Exploring the Balance between DNA Pressure and Capsid Stability in Herpesviruses and Phages

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ABSTRACT

We have recently shown in both herpesviruses and phages that packaged viral DNA creates a pressure of tens of atmospheres pushing against the interior capsid wall. For the first time, using differential scanning microcalorimetry, we directly measured the energy powering the release of pressurized DNA from the capsid. Furthermore, using a new calorimetric assay to accurately determine the temperature inducing DNA release, we found a direct influence of internal DNA pressure on the stability of the viral particle. We show that the balance of forces between the DNA pressure and capsid strength, required for DNA retention between rounds of infection, is conserved between evolutionarily diverse bacterial viruses (phages λ and P22), as well as a eukaryotic virus, human herpes simplex 1 (HSV-1). Our data also suggest that the portal vertex in these viruses is the weakest point in the overall capsid structure and presents the Achilles heel of the virus's stability. Comparison between these viral systems shows that viruses with higher DNA packing density (resulting in higher capsid pressure) have inherently stronger capsid structures, preventing spontaneous genome release prior to infection. This force balance is of key importance for viral survival and replication. Investigating the ways to disrupt this balance can lead to development of new mutation-resistant antivirals.

IMPORTANCE

A virus can generally be described as a nucleic acid genome contained within a protective protein shell, called the capsid. For many double-stranded DNA viruses, confinement of the large DNA molecule within the small protein capsid results in an energetically stressed DNA state exerting tens of atmospheres of pressures on the inner capsid wall. We show that stability of viral particles (which directly relates to infectivity) is strongly influenced by the state of the packaged genome. Using scanning calorimetry on a bacterial virus (phage λ) as an experimental model system, we investigated the thermodynamics of genome release associated with destabilizing the viral particle. Furthermore, we compare the influence of tight genome confinement on the relative stability for diverse bacterial and eukaryotic viruses. These comparisons reveal an evolutionarily conserved force balance between the capsid stability and the density of the packaged genome.

A virus can generally be described as a nucleic acid genome contained within a protective protein shell, termed the capsid. The viral replication cycle is composed of two distinct processes: delivery of the genetic material to the host (infection) and the subsequent production of new virions (replication). Between rounds of replication, the virion must be sufficiently stable to ensure that the packaged genome is retained within the capsid. Conversely, during infection it must be unstable enough to allow efficient genome release into the host cell. This balance between genome retention and release is described as viral metastability (1). Viral particles are therefore not inert structures and have not attained the minimum free energy conformation, separated by an energetic or kinetic barrier, prior to cell attachment and entry (1). The physical properties of viruses that underlie metastability also relate to the molecular mechanisms leading to tradeoffs between viral reproduction and survival (2). To facilitate genome packaging and its subsequent release for infection, many bacterial viruses (phage) and eukaryotic double-stranded DNA (dsDNA) viruses (e.g., herpesviruses) utilize a specialized portal complex at a unique vertex, which forms a narrow channel through the capsid wall. Packaged DNA experiences bending stress and repulsive electrostatic and hydration forces resulting from the extreme confinement of the encapsidated genome (3–6). This stressed state of

DNA creates tens of atmospheres of internal capsid pressure and has been characterized for viruses that infect hosts from all three domains of life (7–9).

The viral capsid plays a crucial role in retaining packaged DNA and regulating initiation of its release upon interaction with an appropriate host cell receptor (10, 11). This step must be tightly controlled as premature ejection of the packaged genome results in loss of viral infectivity. Here, we investigate pressurized genome release from viral capsids using differential scanning microcalorimetry (DSC). We analyze DNA release from bacteriophages (λ and P22), as well as a eukaryotic dsDNA virus, herpes simplex

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virus 1 (HSV-1). These studies provide direct thermodynamic measurements for genome release from viral particles.

In addition, investigating thermal stability of complex macromolecules provides insight on structural integrity and predicts stability at lower, physiological temperatures (12, 13). We designed a new DSC assay specifically monitoring the temperature of heat-induced DNA release from viruses to describe the influence of internal DNA pressure on viral particle stability. Specifically, we investigated the effects of internal DNA pressure (by varying packaged DNA lengths and ionic conditions [7, 14]) on the ability of the capsid to retain its packaged genome, measured by the temperature of DNA ejection. A delicate balance between internal DNA pressure and capsid strength results in a strong effect of the genome pressure on the temperature required to induce DNA ejection. We show that this effect is conserved between bacterial viruses that regulate their packaged DNA length utilizing either site-specific (phage λ) or headful genome packaging (phage P22) mechanisms (15). This interplay between internal pressure and capsid stability is also observed for HSV-1. Furthermore, comparison between these diverse viral systems shows an increase in the intrinsic capsid stability for viruses that have higher genome packaging densities and correspondingly higher internal pressures. This relation suggests an evolutionary optimization in the capsid strength to withstand the outward force of the packaged genome balanced against the requirement of efficient DNA release during infection. Finally, our results also indicate that the portal vertex is thermally and mechanically the weakest point in the capsid stability of these viruses, limiting their ability to withstand the internal pressure associated with genome packaging.

MATERIALS AND METHODS

Virus sample preparation. Procedures were performed as previously described for isolation of wild-type (wt) P22 (c1-7 13-amH101) (16) and λ virions containing wild-type (λ c160) or truncated (λ b221) genome lengths (17). The His73Leu change in the P22 tail needle protein (gp26) was created by recombinantly replacing the His73 codon with CTC in the P22 prophage of *Salmonella enterica* strain UB-1790, as described previously (18, 19). HSV-1 capsids containing wild-type (KOS strain) or truncated (recombinant R7023 F strain [20]) genomes were isolated as previously described (9).

DSC. Differential scanning calorimetry (DSC) experiments were done with cylindrical-cell VP-DSC and capillary-cell DSC (Cap-DSC) microcalorimeters from GE Healthcare-MicroCal. Cap-DSC scans were performed at 90, 150, or 200°C per hour. All experiments utilizing the cylindrical-cell DSC were performed at 90°C/h. Scans were generally reproducible to within less than 0.2°C in comparisons between duplicate sample preparations.

SAXS. Small-angle X-ray scattering (SAXS) measurements were conducted at the 12-ID B station at the Advanced Photon Source (APS), Argonne National Laboratory, using a 14-keV X-ray beam as the photon source and a sample-to-detector distance of 2,027.66 mm. DNA diffraction peaks were fit with a Gaussian curve plus a linear background. Assuming hexagonal packing of DNA arrays, DNA-DNA interaxial spacing (a_H) values were calculated as $a_H = 4\pi/(\sqrt{3})q$, where q is the scattering vector.

Southern blotting. Capsids were heated at 70°C in the presence of 10 and 12% polyethylene glycol (PEG) 8000 (molecular weight [MW], 8,000 g/mol), followed by incubation at 37°C with DNase to degrade ejected DNA. Pulsed-field gel electrophoresis (PFGE) was then performed on the nonejected partial genomes extracted from capsids, followed by blotting with probes specific to the L and S segments of the HSV-1 genome (GenBank accession number JQ780693).

RESULTS AND DISCUSSION

Measuring the energy of DNA ejection from phage: calorimetric detection of DNA ejection enthalpy. During a DSC temperature scan, the calorimeter records the differential power (Dp) required to heat the viral sample cell relative to the reference cell containing buffer solution alone. The change in sample cell temperature due to transformations in the viral particles causes the DSC feedback system to reduce or increase the heating of the sample cell in order to keep the temperatures of the sample and the reference cells equal. This leads to a negative or a positive peak in the differential power. The peak provides direct measurement of the energetics and transition temperatures for heat-induced processes. For conventional analysis the raw Dp data are converted to heat capacity (Cp) as a function of temperature via normalization by the scanning rate. Endothermic transitions—such as protein denaturation or DNA melting—result in positive peaks in the heat capacity-versus-temperature profile, while exothermic events yield downward peaks. The area of the peak observed in the DSC scan represents the enthalpy change for the heat-induced process. Several advancements in differential scanning calorimeters over the past few decades provided enhanced sensitivity and signal detection (21–23). Such developments allow a broader range of applications and interrogation of more diverse experimental systems.

DSC scans of bacteriophage λ in 10 mM MgSO₄-Tris buffer performed with a newer-generation capillary microcalorimeter (Cap-DSC) at 90°C/h (Fig. 1, green line) reveal three prominent thermal transitions. The higher-temperature endothermic peaks, representing capsid protein denaturation (88°C) and DNA melting (91°C), have been previously investigated for bacteriophage λ (24) as well as HK97 (25). Prior to these endothermic peaks, we observed an exothermic event at ~70°C. The temperature of this exothermic peak coincides with the temperature of heat-induced DNA release from λ virions, determined by the accessibility of the viral genome to DNase digestion as a function of temperature (DNase protection assay) (9). This is shown in the inset panel of Fig. 1 by gel electrophoresis of DNA extracted from the capsids after DNase I treatment as a function of incubation temperature (DNase was added after the high-temperature incubation). Electron microscopy (EM) images of λ virions after brief incubation at 75°C show that the overall capsid structure and tail appear intact (Fig. 1). The temperature for heat-induced DNA ejection observed here coincides with previous experiments on phage λ utilizing light scattering to monitor genome release (24). Also, by measuring receptor-triggered genome release using isothermal titration calorimetry (ITC), we previously confirmed that DNA ejection from viral capsids does indeed have an exothermic enthalpy component (26). However, the DSC scans in Fig. 1 show that the DNA ejection peak is not fully resolved in temperature from the larger endothermic transitions from capsid denaturation and DNA melting (i.e., the heat capacity signal does not return to the baseline value in between the two thermal events). This peak overlap hinders thermodynamic analysis of the DNA ejection process. Therefore, further optimization of the DSC measurements is required in order to obtain well-resolved thermodynamic events.

In general, a lower-temperature scanning rate typically enhances resolution between overlapping peaks in a DSC scan. However, the energy of viral genome ejection is considerably smaller than the endothermic peaks of capsid denaturation and DNA melting, as observed in Fig. 1. It is important to note that

FIG 1 DSC scans of bacteriophage λ performed using a capillary-cell geometry at scan rates of 90 (green), 150 (red), and 200 (black) $^{\circ}\text{C}/\text{h}$. The bottom panels show separate enhanced views of the lower- and higher-temperature portions of the scan, focusing on the peaks of DNA release as well as capsid denaturation and DNA melting peaks, respectively. The contribution of the capsid proteins and DNA melting for scans performed at $90^{\circ}\text{C}/\text{h}$ are indicated. The dotted line reveals a capsid protein denaturation event alone scanned at $200^{\circ}\text{C}/\text{h}$, further revealing the scan rate-dependent shift of capsid denaturation. The central inset illustrates that the amount of DNase-protected viral DNA in phage λ capsids as a function of temperature coincides with the temperature of the exothermic event observed by DSC. The electron microscopy images in the upper panels show virions before (left) and after (right) incubation at 70°C . The heat-treated virions are devoid of their packaged DNA (evidenced by their darker, hollow appearance) and still preserve intact capsid and tail structures.

once initiated, *in vitro* DNA ejection from phage completes on the time scale of seconds (27, 28), while the observed duration of the exothermic signal is measured on the time scale of minutes (29). This is due to desynchronized initiation of DNA ejection events among the individual viral particles in the sample population (27) (i.e., the observed duration of the DNA ejection peak is longer than the duration of DNA ejection from individual particles [29]). This desynchronization reduces the calorimetric signal intensity by spreading it over a longer period of time. Similar kinetic barrier effects have been previously investigated for DSC studies of capsid

maturation and denaturation (30) but has yet to be described in regard to calorimetric detection of DNA release from viral capsids.

DNA ejection from virions is inherently a nonequilibrium event (once DNA ejection is triggered, it is spontaneous and irreversible). In this case a higher scan rate results in a more rapid increase in available thermal energy to surmount the opposing kinetic barrier of initiating DNA ejection, which leads to more synchronized ejection events throughout the sample population. Synchronization thus becomes increasingly important for detecting transitions with smaller enthalpy changes. Although less readily illustrated in the C_p -versus-temperature scans (Fig. 1), the effect of such a kinetic barrier on DSC experiments is more clearly demonstrated in plots of the raw differential power (Dp) signal as function of time (Fig. 2a). Plotting the raw Dp-versus-time data shows that increasing the scan rate from 90 to $200^{\circ}\text{C}/\text{h}$ shortens the duration of the DNA ejection peak from 500 s to 150 s, which noticeably increases the raw calorimetric signal intensity. This effect (along with technical enhancements in newer-generation calorimeters [21–23]) may explain why previous DSC studies on phage λ did not capture the comparatively smaller enthalpy of genome release relative to protein denaturation and DNA melting (24).

Thus, we sought to use higher scanning rates in order to increase instrument sensitivity (31) to better resolve the differential power change associated with DNA ejection from phage λ . Experiments performed using higher scanning rates (Fig. 1, $150^{\circ}\text{C}/\text{h}$, red line; $200^{\circ}\text{C}/\text{h}$, black line) resulted in shifting both the DNA ejection and capsid denaturation peaks toward higher temperatures. The observed shift in the peak position for genome ejection and capsid denaturation also results from a kinetic barrier for initiation of these processes (32).

Capsid denaturation at $200^{\circ}\text{C}/\text{h}$ (the highest scanning rate available on our instrumentation) overlaps in temperature with DNA melting, which results in a larger and more complex endothermic event (Fig. 1, black line). However, the position of the capsid denaturation peak alone at this scan rate is readily obtained by first preheating virions near the DNA ejection temperature (T_{Eject}) to induce genome release (70°C , for 1 h), followed by incubation at 37°C with DNase to degrade ejected DNA free in solution. The DSC scan is then performed on this sample containing empty capsids and digested DNA nucleotides (Fig. 1, dotted line). The preservation of the capsid denaturation peak after preincubation at elevated temperature where all DNA has been ejected from the capsids further reveals that heat-induced DNA release is not due to thermal denaturation and disassembly of the major capsid proteins (see also below). This observation is in line with previous experiments investigating the influence of heat treatment on phages λ (24), HK97 (25), and SPP1 (33). In agreement with our results, cryo-EM images of HK97 virions after 70°C incubation show that although the packaged DNA is ejected, the capsid and tail structures remain intact (25).

Regardless of the scan rate used, under the ionic conditions of the experiments above (10 mM MgSO_4 -Tris buffer), the exothermic peak of DNA ejection overlaps in temperature with the larger endothermic events of capsid denaturation and DNA melting (Fig. 1). We predicted that a larger internal DNA pressure would reduce the stability of the viral particle, rendering it more susceptible to heat-induced destabilization. This should cause DNA to release at a lower temperature, better resolving the ejection peak. Since viral capsids are permeable to smaller ions, reducing the

FIG 2 (a) Results of the DSC scans of phage λ shown in Fig. 1 are plotted as the raw differential power versus time from data collected during the calorimetric scan at 90 (green), 150 (red), and 200 (black) °C/h. For lower scan rates, the duration of the exothermic ejection peak increases with a concomitant decrease in signal intensity. (b) DSC scans of phage λ performed in the presence of 2-fold excess EDTA relative to magnesium concentration (black line). Under these buffer conditions, the ejection peak is well resolved from the larger capsid denaturation (~72°C) and DNA melting (~85°C) peaks. The capsid protein peak is revealed by preincubation at 70°C, followed by DNase treatment and subsequent addition of EDTA (red line). (c) Comparison of DSC scans of phage λ performed with calorimeters utilizing either cylindrical-cell (black line) or capillary-cell (green line) geometry (both scans are at 90°C/h in 10 mM MgSO₄-Tris buffer). The cylindrical-cell geometry results in a larger deflection in the apparent signal than that in capillary cells. au, arbitrary units.

Mg²⁺ concentration of the suspending buffer creates larger electrostatic repulsions between neighboring DNA helices in the capsid, resulting in a higher internal pressure (14). However, a reduction from 10 to 2 mM Mg²⁺ yields only a minimal (~1°C) shift in the ejection peak temperature (data not shown). Alternatively, addition of excess EDTA (at a 2-fold concentration compared to that of Mg²⁺) chelates Mg²⁺ ions in the solution and indeed significantly reduces the ejection temperature by nearly 20°C (Fig. 2b, black line). This results in a well-resolved exothermic DNA ejection peak and allows quantification of the peak area to provide the enthalpy of DNA ejection. The scan of phage λ virions in Fig. 2b (black line) yields a DNA ejection enthalpy of 2×10^{-16} J/virion, which is in precise agreement with the value we previously obtained by ITC for receptor-triggered DNA ejection (26). The peak position and resulting enthalpy per virion are independent of virus concentration in the DSC scans, suggesting that nonspecific intermolecular reactions such as protein aggregation (which can create artificial exothermic heats [34]) do not contribute to this peak. Although our focus is on the process of DNA release from virions, it is interesting to note the effect of EDTA addition on thermal stability of the constituent parts of the virus—the protein capsid and dsDNA genome. Capsid protein denaturation and DNA melting occur at lower temperatures with well-resolved endothermic peaks upon EDTA addition. The identity of the capsid protein peak under these buffer conditions is again revealed by preincubating capsids at the DNA ejection temperature, followed by DNase treatment (as described above), which yields only the capsid denaturation peak (Fig. 2b, red line). Reduced thermal stability for viral capsids in the presence of EDTA was previously observed for SPP1 (33) and coincides with reported interactions between λ capsid proteins and magnesium ions (35).

While EDTA addition reduces the temperature for capsid denaturation and DNA melting, the exothermic DNA ejection peak can be successfully resolved from the larger endothermic thermal events. Thus, the enthalpy of DNA release from the capsid can be readily obtained from our DSC measurements. The major advantage of studying genome ejection energetics by DSC is that isolation and purification of a specific cellular receptor are not required to trigger DNA release, unlike in the ITC analysis of DNA ejection

(26). The challenging requirement of receptor purification has restricted earlier *in vitro* ejection studies to a select few viruses for which protein receptors have been identified and isolated. This is especially important for *in vitro* studies of eukaryotic viruses, where genome release occurs in response to multiple, or more complex, cellular interactions.

Besides the enthalpy for DNA ejection, DSC analysis also reveals the temperature at which DNA is released from the capsid. As discussed below, the temperature of DNA ejection is an important parameter providing information on the balance between the inherent capsid stability and the destabilizing effect of internal DNA pressure. However, the measured DSC signal associated with the thermodynamic event of DNA ejection is relatively small. Furthermore, as we have shown above, very specific salt ionic buffer conditions are required in order to separate the DNA ejection peak from the capsid denaturation and DNA melting peaks. This limits the ability of this assay to detect DNA ejection temperatures for viruses with smaller ejection enthalpies (e.g., with lower DNA packing density and therefore smaller internal pressure, such as for HSV-1). The specific salt requirements also limit investigations of the effect of various buffer conditions on capsid stability for genome retention. Therefore, we have designed a new DSC assay that allows accurate detection of the DNA ejection temperature from a wide range of viral systems under diverse ionic conditions.

Force balance between internal DNA pressure and capsid/portal stability in dsDNA viruses. (i) Calorimetric detection of DNA ejection temperature. Using a new calorimetric approach to specifically detect the temperature of genome release, we next investigated the influence of internal pressure on genome retention by the capsid for multiple evolutionarily diverse viral systems, phages λ and P22, as well as herpes simplex 1 virus (HSV-1).

Modern differential scanning microcalorimeters are equipped with sample cells of either a capillary (23) or cylindrical (22) geometry. A major advantage of capillary cells is to reduce thermal convection from inhomogeneities caused by temperature gradients in the sample solution. For cylindrical cells, such effects are capable of producing artificial (i.e., nonthermodynamic) signals in scanning calorimetry experiments (21). Figure 2c shows a com-

FIG 3 (a) DSC scans of phage λ (black), HSV-1 (red), and phage P22 (blue) utilizing cylindrical-cell geometry. (For clarity, the HSV-1 and P22 scans are truncated after the differential power signal drop). (b) DSC scans of DNA-filled HSV-1 C-capsids alone (red) or in the presence of DNase (green), as well as empty HSV-1 A-capsids (purple). au, arbitrary units.

parison of DSC scans on wild-type (wt) DNA length phage λ in 10 mM Mg^{2+} -Tris buffer obtained by two separate DSC instruments utilizing either sample cell design (both experiments performed using a scan rate of $90^\circ\text{C}/\text{h}$). While the protein denaturation and the DNA melting peaks appear identical for both cell geometries, scans performed with the cylindrical sample cells (black line) show an abrupt drop in signal at the DNA ejection temperature. It was previously shown that DNA ejection from viral capsids causes an abrupt change in the solution viscosity (36). This sudden change of the solution viscosity may create thermal inhomogeneities in the cylindrical sample cell that result in the (thermodynamically) artificial signal drop. This effect is not present in the capillary-DSC scans (Fig. 2c, green line) because this cell geometry reduces generation of such inhomogeneities during heating (21). Although the signal drop observed by the cylindrical-cell DSC does not represent a true thermodynamic quantity, it does, however, precisely show the temperature at which genome release occurs. It also provides the advantages of clearly detecting the DNA ejection event under any buffer ionic conditions (which we investigate below) and requiring significantly less sample material than capillary-DSC experiments (due to the much larger DSC signal) (Fig. 2c). In the following investigation of capsid stability, we use DSC measurements utilizing a cylindrical-cell geometry, which produces an abrupt drop in the scan at the temperature of DNA ejection. As this signal drop does not represent a thermodynamic quantity, the DSC scans in the following discussions are reported as the (raw) differential power (Dp) as opposed to the (thermodynamic quantity) heat capacity (C_p).

(ii) Detecting heat-induced DNA ejection from viral capsids. DSC scans in 10 mM Mg^{2+} -Tris buffer (utilizing the cylindrical-cell geometry) of phage P22 (Fig. 3a, blue line) and HSV-1 (red line) show signal intensity drops analogous to the signal drop for phage λ (black line). (For clarity, the HSV-1 and P22 scans are truncated after the drop in signal intensity.) To verify that this signal drop is strictly dependent on the process of DNA ejection, we compared DSC scans of HSV-1 capsids with and without their packaged genome. During HSV-1 replication, a packaging motor (37) inserts the viral genome into empty procapsids, analogous to

dsDNA bacteriophages (38). Successful packaging results in DNA-containing particles (C-capsids) that continue on the maturation pathway. However, a percentage of capsids fail to complete packaging, resulting in naturally occurring empty capsids (A-capsids) that have an overall morphology and protein composition similar to those of C-capsids (39). DSC scans of these empty HSV-1 A-capsids (Fig. 3b, purple line) do not show any signal drop compared to scans of C-capsids (red line) and yield only the capsid denaturation peak at $\sim 81^\circ\text{C}$. Additionally, scans of DNA-containing HSV-1 C-capsids in the presence of DNase (which rapidly digests ejected DNA [40]) also eliminate this signal drop (green line). These measurements confirm that viral genome release is specifically responsible for the observed signal drop in the DSC scans and that this effect is dependent on genome release occurring as an intact DNA molecule. Our results show that DSC not only allows the investigation of thermal denaturation of viral components but also provides insight into the process of genome release from viral capsids. Thus, the precision and reproducibility of this differential power signal drop provide a new and convenient method for investigating the influence of internal DNA pressure on genome retention in the capsid, which is analyzed below.

(iii) Force balance between internal DNA pressure and capsid stability. DNA inside the viral capsid experiences bending stress and repulsive forces between neighboring helices (3, 4). These forces—and the resulting internal pressure—vary depending on the packaged genome length within the capsid (17). The internal pressure rapidly increases with the amount of DNA packaged inside the capsid, evidenced by the force required by the viral DNA packaging motor throughout the packaging reaction (41, 42). In addition to changing the packaged DNA length, ionic conditions of the suspending buffer also regulate internal genome pressure due to the capsid's permeability to water and small ions. Polyvalent ions (e.g., Mg^{2+}) have a strong influence on the magnitude of DNA-DNA repulsion by screening the negative charges on the encapsidated DNA (5, 7, 14, 43, 44). Using genome length and buffer ionic conditions as two experimental parameters, we

FIG 4 (a) DSC scans of wild-type and 78% genome length λ virions along with wild-type and 91% genome length HSV-1 capsids performed in 10 mM MgCl₂–10 mM Tris buffer. Reduced packaged genome lengths result in ~ 2 to 3°C increases in the temperature of genome release. (b) Comparison of DSC ejection temperatures (T_{Ej} s) for HSV-1 and phages λ and P22 in the presence of 10 mM MgCl₂-Tris and 1 mM MgCl₂-Tris buffers. (c) DNA-DNA interaxial spacings (a_H values) of packaged viral DNA in 10 mM MgCl₂-Tris buffer obtained by SAXS experiments on phage λ (black), phage P22 (blue), and HSV-1 (red) are indicated in parentheses for the 10 mM MgCl₂-Tris buffer DSC scans in panel b. au, arbitrary units.

investigated the role of internal pressure on viral particle stability in relation to stable retention of the packaged genome.

The influence of genome packaging density (volume of the packaged DNA molecule divided by the internal capsid volume) is shown in Fig. 4a by comparing DSC scans of two different phage λ strains that package either 100% (solid black line) or 78% (dashed black line) of the wild-type (wt) genome length. While the protein compositions between these strains remain identical, there is, however, a significant reduction of the internal pressure from 25 atm (wt λ DNA length) to 15 atm (78% genome length mutant) (17). In 10 mM MgCl₂-Tris buffer, this decreased internal pressure results in a nearly 5°C increase in the temperature required to induce ejection from an otherwise identical λ capsid. Since the inherent capsid strength is identical for these two viral strains, this result clearly demonstrates that internal pressure directly influences viral particle stability. The lower internal pressure of the 78% genome mutant results in a smaller outward force acting against the inner capsid wall relative to that of the wt genome strain. Consequently, more thermal energy is required to disrupt the capsid structure and initiate genome release. We observed an analogous effect of internal DNA pressure for HSV-1 capsids containing 100% (Fig. 4a, solid red line) or 91% (Fig. 4a, dashed red line) of the wt genome length. Using atomic force microscopy (AFM) nano-indentation measurements, we have recently measured the stiffness of the HSV-1 capsids as a function of temperature (45). The stiffness of empty HSV-1 capsids decreases with increasing temperature, suggesting that the inherent mechanical stability of viral particles decreases with elevated temperature. Thus, the observed pressure-dependent variation in ejection temperature reveals that heat-induced ejection does not simply measure the inherent capsid thermal stability. Rather, it assesses the balance between the outward force of the packaged genome and the inherent stability of the viral capsid. Therefore, DSC experiments comparing genome release temperatures between identical capsids reveal that increasing temperature and internal DNA pressure both contribute to viral particle destabilization, resulting in loss of the packaged genome.

Along with genome packaging density, we also investigated the influence of variations in internal pressure due to changes in ionic conditions. We observed an equivalent pressure dependence for

the stability of genome retention in response to varying the Mg²⁺ concentration. Ejection temperatures in the presence of 1 mM- and 10 mM MgCl₂-Tris buffers were determined for all three viral systems (phages λ and P22 and HSV-1), as shown in Fig. 4b. As explained above, higher Mg²⁺ concentrations yield a lower internal capsid pressure (14). In agreement with the pressure-dependent stability observed for the genome length mutations, the presence of 10 mM Mg²⁺ leads to capsid fatigue, and the resulting DNA ejection, at a higher temperature than 1 mM Mg²⁺ (Fig. 4b). Indeed, changes in buffer composition can influence protein-protein interactions and their inherent stabilities. However, the inherent thermal stability of the viral capsid, assessed by the protein denaturation peak temperature (measured by DSC), does not significantly change within this range of Mg²⁺ concentrations (1 to 10 mM) for the viruses studied here (data not shown) and for other viral systems (25, 33). Furthermore, our previous AFM experiments show that the mechanical strength of HSV-1 capsids is not significantly affected by changes in the Mg ion concentration (45). Together, these results strongly suggest that it is the reduced internal pressure (due to a higher Mg ion concentration) that gives rise to the enhanced stabilization of the viral particle. This also agrees with earlier observations showing that viruses are less susceptible to heat-induced inactivation upon addition of polyethylene glycol (PEG) in the suspending buffer (46). The presence of PEG reduces the osmotic pressure difference across the capsid wall, resulting in reduced DNA pressure.

In summary, as demonstrated above, DNA ejection temperatures are sensitive to variations in the internal pressure of the packaged viral genome. Thus, although our assay utilizes heat to induce DNA ejection, it is not simply a measure of capsid thermal stability. This calorimetric study reveals the interplay between internal pressure and the inherent capsid stability required to withstand the outward force of the tightly packaged genome. Viruses must often sustain infectivity over extended periods of time between rounds of infection, which requires stable retention of the packaged genome. Correspondingly, loss of infectivity in response to harsh environmental conditions strongly correlates with genome release from the viral particle (33, 47, 48). Although the viruses studied here may not normally experience elevated temperatures, heating appears to provide the energy to induce struc-

tural and mechanical transformations that activate the process of genome ejection (49, 50). Furthermore, a comparative analysis of capsid stabilities between different viral systems, using our new DSC assay, reveals important information on evolutionary adaptation of viral capsid stability to withstand the internal DNA pressure. Below, we perform such a comparison between the DNA ejection temperatures for wild-type virions of phages λ and P22 as well as HSV-1.

(iii) Evolved dependence of capsid stability on packaged genome density for dsDNA viruses. The concept of viral metastability describes how a virus has to be sufficiently unstable to release its genome during infection and yet sufficiently stable to protect and retain the DNA between infections. Failure of the capsid to retain the viral genome is responsible for virus mortality. Thus, the trade-off between survival and infectivity is of key importance to the infectious cycle. This raises a central question pertaining to viral evolution, namely, whether stability of viral capsids varies between viral systems depending on the genome packing density and internal capsid pressure.

The three viral systems studied here differ in multiple aspects, including evolutionary diversity of hosts and genome packaging density. Small-angle X-ray scattering (SAXS) provides structural details of genome packaging density in terms of the average Bragg spacings for neighboring DNA helices. At the spacings measured for the packaged viral genome, DNA adopts a local hexagonal lattice, which can be described by the interaxial DNA-DNA spacings between neighboring hexagonally packaged helices (a_H) for phages (43, 51) as well as herpesviruses (52). Thus, we report the average interaxial DNA spacings (a_H values), which are directly related by geometric conversion (52) to the “interlayer” DNA spacings observed by electron microscopy reconstructions (53). Tighter DNA-DNA spacings within a given viral capsid (43) create larger repulsive forces and yield a higher internal DNA pressure (14, 17). A comparison between different viral systems shows that intracapsid DNA spacings correlate with internal genome pressure (9). Using SAXS, we determined intracapsid DNA-DNA spacings for all three viral systems in 10 mM MgCl_2 -Tris buffer, as shown in Fig. 4c. The interaxial DNA-DNA spacings (Fig. 4c) suggest that the internal DNA pressures for these viruses (with wt packaged DNA length) would scale from lowest to highest as HSV-1 ($a_H = 31.1 \text{ \AA}$) < λ (27.5 $\text{\AA}$) < P22 (26.7 $\text{\AA}$), which agrees with direct pressure measurements for HSV-1 (18 atm [9]) and λ (25 atm [7]). Interestingly, the observed DNA ejection temperatures in 10 mM MgCl_2 -Tris buffer (Fig. 4b, upper portion) follow the same relative dependence: HSV-1 (T_{eject} of 52°C) < λ (68°C) < P22 (84°C). The equivalent trend in DNA ejection temperatures is also observed in the presence of 1 mM Mg^{2+} , illustrated in the lower portion of Fig. 4b.

It is important to stress that the measured DNA ejection temperature reflects the balance between internal DNA pressure and intrinsic capsid stability. Therefore, the ejection temperature does not represent the capsid strength on an absolute scale but, instead, reveals capsid strength relative to the internal DNA force. Nevertheless, the above comparisons between the ejection temperatures do suggest that HSV-1 capsids are intrinsically weaker than phages λ and P22 capsids (in regard to genome retention). This conclusion is summarized by the following. Phage λ , with 25 atm DNA pressure, releases its genome at a significantly higher temperature than HSV-1, which has only 18 atm internal pressure. As we have described above, a reduced internal pressure results in an in-

creased ejection temperature, based on findings comparing identical viruses with various packaged DNA lengths (Fig. 4a). Thus, if the intrinsic capsid strengths were equal for both HSV-1 and phage λ , we would anticipate the lower DNA pressure within HSV-1 to cause a higher ejection temperature than that of phage λ . However, we observed the opposite trend: HSV-1 ejects its genome at a much lower temperature than phage λ under the same buffer condition (Fig. 4b). Thus, given that phage λ has a larger internal pressure, this suggests that the intrinsic capsid strength of HSV-1 is indeed lower than that of phage λ (and similarly of phage P22). The observed correlation of increasing ejection temperature with increasing internal DNA pressure, as determined by comparisons of different viral systems (as opposed to the same virus with different packaged DNA lengths), suggests an evolutionary adaptation of increased capsid stability for viruses that package their genome to higher densities. This adaptation has been previously proposed based on our AFM results probing the mechanical strength of DNA-filled versus empty viral capsids (54, 55). These combined DSC and SAXS measurements provide experimental support for this hypothesis of capsid strength matching packaged genome density, which facilitates retention of the highly pressurized genomes between rounds of infection.

(iv) Portal vertex, the Achilles heel of viral capsid stability: mechanism of DNA loss upon heating. In addition to the average genome spacings, the DNA scattering peak of SAXS experiments directly reveal the presence (or absence) of packaged DNA inside the capsid. Further, the scattering curves also show a separate set of peaks that correspond to the icosahedral structure of the intact viral particle. Using SAXS, we confirmed for both phage λ and HSV-1 that heat treatment results in DNA release without thermal disruption of the overall capsid structure. The specific temperatures required for DNA loss observed in DSC scans are in precise agreement with the SAXS experiments. Furthermore, loss of capsid structural integrity is observed only upon heating at further elevated temperatures, as predicted by the DSC results described above.

Figure 5a shows SAXS results for wt DNA length λ in 10 mM Mg^{2+} -Tris buffer for three different temperature incubations. The black curve corresponds to λ virions prior to heat treatment. The distinguishing features of the scattering curves are the packaged DNA peak at higher q values (inset) and the broader set of peaks corresponding to the icosahedral viral capsid structure. Phage λ virions were incubated at 78°C (Fig. 5a, red curve), which is above the temperature of DNA ejection observed by DSC (Fig. 1). After incubation at 78°C, the SAXS peak corresponding to packaged DNA is no longer present (inset). This confirms that DNA is released from the virus after a 30-min incubation at 78°C. Additionally, this incubation is below the temperature of the capsid protein denaturation event determined by DSC. As anticipated, the SAXS peaks representing the icosahedral capsid are still conserved (Fig. 5a, red curve). Conversely, viral particles heated at 88°C (which is above the temperature of capsid denaturation) (Fig. 5a, blue curve) no longer show the SAXS peaks that correspond to the intact capsid structure. These results confirm that heat incubation at 78°C causes DNA release from phage λ without disrupting the overall capsid structure.

Analogous SAXS experiments were also conducted for wt phage λ in the presence of EDTA (Fig. 5b). These results in the presence of EDTA demonstrate the same findings as observed above for wt DNA length λ in 10 mM Mg^{2+} -Tris buffer. In the

FIG 5 SAXS experiments demonstrating that heat treatment results in DNA release without loss of the overall capsid structure. The peaks at higher q values (insets) indicate the presence (or absence) of packaged DNA. The broader set of peaks from q values of ~ 0.01 to $0.1 \text{ \AA}^{-1}$ represents the intact viral capsid structure. Experiments were done on phage λ in 10 mM Mg^{2+} buffer (a) and in buffer plus EDTA (b), as well as on HSV-1 capsids in 10 mM Mg^{2+} -Tris buffer (c). In all cases, milder heat treatment (red curves) resulted in loss of the DNA peak without disruption of the overall capsid structure.

presence of EDTA, DSC scans reveal that DNA release occurs by 60°C and that thermal disruption of the capsid occurs at $\sim 72^\circ\text{C}$ (Fig. 2b). To further confirm that DNA release occurs without capsid disassembly under these buffer conditions, λ virions were incubated at 60°C , and their SAXS scattering profile was determined (Fig. 5b). As anticipated, heated virions (Fig. 5b, red curve) no longer produce the peak for packaged DNA (inset), which is clearly present for nonheated samples (black curve). Alternatively, the broad set of peaks that correspond to the overall capsid structure are still present for these particles after they are heated to 60°C . Incubation at 78°C (above the predicted capsid denaturation temperature) results in a loss of the broader SAXS peaks (Fig. 5b, blue curve) that demonstrate overall particle structure. These results confirm that heat-induced genome release from phage λ results in DNA loss without disruption of the overall capsid structure. This is true for phage λ in both 10 mM Mg^{2+} -Tris buffer (Fig. 5a) and in the presence of excess EDTA (Fig. 5b).

Using this same approach, we also confirmed that heat treatment of HSV-1 viral particles results in DNA loss without disruption of the capsid (Fig. 5c). DSC experiments show that DNA loss occurs at about 55°C in the presence of 10 mM Mg^{2+} -Tris buffer, and the subsequent capsid denaturation occurs at $\sim 82^\circ\text{C}$ (Fig. 3b). HSV-1 capsids prior to heat treatment (Fig. 5c, black curve)

show the scattering peaks corresponding to the intact capsid and the packaged DNA (inset). After incubation at 65°C , HSV-1 particles show a loss of the packaged DNA peak (inset, red curve). As also demonstrated above for phage λ , the scattering peaks corresponding to the intact capsid structure are still observed after genome loss occurs. The presence of these peaks after particle incubation at 65°C confirms that heat-induced DNA release from HSV-1 capsids does not result in loss of the overall capsid structure.

It is well established that harsh conditions, such as heating, result in loss of viral infectivity in proportion to the amount of virions that have lost their packaged genome (47, 48). However, the mechanism governing genome release remained undefined. It is of interest to speculate how DNA might be released from the viral capsid under these conditions.

The intrinsic stability of the HSV-1 capsid (in regard to DNA retention, described above) is lower than that of phage λ , despite HSV-1 capsids being thicker than λ capsids (smallest thickness is 4 nm between HSV-1 capsomer subunits [56] versus 1.8 nm in λ [57]). We also note that λ virions imaged by electron microscopy (EM) show intact capsid and tail structures after heat-induced genome release (Fig. 1). Together, these observations suggest that DNA loss may occur as a result of a small perturbation in the

FIG 6 (a) Slice through a three-dimensional reconstruction of P22 showing the packaged DNA (green) and the gp26 tail needle protein (yellow) (top). The approximate location of the gp26 tail needle trimer (green) is shown relative to the electron density of gp10 (gray) (bottom). His73 is highlighted in red (structure from reference 58). (b) DSC scans of P22 virions possessing wild-type (solid lines) or mutant (dashed lines) gp26 in 1 mM $MgCl_2$ -10 mM Tris buffer and upon addition of 2 mM EDTA. au, arbitrary units.

virion's structure rather than from large deformations in the capsid wall. Cryo-EM images of HK97 also showed intact virions after heat-induced ejection, and it was proposed that the portal vertex, which acts as the normal passageway for DNA into and out of the capsid, also serves as the conduit for DNA ejection upon heating (25). To investigate whether heat treatment might result in ejection through the portal-tail vertex (as opposed to random breaks in the capsid wall), we compared DSC scans of wild-type phage P22 to those of P22 virions that have a single histidine-to-leucine (H73L) mutation in the tail needle protein (gp26), illustrated in Fig. 6a. A homotrimer of this protein acts as a "plug" sealing the portal-tail complex opening to retain the packaged viral genome (58). This histidine residue is buried within the binding interface of the gp26 trimer. Mutation to leucine likely causes steric hindrance or loss of stabilizing hydrogen bonds, resulting in destabilization of the portal-tail complex. Indeed, in the presence of 1 mM $MgCl_2$ -Tris buffer, there is an $\sim 5^\circ C$ reduction of the DNA ejection temperature for P22 virions that incorporate the H73L gp26 mutant relative to that of the wild-type P22 (Fig. 6b, blue lines). Additionally, we performed the same comparison in the presence of 2-fold excess EDTA, which chelates Mg^{2+} ions in solution (and those bound to viral DNA within the capsid), further reducing the electrostatic screening between packaged DNA strands, resulting in an increased DNA pressure. Under these buffer conditions the destabilizing effect of pressurized DNA on the mutant tail needle protein is even more pronounced, resulting in nearly a $10^\circ C$ decrease in the ejection temperature compared to that of wt phage P22 (Fig. 6b, black lines). Reduced ejection temperatures for the gp26 P22 mutant strongly suggest that heat-induced DNA release relates specifically to destabilization and failure of the portal-tail vertex. Recent atomistic characterization of the viral portal suggests that it is specifically structured for enhancing mechanical reinforcement at this unique capsid vertex (59), in a manner analogous to that of proteins that provide structural support.

In addition to the measurements above, we have also demonstrated that *in vitro* DNA ejection from HSV-1 capsids triggered by heating remains directional, where the last genome end packaged

is released first (60). This conserved directional ejection further supports the notion of heat-induced DNA release occurring through the portal vertex, as anticipated for genome ejection *in vivo* (60). In these experiments, partial genome ejections were interrogated by Southern blot analysis utilizing nucleic acid probes complementary to specific sites of the HSV-1 genome. We previously adapted this approach to trypsin-induced HSV-1 genome ejections in the presence of an external osmotic pressure (created by the presence of PEG) (9). Under these conditions, only partial genome lengths are ejected due to the balance of internal DNA pressure and that of the external PEG solution. The partial genome lengths remaining within capsids are then extracted and subjected to Southern blot analysis. We previously found that trypsin-induced ejection results in the same conserved directionality for HSV-1 genome release (9). Here, we applied this approach to heat-induced genome release from HSV-1. Capsids were heated at $70^\circ C$ in the presence of 10 and 12% PEG 8000 (MW, 8,000 g/mol) (the temperature inducing DNA release from HSV-1 is higher in the presence of PEG since it stabilizes the capsid), resulting in partial genome ejections (9). After incubation at $37^\circ C$ with DNase to degrade ejected DNA, pulsed-field gel electrophoresis (PFGE) was performed on the nonejected partial genomes extracted from capsids (Fig. 7). Southern blot analysis of the resulting PFGE DNA bands clearly shows that heat-induced genome release follows the expected directionality for HSV-1 genome ejection, with ejection beginning at the S end of the HSV-1 genome (60).

In conclusion, we have used a new calorimetric assay to study the role of internal genome pressure for viral particle stability in terms of genome retention. The balance between viral capsid stability and its ability to withstand internal pressure directly influences the success of viral replication. An evolutionarily conserved correlation between capsid strength and the packaged DNA density in diverse viral systems provides new insight into viral metastability and fitness (61). Further understanding and manipulation of this delicate force balance offer new approaches for interfering with viral replication as well as designing viral vectors that can stably retain foreign nucleic acid.

FIG 7 Directionality of heat-induced HSV-1 genome ejection. (a) Ethidium bromide-stained gel of partially suppressed DNA remaining within HSV-1 capsids in the presence of 12% PEG 8000. Capsids were heated at 70°C to induce genome release, while the presence of PEG results in ejection of partial genome lengths. The lane on the left represents DNA molecular size markers. (b) The gel lanes from the experiment shown in panel a after Southern blot analysis using probes originating from the unique long (U_L; left two lanes; probe 1) or unique short (U_S; right two lanes; probe 2) genome ends. (c) Linear map of HSV-1 genome indicating the positions of probes 1 (16 to 18 kbp) and 2 (139 to 141 kbp) used in the experiment shown in panel b.

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