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A Free Energy Cascade with Locks Drives Assembly and Maturation of Bacteriophage HK97 Capsid.

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Summary

We investigated the thermodynamic basis of HK97 assembly by scanning calorimetry and cryo-electron microscopy. This pathway involves self-assembly of hexamers and pentamers of the precursor capsid protein gp5 into procapsids; proteolysis of their N-terminal Δ domains; expansion - a major conformational change; and covalent crosslinking. The thermal denaturation parameters convey the changes in stability at successive steps in assembly, and afford estimates of the corresponding changes in free energy. The procapsid represents a kinetically accessible local minimum of free energy. In maturation, it progresses to lower minima in a cascade punctuated by irreversible processes ("locks") - i.e. proteolysis and crosslinking - that lower kinetic barriers and prevent regression. We infer that Δ -domains not only guide assembly but also restrain the procapsid from premature expansion; their removal by proteolysis is conducive to initiating expansion and to its proceeding to completion. We also analyzed the mutant E219K, whose capsomers reassemble *in vitro* into procapsids with vacant vertices called "whiffleballs". E219K assemblies all have markedly reduced stability compared to wildtype gp5 ($\Delta T_p \sim -7$ to -10°C ; where T_p is the denaturation temperature). As the mutated residue is buried in the core of gp5, we attribute the observed reduction in stability to steric and electrostatic perturbations of the packing of sidechains in the subunit interior. To explain the whiffleball phenotype, we suggest that these effects propagate to the capsomer periphery in such a way as to differentially affect the stability or solubility of dissociated pentamers, leaving only hexamers to reassemble.

Keywords

Differential scanning calorimetry; cryo-electron microscopy; virus capsid structure; morphogenesis; conformational changes

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Introduction

To assemble a viral capsid, hundreds of protein subunits must be coordinated precisely in three dimensions to produce a shell of predetermined size, shape, and complexity. For the smallest and simplest viruses, all the information needed for assembly is encoded in the subunit fold. Such particles may be passed through cycles of assembly and disassembly *in vitro*¹. For more elaborate viruses, larger numbers of subunits are involved and transient assembly factors – chaperones, scaffolding proteins, and a maturational protease – may be required². Viruses of this kind include the tailed bacteriophages and the herpesviruses which, despite their very different hosts, follow similar pathways of capsid assembly^{3; 4; 5}. In these systems, assembly proceeds in two stages: formation of a precursor particle (procapsid) and its maturation^{6; 7}. Maturation renders the capsid so robust that its subunits may only be dissociated, if at all, with strong denaturants and, typically, the dissociation products are unable to refold and reassemble. At least some procapsids are capable of disassembly/ reassembly reactions, but maturation is an essentially irreversible process.

Mechanisms that govern capsid assembly are likely to recur in the assembly of macromolecular complexes in general⁸. HK97 affords an advantageous system in which to study them. Its pathway (Fig. 1A) has been extensively characterized. The capsid protein precursor, gp5, is a fusion of the scaffolding protein - its Δ -domain (residues 2 to 103) - with the major capsid protein, gp5* (residues 104 to 385). Gp5 forms pentameric and hexameric capsomers that self-assemble *in vivo* or *in vitro* into a T=7I procapsid called Prohead I. If the viral protease gp4 is co-expressed with gp5, ~ 50 copies are incorporated into Prohead I. The protease is not needed for assembly but is required for maturation which begins with proteolysis of the Δ -domains. The resulting particle, Prohead II, is metastable and undergoes a major structural transformation – "expansion"^{9; 10} - that is triggered *in vivo* by DNA packaging and may be induced *in vitro* by acidification.

Expansion proceeds to the final Head II state via three intermediates, called Expansion Intermediates I, II and III¹¹ (Fig. 1A). Conversion of Prohead II to Head II involves large rotations (~ 40°) and translations (~ 40 Å) of the subunit cores that radically alter the capsid structure^{12; 13}. (We use the term "core" for the portion of the molecule that appears to move as a single rigid body throughout capsid assembly. It consists of the A- and P-domains but not two protruding motifs - the E-loop and the N-arm – see Fig. 6A). These movements are accompanied by remodeling of the E-loop, which is a β -hairpin, and the N-arm, which has an extended conformation in Head II but is disordered in Prohead II. Expansion is accompanied by the formation of a network of covalent crosslinks, connecting residue K169 in the tip of the E-loop with N356 on a neighboring subunit^{14; 15}. Crosslinking is sterically controlled by the subunit rotations that bring crosslinkable residues, initially far apart, within reach. The first crosslinks form at the EI-II stage and the final ones in the last step to Head II. These interactions effect an essential stabilization of the capsid¹⁶.

Differential scanning calorimetry (DSC) affords a quantitative method to assess the stability and energetics of interactions in macromolecular systems¹⁷. In a previous study, we used DSC in combination with cryo-EM and mutagenesis to examine the effects of crosslinking and expansion on HK97 capsid stability¹⁶. We found that crosslinking is entirely responsible for the greater stability of the mature capsid; expansion allows crosslinking to take place, with the added benefit of doubling the capsid volume and hence its capacity for DNA. Here, we follow a similar approach to investigate the role of proteolysis in capsid assembly and a point mutation with altered assembly properties. Specifically, we analyze the distinct assembly states of uncleaved gp5 - unassembled capsomers, Prohead I, and capsids that were induced to expand without prior cleavage by treatment with urea - and compare them with earlier data on gp5*-containing particles. In Prohead I, the capsomers are shaped like parachutes whose canopies,

comprising gp5* plus the C-terminal segments of the Δ -domains, form the capsid's contiguous shell, while the underlying gondolas are composed of the remaining portions of the Δ -domains¹⁰. We also investigate the morphogenic mutation, E219K. Whereas capsomers of wild-type gp5 reassemble *in vitro* into Prohead I, E219K capsomers reassemble into "whiffleballs" lacking pentamers at their vertices¹⁸ (Fig. 1B). The present study complements and extends earlier calorimetric work on the capsids of T4^{19; 20} and P22^{21; 22}. It was undertaken because, although T4 and P22 subscribe to the same paradigm as HK97 and their capsid proteins are now known to have similar folds^{23; 24; 25}, there are also significant differences between the respective systems. P22 maturation is not accompanied by proteolysis. With T4, unlike HK97, capsid expansion *per se* effects a sufficient stabilization of the capsid²⁶. An additional incentive to analyze the HK97 system in this way was the availability of a crystal structure for Head II^{23; 27} as well as detailed structural information on other states^{12; 13} that allow the thermodynamic data to be considered in terms of inter- and intramolecular interactions.

Results

As a macromolecular complex is heated, the interactions that stabilize it are disrupted at temperatures that are characteristic for a given specimen. In DSC, samples are heated at a constant rate and the input of energy required to effect such transitions is recorded in the thermogram (e.g. Fig. 2A). With proteins, the major event is denaturation and it may be preceded by minor events that represent thermally induced conformational changes. If a specimen has multiple conformations – as in sequential stages of capsid assembly - their energetics may be compared from the thermograms.

Thermodynamic parameters measured from these scans are compiled in Table I. Thermal stability is measured by T_p , the peak temperature of a denaturation endotherm. The enthalpy change accompanying denaturation, ΔH_m , is the area under the peak and represents the energy required to disrupt interactions within and between subunits. The entropy change, ΔS_m , is given by $\Delta H_m/T_p$. The breadth of a thermal event is expressed as ΔT_{FWHH} , its full width at half-height. For a two-state transition, this parameter represents cooperativity. Thermodynamic stability, ΔG° , is the difference in free energy between the folded and unfolded forms. This quantity may be calculated from the denaturation parameters obtained by calorimetry (see Methods). The effect of a structural transition or a mutation on thermodynamic stability may be expressed in terms of $\Delta\Delta G^\circ$.

Assembly of wild-type capsomers into Prohead I enhances their thermal stability by ~ 20°C

To assess the contribution of inter-capsomer interactions to stabilizing Prohead I, we compared thermograms recorded for unassembled capsomers with those of assembled particles (Fig. 2A). Capsomers were prepared by dissociating Prohead I in high ionic strength at alkaline pH, and then dialyzing back into the original buffer to eliminate any effect of solution conditions on the comparison. Material prepared in this way is a mixture of hexamers and pentamers, mainly hexamers²⁸. The resulting thermograms (e.g. Fig. 2A, 4th curve) exhibit a single, quite sharp, endotherm with a T_p of 63°C. In contrast, native Prohead I (i.e. capsids isolated directly from cells in which gp5 was expressed) denatures at 82°C, almost 20° higher; moreover, this event is substantially broader (Fig. 2A, 3rd curve).

To confirm the assembly competence of these capsomers as well as the authenticity of Prohead I reassembled *in vitro*, we prepared the latter particles and analyzed them by calorimetry. The resulting thermograms (e.g. Fig. 2A, 2nd curve) were indistinguishable from those of native Prohead I except for a vestigial event at 62°C attributable to a small amount of unassembled capsomers.

Prohead I, but not capsomers, exhibits a minor thermal event at 54°

This event, well separated from the denaturation endotherm and involving about 8% as much enthalpy (Fig. 2A, 2nd and 3rd curves), is exhibited by both native and reassembled Prohead I but not by capsomers. Unlike thermal denaturation, this transition is reversible (data not shown). Since the capsids remain intact after this transition, its structural basis may be tested by cryo-EM of Prohead I that is heated past 54°C and then rapidly frozen before the structure can revert (Conway et al., Ms in submission).

Conversion of Prohead I to Prohead II involves a structural change in gp5*

As visualized by cryo-EM, the cleaved and uncleaved procapsids are similar in size and structure¹⁰. Evident differences are confined to the inner surface where a "gondola" of density underlies each Prohead I capsomer (Fig. 4A). The gondolas are absent from Prohead II, implying that they are clusters of Δ -domains. The T_p of Prohead II is 4 - 5° higher than that of Prohead I, while these denaturation events are of similar width (Fig. 2A, 1st curve; cf. ¹⁶). The simple "excision-of- Δ -domains" scenario should not result in enhanced stability, i.e. an increased T_p , whence we infer that conversion of Prohead I to Prohead II is accompanied by a subtle revision of the interactions in the contiguous shell (see Discussion).

Prohead I may be expanded (without cleavage) by treatment with 5M urea

In the normal course of events, degradation of the Δ -domains is a prerequisite for expansion and crosslinking to occur. Cleavage of gp5 primes Prohead II for expansion, which can be triggered *in vitro* by acidification to pH 4⁹. The same treatment does not cause Prohead I to expand (R.L.D., unpublished observations). However, a transition akin to expansion, as judged by mobility in non-denaturing gels, is induced by treating Prohead I with 5M urea (Fig. 3A). Consistent with this inference, the transition is accompanied by substantial crosslinking, as evidenced by SDS-PAGE (Fig. 3B). We call these particles, putatively expanded but retaining their Δ -domains, Expanded Prohead I.

To characterize them further, we performed cryo-EM and image reconstruction. Expanded Prohead I (Fig. 4B) is compared with Prohead I (uncleaved, unexpanded; Fig. 4A) and Head II (cleaved, expanded; Fig. 4C) at the same resolution: it is indeed almost fully expanded, having a vertex-to-vertex diameter of 600 Å, essentially the same as Head II. Also, like Head II, Expanded Prohead I has rather flat facets, although they are buckled in the middle, not planar (cf. right-hand panels in Fig. 4).

The shell of Expanded Prohead I is thicker and has a more pronounced surface relief than that of Head II. However, lacking the Δ -domain gondolas (yellow in Fig. 4A, middle panel), it is markedly thinner than the shell of Prohead I. Compared with Head II, Expanded Prohead I has additional densities on both the outer and inner surfaces (arrows and arrowheads in Fig. 4B, right panel), suggesting that the Δ -domains are partly translocated to the outer surface. However, it appears that these domains are too bulky to be fully translocated, so that the rotations undergone by the gp5* cores en route to the Head II state¹² cannot go to completion. Nevertheless, the transition proceeds far enough for crosslinking to take place. Consistent with these data, the E-loops which engage in the crosslinks and protrude outwards on unexpanded capsids (white arrows in Fig. 4B, left and middle panels) are less prominent in Expanded Prohead I, except around the pentamers. A similar effect whereby the penton-proximal E-loops are the last to remain protruding was observed on EI-III capsids¹⁵.

Expanded Prohead I is partially stabilized by crosslinking

In Figure 3C, a thermogram of Expanded Prohead I (2nd curve) is compared with those of Prohead I (4th curve) and Head II (1st curve). Its T_p is 10° higher than for Prohead I. The width

of its denaturation endotherm resembles that of Head II, but its T_p is 5° lower. This thermogram lacks the reversible minor event at 80° exhibited by Head II (Fig. 3C, 1st curve) and the 54° event of Prohead I. Since expanding Prohead II in the absence of crosslinking does not enhance its thermal stability¹⁶, we attribute the increased T_p of Expanded Prohead I to crosslinking.

To test this hypothesis, we prepared Expanded Prohead I with the mutant K169Y which is unable to crosslink, and confirmed by cryo-EM that these particles were morphologically similar to wild-type Expanded Prohead I (data not shown). Their thermogram is shown in Fig. 3C (3rd curve). Expanded Prohead I (K169Y) melts at 77°, ~ 4° lower than Prohead I (wild-type). If we allow a 2° decrease in T_p for the K169Y substitution¹⁶, it follows that inducing Prohead I to expand without prior cleavage results in a 2°C reduction in its T_p , i.e. a net destabilization. However, when wild-type Prohead I is expanded, most crosslinks form (Fig. 3B), resulting in a ΔT_p of +12°C; this increment is the same as that effected by crosslinking the cleaved and expanded surface lattice.

Capsomers and Proheads of the whiffleball mutant E219K have reduced stability

The E219K mutation was initially identified in a screen for mutant gp5s with altered assembly properties. As outlined above (Introduction), E219K capsomers – which appear to be exclusively hexamers after dissociation from Prohead I - reassemble only into whiffleballs. To investigate the thermodynamic consequences of this amino acid substitution, we scanned capsomers, Prohead I, and Prohead II of the E219K mutant protein (Fig. 2B). These data show a similar trend as with wild-type gp5 (Fig. 2A), but there are some differences. First, there is a systematic reduction in thermal stability. The T_p 's are displaced to lower temperatures, by 7 – 10°, depending on the state of assembly. Second, the E219K Prohead I endotherm is much sharper than for wild-type, i.e. $\Delta T_{FWHH} \sim 2^\circ$ vs. 6° (Table 1); in contrast, the wild-type and E219K capsomer endotherms have the same width, $\Delta T_{FWHH} \sim 3.5^\circ$. Third, the minor 54° event of wild-type Prohead I is absent.

To investigate the consequences of the absence of gp5 pentamers at the procapsid vertices, we assembled whiffleballs according to Li *et al.*¹⁸ and scanned them also (Fig. 2B, 2nd curve). Their denaturation endotherm is as sharp ($\Delta T_{FWHH} \sim 2^\circ$) as that of Prohead I (E219K), but their T_p is 4° lower. This thermogram also has an event at 56° that reflects the presence of some unassembled capsomers. From the size of this peak, they should account for ~ 25% of the gp5 present.

We conclude that the E219K mutation results in a general destabilization relative to assemblies of wild-type gp5. Nevertheless, the mutant Prohead I (with pentamers at its vertices) is more stable than mutant capsomers by about the same margin in T_p (+ 15°–18°) as with wild-type gp5. Despite lacking pentons, whiffleballs are only slightly less stable than Prohead I (E219K), their T_p 's differing by 4°.

With wild-type gp5, pentamers are no less stable than hexamers

The failure to recover pentamers from the dissociation products of Prohead I (E219K)¹⁸ suggests that the mutant pentamers may be less stable than hexamers. Since E219K assemblies are generally less stable than wild-type (cf. Figs. 2A and 2C), it may be that E219K pentamers, once dissociated, simply fall apart. Alternatively, they may precipitate or even reorganize into hexamers. In fact, Xie and Hendrix²⁸ found that wild-type capsomers may be converted from mostly hexamers to mostly pentamers under appropriate solvent conditions, (they were able to distinguish the two kinds of capsomers on the basis of their differing mobilities in non-denaturing gel electrophoresis).

To test the "labile pentamer" hypothesis, we sought to compare pentamers with hexamers by calorimetry; however, the E219K protein precipitates under the buffer conditions that favor pentamers¹⁸. Nevertheless, we were able to produce wild-type pentamers. To confirm their oligomeric status, we examined this material by negative staining EM and image analysis (Fig. 2C-E) and, in this way, estimated that > 80% of these oligomers were indeed pentamers. Calorimetrically, this preparation was indistinguishable from capsomers from dissociated Prohead I, which are mostly hexamers (cf. curves 5 and 4 in Fig. 2A). Thus, we conclude that any differential lability of pentamers should be specific to the E219K mutation.

Discussion

The progressive stabilization of the maturing HK97 capsid is plotted in Fig. 5A in terms of T_p , the denaturation temperature. Assembly of capsomers into Prohead I enhances their thermal stability by 20°C. Prohead I is already a robust particle whose T_p (82°C) is comparable to some proteins of thermophiles. Conversion to Prohead II effects a further stabilization (ΔT_p , +4.5°C), reflecting a subtle structural change in the contiguous shell (Fig. 5B). Assembly of the E219K mutant up to the Prohead II stage follows a similar course, but at each stage the mutant particle is ~ 10° less stable than wild-type. When Prohead II expands to Head II, further stabilization to a T_p of 96°C is conferred by crosslinking (Fig. 5A). Unlike Prohead II, which may be induced to expand by relatively mild stimuli, Prohead I expands only when exposed to a strong denaturant, i.e. 5M urea, but this transition also results in substantial crosslinking and a concomitant stabilization.

Cooperation among capsomers lends stability to Prohead I

Capsomers and pentamers denature in single endotherms (Fig. 2) whose asymmetry is characteristic of oligomeric dissociation processes²⁹: the best fits obtained for these curves were for hexamer and pentamer dissociation processes, respectively (data not shown). With wild-type gp5, we observed no significant difference between hexamers and pentamers (Fig. 2). Prohead I differs from unassembled capsomers in (i) a 20° increment in T_p ; and (ii) the appearance of a minor endotherm at 54°. A similar assembly-related stabilization was observed with the enzyme, riboflavin synthase, for which, assembly of pentamers into paired pentamers was found to be accompanied by a 20° increase in T_p ³⁰. In the case of gp5, the stabilization of capsomers assembling into Prohead I corresponds to a $\Delta\Delta G^\circ$ of ~ 1.5 kcal/mol (of monomer) at 37°C. This appears to represent a packing effect whereby isolated capsomers may have a tendency to undergo local unfolding around their peripheries that then leads to complete conformational breakdown; in procapsids, this tendency is suppressed by inter-capsomer interactions. We call this effect *cooperation* to distinguish it from cooperativity.

The assembly-related stabilization of gp5 capsomers does not reflect enhanced cooperativity because the width of the endotherm increases (Fig. 2). If the transitions are interpreted as simple two-state processes (folded/unfolded), the observed broadening would indicate a decrease in cooperativity. However, thermal denaturation of protein assemblies involves the disruption of multiple interactions – within subunits, between subunits, and between capsomers. If these interactions are similarly but not equally resistant to thermal stress, they should be disrupted in a single composite endotherm. Thus, the broadening of the denaturation endotherm when capsomers assemble into Prohead I reflects a widening of the distribution of T_p 's of the various subprocesses. Similarly, we attribute the threefold difference in ΔT_{FWHH} between wild-type and E219K Prohead I to the respective endotherms being composed of sets of more (E219K) or less (wild-type) coincident sub-endotherms.

Role of Δ -domain cleavage in facilitating expansion

As Prohead II matures to Head II, the gp5* subunit cores undergo rigid-body rotations and the N-arm and E-loop are refolded (Introduction). Do comparable structural changes take place earlier in the pathway, and, in particular, how are the Δ -domain and the N-arm affected? In Prohead I, the Δ -domain moiety adjoining the N-arm lines the inner rim of a capsomer¹⁰, and the N-arm itself is presumably folded and in this vicinity, i.e. on the inner surface. As Prohead I expands and the subunit cores rotate, the Δ -domains must also be drawn into this region where they impede the formation of inter-capsomer contacts (Fig. 5B). Thus we attribute the reduced stability of the Expanded Prohead I conformation *vis-à-vis* the Head conformation to this effect. To make such a comparison without the complication of crosslinking, we refer to the K169Y mutant: the T_p of Expanded Prohead I (K169Y) is 77° vs. 83° for Head I, the fully expanded (and cleaved) K169Y capsid. Thus, the incomplete expansion (Fig. 4B) and crosslinking (Fig. 3B) of Expanded Prohead I (wild-type) probably have similar origins. It follows that one function of cleavage in the HK97 system is to remove this “chock” - the Δ -domain - so that expansion and crosslinking may go to completion.

The major capsid protein of T4 also has a sizable Δ -domain, 65 residues²⁶. In the T4 system, expansion has been studied on tubular capsid analogs called polyheads. Uncleaved polyheads may be induced to expand by the relatively mild treatment of exposure to 0.2M guanidine-HCl, giving rise to a variety of partially expanded structures³¹. Here, also, incomplete expansion was attributed to the trapping of Δ -domains at inter-subunit interfaces. Moreover, it has been shown by immuno-gold electron microscopy that the T4 Δ -domains, if still present, are translocated from the inner to the outer surface in expansion³², as we infer to be the case for HK97 (Figs. 4B & 5B).

In Expanded Prohead I, the gondolas disappear (cf. Figs. 4A & 4B) and it appears likely that the corresponding portions of the Δ -domains – putatively¹⁰, residues 1 to ~85 - are dissociated and, perhaps, unfolded. To investigate the Δ -domain alone, we expressed and purified it, and subjected to calorimetry, whereupon we observed a very broad endotherm between 20°C and 50°C (data not shown). Its low temperature and the absence of such an event with Prohead I or capsomers suggest that isolated Δ -domains are not fully folded and these domains only assume their proper conformation and oligomeric state(s) in the context of folded and assembled capsomers.

Role of the Δ -domain in promoting assembly and stabilizing Prohead I

Structural information on isolated capsomers is limited to negatively stained projections (Fig. 2B) which indicate that the capsomers have essentially the same arrangements as in Prohead I, although the hexamer image is noisier, being based on fewer particles. The stain-excluding features at the centers of both capsomers presumably represent Δ -domain gondolas. We posit that the propensity of Δ -domains to self-associate in clusters of five and six (a double trimer – see Fig. 4A) helps to guide the gp5 subunits in forming assembly-competent capsomers; moreover, as long as these interactions between Δ -domains are maintained, they restrain the gp5* moieties from switching to their Prohead II state. When the Δ -domains are proteolyzed, this constraint is eliminated and the contiguous shell undergoes a subtle structural change that results in a ΔT_p of + 4.5° when Prohead I is converted to Prohead II and a stabilization of $\Delta\Delta G^\circ \sim -2.5$ kcal/mol. Presumably, exposing Prohead I to 5M urea has a similar effect, disrupting the Δ -domain interactions and driving the capsid as far along the expansion pathway as the still-attached Δ -domains allow. In the P22 and HSV systems, which have separate scaffolding proteins, a similar restraining effect has been attributed to the scaffold-capsid protein interaction in the procapsid^{33; 34}.

Successive states of the N-arm during capsid maturation

Our working hypothesis for the structural basis of HK97 maturation envisages the following conformational history for the N-arm. In capsomers and Prohead I, it should have a definite conformation: otherwise, it is hard to see how the Δ -domain could influence aggregation of the gp5* core, if connected to it only by a disordered polypeptide. This putative conformation is probably context-specific as the N-arm is rather short (33 amino acids) to fold into a separate domain. In Prohead II, the N-arm is disordered (12 and I. Gertsman, L. Gan & J.E. Johnson, personal communication), and the switch undergone by the contiguous shell between the Prohead I and II states presumably involves unfolding of the N-arm (see also ³⁵). In Head II, it again assumes a definite structure – in this case, an extended one that is stabilized only by interactions with other subunits ²⁷. This arrangement is reminiscent of the C-terminal arm of the SV40 capsid protein invading neighboring capsomers ³⁶, and represents a manifestation of the principle of domain-swapping ³⁷ in higher-order structures.

Destabilizing effects of the E219K mutation

How does this amino acid substitution reduce the stability of procapsids? Essentially the same value for $\Delta\Delta G^\circ$ between mutant and wild-type (5.5 kcal/mol) was obtained for Prohead I and Prohead II. Similarly, E219K capsomers are less stable than wild-type by 3.7 kcal/mol. Since the denaturational enthalpy ΔH_m is essentially the same for wild-type and mutant Proheads (Table 1), it follows that the observed destabilization of the mutant capsids is mainly if not wholly entropic.

The T_p of whiffleballs is 4° lower than that of Prohead I (E219K). That this change reflects their lack of pentamers and not a different packing of hexamers after reassembly is implied by the fact that native and reassembled Prohead I (wild-type) have identical thermal denaturation profiles (Fig. 2A). Moreover, the hexameric frameworks of whiffleballs and Prohead I are superimposable ¹⁸. We attribute the observation that the same hexamers in whiffleballs melt 4° earlier than in Prohead I to the absence of inter-capsomer "cooperation" around the whiffleball vertices.

Assembly-competence and capsomer stability

Is it possible to rationalize the effects of the E219K substitution on assembly and stability in terms of this residue's location in the capsid? Since the gp5* subunit structure from Head II fits snugly into the cryo-EM density map of Prohead II ¹², and the contiguous shell of Prohead I closely resembles that of Prohead II, we take the latter model as the best current guide for interpreting the effects of the mutation. The E219 side-chain is buried inside the subunit core, between the major α -helix and the capsid-interior surface (Fig. 6A). There, it is saltbridge-linked to R135 and close enough to E364 to form a hydrogen bond, and thus appears to be part of a network of charged residues (Fig. 6B). Changing E219 to K should induce re-arrangements in side-chain packing arising from the extra bulk of lysine and the charge reversal at this position. In general terms, therefore, the decreased stability of E219K Proheads and capsomers appear to arise from an inability to achieve a comparably stable packing of side-chains in the subunit interior. The inferred remodeling of E219's electrostatic network may be an important factor in the observed decrease in stability, as electrostatic networks - and salt bridges in particular - appear to contribute to the increased stability of proteins from thermophiles as compared to their homologs from ambient-temperature organisms ³⁸.

In Prohead I capsomers, residue E219 is far from subunit interfaces (Fig. 6C & D); however, mutation-induced side-chain rearrangements around E219 may propagate outward to affect more distant residues and may thus be responsible for the altered reassembly properties of the mutant. For example, the two close E219 neighbors, E364 and L366, are on β -sheet J in the P-domain on the peripheries of both hexamers and pentamers. On the opposite side of this strand,

residues E363 and R365 appear to contact neighboring capsomers (Fig. 6C & D). We speculate that rearrangements of the peripheral residues E363 and R365 in E219K pentamers alter their properties sufficiently to account for the absence of pentamers in the dissociation products of Prohead I and hence, the vacant vertices of whiffleballs. Such rearrangements could also explain why E219K Prohead I and whiffleballs have an altered electrophoretic mobility, relative to wild-type Prohead I and why whiffleballs shift towards normal electrophoretic mobility when wild-type capsomers are added ¹⁸.

Thermodynamic forces that drive capsid assembly

To estimate the free energy differences between states of assembly at the physiological temperature of 37°C, which is far from the observed denaturation temperatures, the full form of the Gibbs-Helmholtz equation must be used (Methods). The resulting free energy curves indicate that wild-type capsids have maximum stability in the physiological temperature range (Supp. Figure 1). At 37°C, the difference in free energy between gp5 capsomers and Prohead I, amounting to about 2 kcal/mol (of monomers) - Fig. 5C - is quite small in view of the large difference in T_p (Table 1). However, although subject to substantial experimental uncertainty (Supp. Table 1), it is similar to the value obtained by other, quite different methods, for the change in free energy when dimers of the hepatitis B virus capsid protein assemble into capsids ^{39; 40}. There is a similar decrement, i.e. ~ 2 kcal/mol, between Prohead I and Prohead II. On the other hand, a massive stabilization of ~ 13 kcal/mol accompanies the transition of Prohead II to Head II (Fig. 5C). This large change is attributable to the network of crosslinks, as there is little change in free energy associated with HK97 capsid expansion in the absence of crosslinking (16 and this study).

Materials & Methods

Preparation of Proheads

Proheads were made using plasmids which express HK97 gene 5 (or genes 4 and 5) under the control of the T7 ϕ 10 promoter, and purified by a combination of differential centrifugation, polyethylene glycol precipitation and velocity sedimentation in glycerol gradients, as described previously ^{18; 41}. Plasmid pV0 (pT7-5Hd2.9 41) was used to produce Prohead II. Preparations of Prohead I were made using protease-knockout plasmids pVB (pT7-5Hd2.9(fsBstB1) ⁴¹) or pVK ¹⁸. Derivative plasmid pVK-E219K was used to make E219K Prohead I and plasmid pV0-E219K was used to make E219K Prohead II (both contain the mutation E219K: codon 219 changed from gaa (Glu) to aaa (Lys) ¹⁸. *E. coli* strain BL21(DE3)pLysS was used as host. Cells were induced for ~ 16 hrs at 28°C and used immediately.

Prohead I purification steps prior to chromatography were done using TKG buffer (20 mM Tris HCl, pH 7.5, 100 mM potassium glutamate). Proheads were further purified by adsorption to Poros HQ20 quaternary amine ion exchange columns (ABI, Foster City CA) in 20 mM tris (hydroxymethyl)amino-methane hydrochloride (Tris HCl)- bis-tris-propane buffer (Sigma) at pH 7.5 with 20 mM NaCl and elution with a linear gradient of NaCl to 0.5 M at 13-33 ml/min using a BioCAD chromatography system (ABI, Foster City CA). Prohead samples were concentrated by ultracentrifugation, resuspended in a small volume, and dialyzed into a buffer appropriate for the next step.

The E219K Prohead II-producing strain actually yields a mixture of Prohead I and Prohead II. Accordingly, E219K Prohead II was purified by dissociating the unwanted Prohead I using the glucose method described below. After Prohead I dissociation, the E219K Prohead II was separated from bulk capsomers by pelleting via ultracentrifugation and then repurified using Poros HQ20 anion exchange chromatography as described above.

Expansion of Prohead I

Prohead I was induced to expand by a 2 hr treatment treated with 5 M urea in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) - KOH buffer at pH 5.5. After two hrs, the buffer was exchanged to TAMg (40 mM Tris base, 20 mM acetic acid pH 8.1, and 1 mM magnesium sulfate) using centrifugal gel filtration in small spin columns. 0.2 ml samples were loaded onto 2.2 ml columns of washed, pre-equilibrated and pre-centrifuged Sephadex G25 (coarse) (Pharmacia), and centrifuged for 2 min at 1000 rpm in the swinging bucket rotor of a large clinical centrifuge (International PR-J).

Dissociation of Prohead I into capsomers

Wild-type capsomers were made by dissociating Prohead I by treatment at room temperature with 2 M KCl in 20 mM Tris HCl pH 9.5 for ~16 hrs, followed by dialysis into 5 mM Tris HCl pH 7.5. E219K Prohead I was dissociated by treatment with 40% glucose in 20 mM Tris HCl pH 9.5 for ~16 hrs. The extent of dissociation was checked using agarose gel electrophoresis. Before use, capsomer preparations were dialyzed into our standard calorimetry buffer (below).

Purification of capsomers and re-assembly into Whiffleballs

Capsomers produced by dissociation of E219K Prohead I were dialyzed to remove glucose and purified using Poros HQ20 anion exchange chromatography as described above. Capsomers (which elute before proheads) were assembled into whiffleballs by concentrating the protein about 100-fold and transferring to PBS buffer as described previously¹⁸. Purified E219K hexamers were precipitated with 70% (w/v) (NH₄)₂SO₄ at 4°C, resuspended at ~ 35 mg/ml and dialyzed against PBS, containing 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄ • 7H₂O, 2 g KH₂PO₄ per liter.

Assessment of purity

In a final step, all capsid and capsomer samples were dialyzed into calorimetry buffer. Purity and homogeneity were assessed by SDS-PAGE and agarose gel electrophoresis. Many samples were also checked by electron microscopy. Native agarose gels were run in DNA mini-gel equipment using TAMg buffer and stained for protein after drying⁴¹. SDS-polyacrylamide gel electrophoresis methods were modified from⁴² using a low-crosslinker stock (33.5% acrylamide plus 0.3% methylene bis acrylamide); all samples were TCA-precipitated prior to heating in SDS⁴¹.

Differential Scanning Calorimetry

We analyzed various assemblies of uncleaved gp5 under standard conditions, i.e. in our "calorimetry buffer" (17.2 mM K₂HPO₄, 2.8 mM KH₂PO₄, 0.1 M KCl, pH 7.5 at a protein concentration of ~ 1 mg/ml, and a heating rate of 120 deg/hr. Protein concentrations were determined and calorimetry was performed as described previously¹⁶, using a Micro-Cal (Springfield, MA) MC-2 calorimeter equipped with 0.6 ml cells and operated at a scan rate of 1.98 K/min. Scans of buffer vs. buffer were subtracted to minimize systematic differences between the cells. The T_ps observed have some dependence on scan rate. In this context, we found the dependencies of capsomers, Prohead I and Prohead II, both wild-type and mutant, to be very similar (data not shown). Accordingly, the ΔT_p values that we recorded are a very close approximation to the values that would be obtained at zero scan rate where kinetic effects would be absent.

Determination of Changes in Free Energy

ΔG°, the difference in standard free energy of the folded and unfolded forms of a capsid, may be calculated for any temperature, T, from the thermodynamic parameters of denaturation

evaluated by DSC (i.e. the denaturational enthalpy, entropy and heat capacity - ΔH_m , ΔS_m , and ΔC_p), with the integrated form of the Gibbs-Helmholtz equation

$$\Delta G^0(T) = \Delta H_m - T\Delta S_m + \Delta C_p[(T - T_m) - T \ln(T/T_m)] \quad (1)$$

T_m is the median temperature of the denaturation endotherm which, except for highly asymmetric transitions, is very close to the peak temperature, T_p . Accordingly, we substituted the determined values of T_p for T_m in the calculations. The values used for ΔC_p and the resulting values of ΔH , ΔS at 37°C are given in Supplementary Table 1. For the difference in the free energy of unfolding between a reference state (state A, e.g. a wild-type (wt) capsid) and an altered state (state B, e.g. a later conformation or a mutant) evaluated at T_A , the T_p of state A, is denoted by $\Delta\Delta G^0(T_A; AB)$. For our data, the ΔC_p term of Eqn. (1) does not make a significant contribution for temperatures within ~ 15° of T_A . Accordingly, in this region, $\Delta\Delta G^0$ may be evaluated using the following approximation⁴³,

$$\Delta\Delta G^0(T_A; AB) = (T_B - T_A)(\Delta H_{m,B}/T_B) = \Delta T_p \cdot \Delta S_{m,B} \quad (2)$$

It may be seen from Eqn. (2) that because $\Delta S_{m,B}$ is a constant, $\Delta\Delta G^0$ scales with ΔT_p , the difference in T_p . In this approximation, the thermal stability of a particular capsid, given by its T_p , is also indicative of its thermodynamic stability, providing the rationale for Fig. 5A.

Cryo-EM and Image Reconstruction

Expanded Prohead I was imaged at 50,000x magnification on a CM200-FEG electron microscope (FEI, Mahwah, NJ) equipped with a Gatan 626 cryo-holder⁴⁴. 4 focal pairs, selected on the basis of their optical diffraction patterns, were digitized on a SCAI scanner (Z/I Imaging, Huntsville, AL) at a step size of 7 m, corresponding to 1.4 Å pixels. Image reconstruction, including contrast transfer function (CTF) correction, was performed as described⁴⁵. The micrographs yielded 1724 particles (focal pairs), of which 1156 were included in the final map. Its resolution, assessed in terms of the Fourier Shell Correlation coefficient⁴⁶ with a threshold of 0.3, was 11.1 Å. Analyses were performed on Macintosh G5 computers (Apple Computer, Cupertino CA), and panels in Figure 4 were prepared on Linux workstations (Dell, Austin TX) with Amira 3.1 software (Mercury Computer Systems/3D Viz group, San Diego CA and Merignac, France).

Negative staining of pentamers

A sample was negatively stained with uranyl acetate and imaged on a CM200-FEG electron microscope at 120 keV and 66,000x magnification. Micrographs were digitized as described above. From these fields, round-appearing particles were extracted as representing axial views. These were sorted, initially by visual criteria, into pentamers (N = 190) or hexamers (N=35). The proportion of pentamers, ~ 85%, was subsequently confirmed by a quantitative, iterative, correlation-based classification procedure. In each cycle, all the data were compared against the current average pentamer and hexamer images and re-assigned according to which gave the higher correlation coefficient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HK97 capsid assembly with and without the maturational protease

The pathway (A) is for the expressed capsid protein in the absence of the portal/connector protein and DNA packaging. Pathway (B) refers to *in vitro* assembly of Prohead I and how this assembly pathway is altered in the mutant E219K which reassembles into whiffleballs.

Figure 2. Calorimetry of various assembly states of wild-type HK97 capsid protein and the E219K mutant

(A) Thermograms of five states of wild-type gp5 or the cleaved form gp5* in the case of Prohead II. P-I = Prohead I, etc. Capsomers prepared by dissociating Prohead I are predominantly (> 80%) hexamers and the remainder, pentamers. In pentamer preparations, the proportions are reversed. (B) Thermograms of four assembly states of the mutant capsid protein, gp5 (E219K). The minor peak at 57° in the whiffleball curve is contributed by unassembled capsomers. (C–E) Negative staining electron microscopy of a gp5 "pentamer" preparation confirms that the majority of these oligomers are indeed pentamers, but a minority (~ 15%) of hexamers is also present. (C) a field of oligomers. Bar = 500 Å. (D) left - a typical single pentamer; middle - averaged pentamer after rotational and translational alignment; right - averaged pentamer after 5-fold symmetrization; (E) left - a typical single hexamer; middle - averaged hexamer after rotational and translational alignment; right - averaged hexamer after 2-fold symmetrization. Since hexamers are fewer than pentamers, less averaging was done, so these images are noisier. Bar = 50 Å.

Figure 3. Gel electrophoresis and calorimetric analysis of Expanded Prohead I

(A) shows that the mobility of Prohead I in native agarose gels, after treatment with 5M urea (Expanded Prohead I), is markedly altered and perceptibly different from that of unassembled capsomers. (B) SDS-PAGE of Expanded Protein I shows a ladder of bands with mobilities corresponding to multiples of 42 kDa, indicative of a substantial degree of crosslinking of the uncleaved gp5. Also shown are a ladder of partially crosslinked gp5* (bands at multiples of 31 kDa), and gp5 monomers (not crosslinked). (C) Thermograms showing calorimetric scans of Expanded Prohead I (2nd curve) with wild-type gp5 which is partially crosslinked (see Fig. 3B) and the non-crosslinking mutant, K169Y (3rd curve). To allow direct comparison, the corresponding thermograms of Head II and native Prohead I are reproduced from ¹⁶ and Figure 2, respectively.

Figure 4. Structural comparison of Expanded Prohead I with native Prohead I and Head II
Cryo-EM reconstructions are shown of (A) Prohead I and (B) Expanded Prohead I. The representation of Head II (C) was calculated by limiting the crystal structure²³ to the same resolution as the cryo-EM data, i.e. 11 Å. In each case, renderings of the outer surface (left) and inner surface (middle) are shown, together with a central section (right) in which protein is dark. The insert between (A) and (B) shows Prohead I at lower magnification and the lower resolution of ~ 25 Å under which conditions, the T=7 *levo* surface lattice and the skewing of the hexamers are more evident. The Δ-domain-associated gondolas of density underlying each capsomer are colored yellow in (A) – middle panel. This density is rather diffuse – (A), right panel, yellow arrows, indicative of some disorder. Expanded Prohead I has the same diameter as Head II but its facets are less flat (black arrows, right panel in (B)). The white arrows in (A), left and middle panels, mark protruding E-loops. The shell of Expanded Prohead I is thicker than that of Head II and it has additional density on the outer surface (empty arrowheads) which we interpret as translocated Δ-domains.

Figure 5. Summary of changes in thermal stability and structure between various assembly states of HK97 capsid protein

A – Summary of denaturation temperatures (T_p). Wild-type gp5 (blue), the E219K mutant (red), and the K169Y mutant (purple). The T_p 's of wild-type capsomers (predominantly hexamers – left data point) and pentamers (right data point) are the same, 63°. Wild-type capsomers reassemble *in vitro* into Prohead I (right data point) which is calorimetrically indistinguishable from native Prohead I (left data point). Cleavage of Prohead I produces Prohead II, the precursor capsid primed for expansion. Expansion *per se* results in slightly reduced thermal stability but crosslinking confers progressively greater stability (gray columns), +12° with complete crosslinking. A similar stabilization is effected with both cleaved

and uncleaved gp5. The open ring at the bottom of each gray column refers to a hypothetical particle of wild-type gp5/gp5* that is expanded but has no crosslinking. At each stage of assembly from capsomers to Prohead II, the E219K mutant is $\sim 10^\circ$ less stable than wild-type. (B) Schematic representation of the HK97 capsid assembly pathway, showing the inferred position of the Δ -domains (yellow), if present; and using different colors to convey different configurations of the gp5 subunit cores in capsomers of assembled surface lattices. (C) Major steps in the free energy cascade that drives HK97 assembly. At 37°C, the ensemble of capsid protein undergoes a net reduction in free energy at each step, as indicated. The heights of the kinetic barriers between successive states are not known but the one between capsomers and Prohead I is presumably low as assembly proceeds readily *in vivo* and *in vitro*. The kinetic barriers between Prohead I and Prohead II and between Prohead II and Head II are presumably high as the initial states in both cases exhibit a negligibly small rate of spontaneous conversion; however, these barriers are circumvented by proteolysis and crosslinking (or, *in vivo*, DNA packaging), respectively.

Figure 6. Location and surroundings of residue E219 mutated to K in the "whiffleball" mutant
 (A) The gp5* subunit (conformer B in the refined Head II structure ²⁷ is shown in a ribbon diagram, as viewed from the capsid interior. The side-chains of E219 and its nearest neighbors are highlighted in space-filling mode (E219, red; R135, blue; E364, orange; L132, A216 and L366, green; and I183, grey). The wedge-shaped A-domains pack around the central axis of a capsomer, while the P-domains form its periphery: see the pentamer model in (C) and (D). The A- and P-domains together form the structural core of the subunit that moves as single rigid body throughout the transition from Prohead II to Head II. Residue E219 is buried within this core. (B) The network of charged residues close to and presumably interacting electrostatically with E219 mostly lie near the capsid inner surface. Side view (C) and bottom view (D) of a Prohead II pentamer from the cryo-EM-derived C α model (PDB ID 1IF0; ¹²) are shown as ribbon diagrams, with E219 (in red) and four other residues highlighted. The positions of the residues, which were approximated by threading the Head II side-chains through the Prohead II model, illustrate possible connections of E219 with the inter-capsomer interface. Residues E364 (orange) and L366 are neighbors of E219 on β -strand J, while residues E363 (magenta) and R365 are adjacent, on the other side of β -strand J and form part of the hexamer-pentamer interface.

Table I
Thermodynamic parameters from calorimetric scans of HK97 capsids

Capsid	T_p (°C)	ΔT_{FWHH} (°C)	ΔH_m kcal/mol	ΔS_m cal/mol/K
capsomers wt	63.3	3.9	159	473
pentamers wt ^a	63.2	4.5	ND	ND
capsomers (E219K)	56.3	3.6	131	398
P- I wt	81.7	6.3	190	535
	54.2 (r)	2.5	14	43
P- I wt ^a (reassembled)	81.6	6.0	ND	ND
	54.2 (r)	2.7	ND	ND
P- I (E219K)	71.5	2.0	192	557
E219K “whiffleballs”	67.4	2.0	145	426
P- II wt ^b	86.3	6.6	188	523
P- II (E219K)	74.6	4.1	175	503
Expanded P- I wt	91.6	3.9	308	844
Expanded P- I (K169Y) ^a	77.6	5.5	ND	ND

For each entry, four scans at 120°C/h were performed, giving average uncertainties (expressed as $\pm$ two standard errors of the mean) of ± 0.2 °C for T_p , and ± 3.5 % for ΔH_m and ΔS_m . ΔH_m and ΔS_m are expressed per mole of gp5 or gp5* protein subunits of which there are 420 copies arranged as hexamers and pentamers on the T = 7I lattice of the icosahedral HK97 capsid. (r) denotes a reversible event; all other event are irreversible.

^aThese runs were carried out at 90 °C/h and the value of T_p has been adjusted upward based upon the observed changes in T_p for capsomers and wt Prohead I at different scan rates of 60–120 °C/h. Values of ΔH_m and ΔS_m are not reported (ND) because these were single experiments.

^bfrom Ross *et al.* 16.