled water. The Eppendorf was then transferred to a beaker containing water at 50°, 70° or 80°C and installed in the environmental chamber pre-equilibrated at the same temperature and 80% humidity. The humidity was increased above 80% just before quenching a grid.

Image reconstruction

Micrographs pre-screened by optical diffraction were scanned on a Zeiss SCAI scanner (Z/I Imaging, Huntsville, AL) at a step size of 7µm, corresponding to 1.4 Å/pixel at the specimen. Particle images were picked with the x3dpreprocess program 50. An initial round of orientation determination and alignment was performed, using a pre-existing cryo-EM reconstruction of Head II (not shown). Subsequent refinement was performed and reconstructions calculated using the PFT2/EM3DR2 software 51. Final refinement was performed with the AUTO3DEM suite 52. The filled (empty) capsid reconstruction was based on 1296 (57) particles from a total of 1396 (72) initially picked from 18 micrographs and had a resolution of 13.9 Å (24.4 Å), according to the FSC criterion with a threshold of 0.3. Surface renditions of the density maps were made with USCF Chimera 53.

Differential scanning calorimetry

DSC measurements were carried out as described 27, using a Microcal MC-2 calorimeter (Springfield, MA) equipped with twin 0.6 ml cells and operated at a scan rate of 1.98°C/min. Two buffers were used in the experiments described here: SCB-Mg buffer, with 0.1 M KCl,

0.02 M KPO₄, pH 7.5, and 1 mM MgSO₄; and a Mg-free buffer containing 5 mM KCl, 5 mM KHPO₄, pH 7.5. The denaturational enthalpy of HK97 heads in SCB was determined from the area under the single endotherm shown in Fig. 4a to be 20.0 mcal for this experiment. We previously determined the denaturation enthalpy of Head-II, which consists of 420 copies of gp5*, to be 278 kcal/mol (of gp5*monomer) 27. The amE1 capsid has 415 copies of gp5* in a Head II-like conformation and 12 copies of the gp3 portal. If we assume that this capsid behaves like Head II, we would expect it to contribute 5.0 mcal from protein denaturation in this experiment. In fact, we adopt the slightly smaller value of 4.9 mcal as our best estimate for this quantity to allow for the possibility that gp3 (which accounts for only 4% of the capsid mass) might have a lower specific denaturational enthalpy than gp5*.

The heat of DNA denaturation, under our conditions, was estimated to be 9140 cal/(mol bp) by two different methods. The first is based upon extrapolating to 88°C the calorimetric value of $\Delta H_{\text{CAL}} = 8293 \text{ cal (mol bp)}^{-1}$ for the denaturation of T7 phage DNA 54 (47.8 % GC) at $T_m = 70^\circ\text{C}$ by using $\Delta C_p = 47 \text{ cal K}^{-1} \text{ (mol bp)}^{-1}$ derived from the plot of ΔH_{CAL} vs T_m in an analysis 55 of Klump's compilation of thermodynamic data of polynucleotide duplexes 56. The second method employs the equation 31 (yielding cal.(mol bp)⁻¹)

$$\Delta H_{\text{DNA}} = 11250 + (2.6 - F_{\text{gc}})(450 \log [\text{Na}^+] - 580)$$

which uses the base composition and counterion concentration. Upon substituting [K+] for [Na+] we obtain the same value for $\Delta H_{\text{DNA}} = 9140 \text{ cal (mol bp)}^{-1}$. We thus estimate 15.4 mcal as the contribution from DNA in the experiment of Fig. 4a. This leads to combined value for DNA and protein of 20.3 mcal, in excellent agreement with our measured value of 20.0mcal, and within the 3.5% margin of error we have customarily assigned to individual calorimetric measurements 27.

The ultraviolet melting curve was recorded with a Cary Model 210 spectrophotometer (Varian, Sugarland, TX) with readings taken at 1-degree intervals and the temperature determined with a calibrated thermistor.

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Assembly pathways of bacteriophage HK97 nucleocapsid

Figure 1.

Schematic diagram of the assembly/maturation pathway of bacteriophage HK97 capsid.

Above: *in vitro* maturation of capsids assembled *in vivo* from co-expression of gp5 and gp4 (the protease) and isolated from these cells. Below: presumed pathway with DNA packaging into portal-containing capsids. It is plausible but unproven that, during packaging, the maturing capsid passes through the same sequence of conformational changes. See 21.

Figure 2.

Cryo-micrographs of tailed and tail-less HK97 heads. (a) tailed virions, imaged at a defocus condition at which the 25 Å inter-duplex spacing is strongly contrasted but the tails are conveyed with low contrast and are barely visible (some are marked with arrows). (b) Tail-less heads. (c) and (d) show, respectively, galleries of side-views (punctate DNA motifs), and (d) axial views (fingerprint motifs). Bars = 50 nm.

Figure 3.

Cryo-EM reconstructions of DNA-filled HK97 capsids (a – c) and empty HK97 capsids (d – f), viewed along an axis of 2-fold icosahedral symmetry. (a) and (d) - the outer surfaces – the centers of the pentamers defining one triangular facet are marked “5” and the hexamers in that facet with “6”; (b) and (e) cutaway views showing, in (b), nested shells of encapsidated DNA (red), and in (e), the interior surface of the empty capsid; (c) and (f) show central sections (positive density is dark). The dashed line defines a frame of reference in which the curvature of the empty capsid wall is apparent. The empty capsid reconstruction is at lower resolution than the filled capsid, being based on fewer particles. To confirm that the observed difference in curvature does not arise from the difference in resolution, the filled capsid structure was recalculated at the same resolution as the empty capsid. Its curvature was unchanged (data not shown). Bar = 10 nm.

Figure 4.

Calorimetric scans of (a) DNA-filled HK97 heads in SCB buffer containing 1 mM Mg^{++} ; (b) DNA-filled HK97 capsids in 5mM KCl, 5 mM $KHPO_4$ buffer (no Mg^{++}); (c) repeat scan of the sample in (b); the signal from the renatured DNA is reproduced. A very small remnant of the high temperature event is recorded at 90-95°. A third scan of the same specimen, after cooling for 18 hrs at room temperature, replicated the DNA melt in complete absence of any endotherm above 90° (data not shown). (d) Head II – a mature, empty, portal-less capsid (reproduced from 27). It may be that the 78°C transition of Head II is also exhibited by filled capsids at a slightly higher temperature but, compared to the DNA melt, it would make a very small contribution to the major thermal event. The stated units are per mole of capsid.

Figure 5. Optical melt of isolated HK97 DNA. Its melting temperature, T_m ($= 88.3^{\circ}C$) is the temperature at which the curve reaches 50% of its total increment.

Figure 6.

(a-c) predicted thermal profiles for melting of the HK97 genome. (a) shows the profile as predicted by the MELTSIM program 32. (b) and (c) show this curve after computational smearing with Gaussian filters (middle), and (bottom). (c) is in good agreement the experimental thermogram - (d), which was reproduced from Fig 4b. The inferred Gaussian parameter of 0.33°C is consistent with an independent estimate of the calorimeter's thermal response time as ~ 10 sec (data not shown). At the scanning rate used, $120^{\circ}/\text{hr}$, a time interval of 10 sec corresponds to a temperature increment of 0.33° .

Figure 7.

(a) Cryo-EM of HK97 heads after heating at 80°C. Almost all heads are empty. An exceptional particle (arrow) remains partially filled. (b) Time course of emptying at virions of their DNA and loss of tails upon protracted heating at 65°C.

Figure 8. Native gel electrophoresis, showing release of DNA from heads and tailed phage. Markers for heads, phage particles and free HK97 DNA are shown at right hand side.

Figure 9. Cryo-EM of HK97 virions after heating at 70°C. In addition to the voiding of DNA from the capsid, the hollow axial lumen of the tail becomes conspicuous. Bar = 50 nm.