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Agarose Gel Electrophoresis Reveals Structural Fluidity of a Phage T3 DNA Packaging Intermediate

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Abstract

We find a new aspect of DNA packaging-associated structural fluidity for phage T3 capsids. The procedure is (1) glutaraldehyde cross-linking of *in vivo* DNA packaging intermediates for stabilization of structure and then (2) determining of effective radius by two-dimensional agarose gel electrophoresis (2d-AGE). The intermediates are capsids with incompletely packaged DNA (ipDNA) and without an external DNA segment; these intermediates are called ipDNA-capsids. We initially increase production of ipDNA-capsids by raising NaCl concentration during *in vivo* DNA packaging. By 2d-AGE, we find a new state of contracted shell for some particles of one previously identified ipDNA-capsid. The contracted shell-state is found when ipDNA length/mature DNA length (F) is above 0.17, but not at lower F . Some contracted-shell ipDNA-capsids have the phage tail; others do not. The contracted-shell ipDNA-capsids are explained by premature DNA maturation cleavage that makes accessible a contracted-shell intermediate of a cycle of the T3 DNA packaging motor. The analysis of ipDNA-capsids, rather than intermediates with uncleaved DNA, provides a simplifying strategy for a complete biochemical analysis of *in vivo* DNA packaging.

Keywords

average electrical surface charge density; bacteriophage DNA packaging motor; chemical cross-linking; effective particle radius; two-dimensional electrophoresis

1 Introduction

Bacteriophage DNA packaging is used as a model for both (1) understanding how biotic motors work (recent reviews: [1–5]), and (2) the related enterprise of determining how best to obtain high-resolution structure for complex assemblies, including assemblies that are at intermediate stages of transcription, protein synthesis, protein degradation, DNA replication, DNA recombination and DNA packaging (reviews of phage DNA packaging: [6–11]). The importance of analysis of intermediate stages is encapsulated in the following statement from Johnson's review of the cryo-electron microscopy (cryo-EM) of the dynamics of the shell of a phage HK97 capsid [12]. "Experimental studies of the transitions between the stable intermediate particles are the next challenge in virus maturation." The HK97 shell dynamics occurred when a HK97 DNA-free procapsid was exposed to non-physiological conditions. However, these dynamics were thought to mimic dynamics that occur *in vivo* for most double-stranded DNA phages when an initially assembled procapsid (capsid I for the

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related phages, T3 and T7; Figure 1a) undergoes DNA-packaging associated conversion to a larger, more angular capsid (capsid II for T3; illustrated in Figure 1b).

Studies of “intermediate particles” (to be called intermediates here, whether or not inactivated during isolation) have potential limitations, beginning with the difficulty of avoiding non-physiological process for the initial generation of the intermediates. Ideally, one obtains intermediates from a lysate of infected cells (*in vivo*) so that the intermediates are initially generated physiologically. The potential limitations continue with the following: (1) instability of intermediates during purification, (2) relatively small amount of any intermediate that is formed at a rate lower than the rate of conversion to a successor, (3) structural fluidity of intermediates, i.e., spontaneous inter-conversion of related intermediates during and after purification and (4) heterogeneity in the chemical and physical properties of intermediates, independent of fluidity. A fractionation-based approach is likely to be difficult-to-impossible to thoroughly implement unless the biological event being studied has properties that assist in either overcoming or bypassing the above limitations. Ideally, the biological event (phage DNA packaging in our case) will, at low frequency, spontaneously undergo premature termination at several stages and, at each termination, produce an intermediate that is “frozen in place” during subsequent purification.

A further advantage is achieved if, during purification, “frozen intermediates” can be fractionated by their extent of progression, i.e., the fraction, F , of the DNA packaged in the case of DNA packaging. In this case, a motor-like assembly can be biochemically/biophysically characterized as a function of a variable (F for DNA packaging) that increases as the motor progresses. That is to say, time is bypassed and the description of the cycle of an internal combustion engine [13] serves as a model for description of the cycle of a biotic motor [14]. F is analogous to the rotation angle of the camshaft of an internal combustion engine. The net result is an ordered series of “snapshots” of some aspects of the process being studied. These snapshots constitute a major constraint for a comprehensive analysis of the mechanism of a biotic motor.

In two previous studies [15, 16], we obtained such snapshots of phage DNA packaging by analysis of DNA packaging intermediates with incompletely packaged DNA (ipDNA). These intermediates were obtained from lysates of phage T3-infected cells; no non-physiological conditions were involved in initial generation of the intermediates. The ipDNA of these intermediates was packaged in a capsid; no external DNA segment was detected (ipDNA-capsids). The ipDNA-capsids were generated by premature cleavage of the DNA being packaged. Packaged T3 DNA begins *in vivo* as an end-to-end concatemer of mature genomes that is twice cleaved during packaging for each mature genome produced. The enzyme that performs the cleavage is called terminase, gp19 for phage T3/T7, the N-terminus of which also serves as a DNA packaging ATPase (reviews: [6, 10]; see Figure 1b,e). T3 proteins are named by gp, followed by the number of the gene (reviewed in [17]) that encodes the protein.

In previous studies, initial detection and characterization of ipDNA-capsids was critically dependent on one-dimensional agarose gel electrophoresis in the absence of a denaturant (1d-AGE). The samples were fractions of a cesium chloride density gradient that had been used for pre-fractionation of the ipDNA-capsids by buoyant density centrifugation. The buoyant density centrifugation fractionated the T3 ipDNA-capsids according to F [15, 16]. The relatively small amount of ipDNA-capsids and large number of fractions made 1d-AGE essential for efficient detection. After fractionation according to F and detection via 1d-AGE, we determined the conformation of ipDNA in capsid II vs. F by cryo-EM with 3D reconstruction [15].

When heterogeneity of electrophoretic mobility (μ) compromised this analysis for ipDNA-capsids relatively minor in amount, we introduced two-dimensional agarose gel electrophoresis in the absence of denaturant (2d-AGE; details are presented below), which produced a clear image of the pattern of heterogeneity [16]. The use of 2d-AGE also enables the separation of the effects on μ of effective radius (R_E) and average electrical surface charge density (σ), the two variables that determine μ in a gel (reviews: [18, 19]). We found that the “heterogeneous” ipDNA-capsids had shells that were larger than the shell of the mature phage T3 (hyper-expanded, or HE ipDNA-capsids) [16].

Further analysis requires either the overcoming or the bypassing of the following limitations of current analysis: (1) As mentioned above, possible structural fluidity of intermediates has not yet been analyzed. That is to say, each intermediate currently known is possibly more than one intermediate with subpopulations that spontaneously inter-convert in solution. (2) The ipDNA-capsids, as currently isolated, are inactivated by the DNA cleavage that makes possible their isolation and characterization. The DNA cleavage implies that we cannot observe the further progression of these intermediates in DNA packaging. We do not directly address the latter limitation here, but will discuss below how the current work builds a foundation for addressing this limitation. The former limitation is potentially overcome by cryo-EM with sorting of single intermediates by state. However, no guarantee exists that the process of freezing for cryo-EM preserves the distribution of inter-converting solution states. We present data below that suggest that this potential limitation of cryo-EM occurs in practice.

In the present communication, we address potential structural fluidity of T3 ipDNA-capsids by using protein-protein cross-linking to block fluidity. We then re-visit the characterization of T3 ipDNA-capsids by 2d-AGE. We find an additional intermediate that fits with expectations based on the hypothesis that a capsid expansion/contraction cycle exists at the latter stages of T3 DNA packaging. We discuss how future studies can be designed to directly test for such a cycle. The principles of the present and projected future analysis of DNA packaging are also applicable to the other biological events mentioned above.

2 Materials and Methods

2.1 Growth and production of phages, capsids and ipDNA-capsids

Phages, their capsids and ipDNA-capsids were prepared and concentrated by the following procedure. *Escherichia coli* BB/1 was grown to a concentration of 4×10^8 per ml with aeration at 30 °C in 6 liters of 2xLB (growth) medium: 20 g Bacto tryptone, 10 g yeast extract, 5 g NaCl in 1 liter of water. The culture was then infected at a multiplicity of 5 with wild type phage T3. The infection was continued with aeration at 30 °C until 21.5 min after infection, at which time 0.25 liters of either growth medium alone or growth medium with NaCl were added and incubation was continued. To initiate lysis, 12 ml chloroform was added to the culture at 39 min after infection and incubation was continued until the culture cleared.

The lysates were then processed by procedures that have been previously described [15, 16] and that included, in brief (1) clarification by low speed centrifugation, (2) precipitation with 9% polyethylene glycol 8,000 at 4 °C for 2–4 days, (3) a second clarification by low speed centrifugation, (4) a second precipitation with polyethylene glycol 8,000, (5) partial digestion of DNA outside of capsids, (6) centrifugation of the concentrated lysate through a cesium chloride step gradient and (7) buoyant density centrifugation in a 5 ml cesium chloride density gradient of the entire ipDNA-capsid region of the step gradient (20 hr; 42,000 rpm; 4 °C; starting density = 1.340 g/ml). The buoyant density centrifugation fractionated ipDNA-capsids by ipDNA length; the longer the ipDNA is (i.e., the higher F

is), the denser the ipDNA-capsid is [15, 16]. The gradient was fractionated by pipeting from the top.

T3 phage and DNA-free capsids were further purified, as follows. In the case of T3 phage, the phage fraction of the cesium chloride step gradient was subjected to buoyant density centrifugation as done for the ipDNA-capsids, but with a higher starting density, 1.499 g/ml, and shorter time of centrifugation, 16 hr. To prepare MLD capsid II for either T3 or T7 phage, the capsid II particles from a cesium chloride buoyant density centrifugation were dialyzed against 0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M MgCl_2 and then further purified by buoyant density centrifugation in a Metrizamide density gradient, as previously described [20]. To prepare capsid I for either T3 or T7, the capsid I from a cesium chloride buoyant density centrifugation was further purified by rate zonal centrifugation in a sucrose gradient [20].

2.2 1d-AGE and 2d-AGE: Determination of the effective radius of ipDNA-capsids

The ipDNA-capsids were initially detected by use of 1d-AGE. A sample of a density gradient fraction was added to a 0.35x amount of the following buffer (electrophoresis buffer), without dialysis: 0.09 M Tris-acetate, pH 8.4, 0.001 M MgCl_2 . A 0.11x amount of 60% sucrose in electrophoresis buffer was then added. The diluted samples were layered in the wells of a horizontal, submerged 0.9 % agarose slab gel (Seakem LE agarose, Lonza, Rockland, Maine). The gel had been cast in and submerged under electrophoresis buffer. The layered samples were allowed to remain in place for 60 min pre-electrophoresis in order to dialyze enough cesium chloride out of the sample so that increased ionic strength at the origin did not interfere with the initial movement of particles during electrophoresis. Electrophoresis was then conducted at 2.0 V/cm, 25 °C for 10 hr. After electrophoresis, the gel was stained for DNA with 1/10,000 diluted GelStar (Lonza, catalogue #50535) in electrophoresis buffer, visualized with an ultraviolet transilluminator and photographed. The gel was then stained for protein with Coomassie Brilliant Blue G-250 [15].

Some fractions of density gradients were also characterized in more detail by use of 2d-AGE. The sample preparation for 2d-AGE was the same as it was for 1d-AGE, except that 100 $\mu\text{g/ml}$ bovine serum albumen was added, just before layering in a sample well, to reduce adherence to the gel origin; this was not done for 1d-AGE because the albumen was not resolved from capsids during 1d-AGE. The first dimension gel was 0.3% agarose and was one of four first dimension gels embedded within an agarose frame that included the second dimensional gel. That is to say, four 2d-AGE fractionations were performed within one composite gel; each fractionation occurred in a separate quadrant. The agarose concentration of the second dimension gel was 2.0% unless otherwise specified; Seakem LE agarose was used for both dimensions. The composite gel was submerged beneath electrophoresis buffer. The first electrophoresis was performed at 2.0 V/cm for 3.0 hr. Unless otherwise indicated, the second electrophoresis was performed at 1.8 V/cm for 8.5 hr. The direction of the second electrophoresis was rotated 90° relative to the direction of the first electrophoresis; the direction was changed without touching the gel (reviews: [18, 19]). In 2d-AGE profiles, the arrows indicate the directions of the first (I) and second (II) electrophoresis. The arrowheads bracket the sample well edge at the first dimension gel.

Previously described procedures [21] were used to R_E values from the position of a band in relation to positions of standards, after 2d-AGE. The 0.3% first dimension gel is the least concentrated gel that essentially never breaks.

Standards were (1) T7 MLD capsid II, for which the radius = 28.2 nm, based on the small-angle x-ray scattering from DNA-free capsid II [22] in a fraction from which MLD capsid II was subsequently isolated, (2) T7 glutaraldehyde cross-linked capsid I, radius = 26.3 nm,

based on small-angle x-ray scattering [22], and (3) T7 phage, radius = 30.1 nm, based on small-angle x-ray scattering [22]. The radius of T3 phage is the same as the radius of T7 phage [20]; the values of R_E for the T3 versions of MLD capsid II and glutaraldehyde cross-linked capsid I were indistinguishable from the T7 versions of these particles, as determined by 2d-AGE (data not shown). Thus, the T3 counterparts were used as the standards.

2.3 Cross-linking with glutaraldehyde

The following solution was used to cross-link (with the intent of freezing the conformation of) particles in an undialyzed fraction from a cesium chloride buoyant density centrifugation: 25% glutaraldehyde (EM grade, Polysciences, Warrington, PA) diluted 1:1 with 1.0 M sodium phosphate, pH 7.4, just before use. One part of the diluted glutaraldehyde was added to 3 parts of a portion of a density gradient fraction. This mixture was incubated at 30 °C for 30 min and then immediately layered in a sample well for 2d-AGE. The Tris in the electrophoresis buffer reacted with the glutaraldehyde in the submerged sample well and stopped the cross-linking. Glutaraldehyde cross-linked standards used in Figure 5, panel i were dialyzed against the following buffer before storage and use: 0.2 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M MgCl₂.

2.4 Electron microscopy

We performed electron microscopy by negative staining, with procedures previously described [16]. The following negative stains were used: 1.0 % sodium phosphotungstate, pH 8.8 and 1.0% uranyl acetate without pH adjustment (pH ~ 4.2). Negatively stained specimens were observed in either a Philips 208S or a JEOL100CX electron microscope in the Department of Pathology at The University of Texas Health Science Center at San Antonio.

3. Results

3.1 Increasing the yield of ipDNA-capsids with minimal inhibition of DNA packaging

To help provide sufficient ipDNA-capsids for the current study, we tested procedures for increasing the yield of the ipDNA-capsids found in a lysate of T3-infected cells. When a procedure to increase yield of intermediates was not used, only approximately 1 HE ipDNA-capsid was found per 10 infected cells, for example [16]. For reasons described in Section 4.2, we predicted that adding NaCl during DNA packaging will increase the yield of ipDNA-capsids.

To test the accuracy of this prediction, we introduced the following modifications to the previous [15] procedure of cellular lysis and pre-purification. First, we infected host cells at a multiplicity of 5, rather than the multiplicity of 0.01 used in [15]. This change increased infection synchronization. Second, we artificially lysed the culture with chloroform at 39 min after infection, rather than allowing spontaneous lysis; the cultures cleared at ~ 50 min after infection. This change both increased infection reproducibility (at 30 °C) and reduced contamination of ipDNA-capsids with cellular envelope fragments. With these modifications in procedure, we tested the effects of additions, described below, of NaCl to T3-infected cells at 21.5 min after infection, a time at which DNA packaging is active [20].

For determining the effects on ipDNA-capsid yields of additions to T3-infected cells during DNA packaging, we initially used 1d-AGE of fractions of cesium chloride density gradients that had been used to fractionate ipDNA-capsids by *F* via buoyant density centrifugation. The 1d-AGE profiles, below, were interpreted by use of assignments that had been previously made [15] via (1) protein composition determined by use of sodium dodecylsulphate polyacrylamide gel electrophoresis/mass spectrometry, (2) morphology

determined by electron microscopy, (3) R_E determined by 2d-AGE and electron microscopy and (4) ipDNA length (and F) determined by gel electrophoresis of DNA expelled from ipDNA-capsids.

Addition of sodium chloride caused an increase in the yield of ipDNA-capsids, as judged by 1d-AGE with GelStar staining (nucleic acid-specific) of the various fractions of a cesium chloride density gradient. Figure 2a shows 1d-AGE analysis for a control lysate with only growth medium added to the infected culture at 21.5 min after infection. The density (g/ml) of the contents of a fraction in Figure 2 is indicated above each lane; we will sometimes name a fraction by the density of its contents. Figures 2b and 2c show 1d-AGE analysis of comparable fractions obtained by cesium chloride buoyant density centrifugation of ipDNA-capsids from parallel infections that differed from the control infection in that the growth medium added at 21.5 min had additional sodium chloride. The final [NaCl] added was either 0.2 M (Figure 2b) or 0.4 M (Figure 2c), as also indicated at the bottom of a panel. The most dramatic NaCl-induced change in staining intensity occurred for the hyper-expanded (HE) ipDNA-capsids. The HE ipDNA-capsids are the GelStar-staining particles that migrated more rapidly than ipDNA-capsid II, in the region of the gel indicated by HE at the left in Figures 2,a–c ([16]; confirmed in the next section). Based on the staining intensities of Figure 2, the 0.2 M and 0.4 M NaCl increased the HE ipDNA-capsid amount by 3–5x and 5–10x. The increase was sufficient to visualize the HE ipDNA-capsids by protein-specific, Coomassie blue staining for the first time; Coomassie staining is not shown for the gels of Figure 2, but is shown below.

As previously found [16], HE ipDNA-capsids were detected only for $F > 0.28$ in Figure 2. A vertical arrow in Figure 2 (above the row of densities) indicates the fraction that has ipDNA-capsids with $F = 0.28$. F increases monotonically as density increases [15, 16].

The remaining ipDNA-capsids, identified as ipDNA-capsid II [15], had a similar response to added NaCl, although smaller in magnitude. This result is seen by inspection of Figures 2,a–c. The ipDNA-capsid II formed two bands in the capsid II region of the agarose gels of Figure 2. The more slowly migrating, GelStar-staining capsid II (called slow ipDNA-capsid II; indicated by S-CII in Figure 2) is an ipDNA-capsid II that has the phage tail; the more rapidly migrating, GelStar-staining ipDNA-capsid II (called rapid ipDNA-capsid II; indicated by R-CII in Figure 2) is an ipDNA-capsid that does not have the tail ([15]; see Figure 1). Even though F increases as density increases in Figure 2, the migration distance of the ipDNA-capsid II's does not detectably vary with density (i.e., with F) during 1d-AGE. This result is expected because σ and R_E do not depend on F (see also [15]).

In summary, the addition of NaCl at 21.5 min after infection had the useful outcome of increasing the rate of premature termination of T3 DNA packaging, thereby increasing the amount of known ipDNA-capsids. However, the perturbation of the infection was small enough that the yield of mature phage particles did not significantly vary among the three infections of Figure 2 (~ 60 phage particles/infected cell; data not shown). Thus, we performed further analysis of ipDNA-capsids from the 0.4 M NaCl-enhanced lysate.

3.2 Further analysis of ipDNA-capsids by 2d-AGE: Preliminary tests

The gel electrophoretic analysis of (intact) ipDNA-capsids eventually required a second dimension of gel electrophoresis because μ is determined by both R_E and σ (reviews: [18, 19]). To distinguish effects of R_E from effects of σ , 2d-AGE is performed with the concentration of the gel for the first electrophoresis (dimension) lower than it is for the second. The concentration of the gel for the first dimension is low enough so that dependence of μ on R_E is minor (although not zero) and fractionation is primarily by σ . The concentration of the gel for the second dimension is much higher. In the second dimension,

the gel exerts a sieving effect that fractionates by R_E , in addition to the fractionation by σ . As the concentration of the second dimension gel increases, the resolution by R_E increases until the effective gel pore becomes so small that the particles do not enter the gel. As illustrated in Figure 3a, the R_E of a particle is a decreasing function of the angle (θ) between the direction of the first dimension and a line (called a size line) from the effective electrophoretic origin (O in Figure 3a) through the center of the band formed by the particle of unknown R_E . The linearity of a size line depends only on the assumption that the following ratio depends only on R_E and not on σ : second dimension μ /first dimension μ [18]. As also illustrated in Figure 3a, values of σ increase in magnitude as distance migrated in the first dimension increases. The 2d-AGE used here is one of several, different gel electrophoretic procedures that include a second dimension [23].

When analyzed by 2d-AGE, the ipDNA-capsids were not qualitatively changed by the addition of 0.4 M NaCl used for the culture of Figure 2c. The 2d-AGE profile for uncross-linked, GelStar-stained (nucleic acid-specific) ipDNA-capsids had both the slow ipDNA-capsid II (has a tail) band and the rapid ipDNA-capsid II (has no tail) band previously observed [15] after 2d-AGE with GelStar staining. Analysis of a fraction with a density of 1.351 g/ml is in Figure 4a, panel iii; analysis of a fraction with a density of 1.367 g/ml is in Figure 4a, panel iv. In figures, slow ipDNA-capsid II is abbreviated S-CII; rapid ipDNA-capsid II is abbreviated R-CII. The capsid protein of both fractions was present in amount sufficient to detect by use of the protein-specific (but, less sensitive) stain, Coomassie Brilliant Blue, as shown for the fraction with a density of 1.367 g/ml in Figure 4b, panel iv. In addition to the ipDNA-capsid II, an arc formed by the previously demonstrated [16] HE ipDNA-capsids is seen in Figure 4a, panels iii and iv (DNA-stained) and Figure 4b, panel iv (protein stained); this arc is indicated by HE.

3.3 Further analysis of ipDNA-capsids by 2d-AGE: Effects of glutaraldehyde cross-linking

Thus, we proceeded with an attempt to determine whether known ipDNA-capsids were fluid in structure. The strategy used was to cross-link the ipDNA-capsids with glutaraldehyde before analysis by 2d-AGE. The rationale was that the cross-linking would prevent inter-conversion of particles in sub-populations of ipDNA-capsids and, therefore, might produce a collection of structurally non-fluid intermediates that would be resolved as separate bands during 2d-AGE and would represent different states of the uncross-linked ipDNA-capsids.

To implement this strategy, we did the following. We adjusted the time of cross-linking to be 50% more than the time needed to obtain saturation. We determined saturation of cross-linking via the end-point of a cross-linking-induced increase in the magnitude of the negative μ of MLD capsid II (a particle described in more detail below) as the time of cross-linking increased; this increase was caused by an increase in the magnitude of σ because R_E was not changed, as shown below. The data for saturation are not shown. This procedure of cross-linking was essentially identical to a procedure previously used to stabilize the procapsid of the T3-related phage, T7, during pelleting for low angle x-ray scattering [22]. If sub-populations exist and this strategy works, during 2d-AGE, we will see (1) an increase in the magnitude of μ and (2) band splitting.

After glutaraldehyde cross-linking, particles of both slow and rapid ipDNA-capsid II did, indeed, undergo both band splitting and the expected (previous paragraph) increase in the magnitude of μ . The increase in the magnitude of μ occurred in both dimensions (as expected), as observed for both slow and rapid ipDNA-capsid II in fractions with densities of 1.251 g/ml (GelStar stain of cross-linked sample: Figure 4a, panel i) and 1.367 g/ml (GelStar stain of cross-linked sample: Figure 4a, panel ii; Coomassie blue stain of the same sample: Figure 4b, panel ii). For particles in fractions of both densities, the increase in the magnitude of μ was accompanied by apparent splitting of both slow and rapid ipDNA-

capsid II bands, so that four bands formed. This splitting, to be shown at higher magnification below, was not observed for the uncross-linked samples, even when the time of electrophoresis was increased so that the distance of migration was the same as that in Figure 4, panels i and ii (not shown).

The four bands observed after cross-linking were all formed by ipDNA-capsid II based on (1) a R_E close to that of capsid II, as shown in detail in Section 3.5, (2) staining with both GelStar (panels i and ii of Figure 4a) and Coomassie blue (panel ii of Figure 4b), (3) capsid II-like morphology of all approximately capsid-sized particles, as shown in detail in Section 3.9, and (4) undergoing of similar cross-linking induced change in μ by purified, DNA-free capsid II, although without forming additional bands (Figure 5 and Legend, below). The two additional bands of cross-linked ipDNA-capsid II are explained by splitting of both slow and rapid ipDNA-capsid II bands to form two bands each. The two products of the splitting of one band are separated almost vertically, as indicated by the brackets labeled S-CII and R-CII, respectively, in Figure 4a, panels i and ii. The ends of the brackets indicate the migration in the first dimension.

If taken at face value, this band splitting indicates (1) difference in R_E , based on size lines, by reference to Figure 3a (size lines are not drawn in Figure 4 to avoid obscuring data) and (2) difference in σ , based on distance migrated in the first dimension, also by reference to Figure 3a. Given the newness of the observation of band splitting of this type, we performed tests of whether the band splitting can be taken at face value or, alternatively, whether it was an artifact, i.e., a phenomenon caused by the process of 2d-AGE, rather than by the properties of the particles fractionated by 2d-AGE.

3.4 Tests of the source and significance of the band splitting

The first test was to compare the 2d-AGE profile of glutaraldehyde cross-linked ipDNA-capsids to the 2d-AGE profile of both glutaraldehyde cross-linked and uncross-linked versions of a previously isolated, DNA-free capsid II that was expected not to vary in either R_E or σ . This latter capsid II was isolated by its relatively high hydration and low density during buoyant density centrifugation in a Metrizamide density gradient (Metrizamide low density, or MLD, capsid II) and is illustrated in Figure 1b. The high hydration is caused by impermeability to Metrizamide ([20]; Materials and Methods Section). The gp10 shell of MLD capsid II has been found by 3D, symmetrized reconstruction of cryo-electron micrographs to have a structure unique enough at 0.35 nm resolution to reconstruct (W. Jiang and P. Serwer, unpublished observations; see also [15]).

In confirmation that the band splitting was not an artifact and is to be taken at face value, the band splitting was not observed after 2d-AGE of glutaraldehyde cross-linked MLD capsid II (band indicated by CII-g in Figure 5, panel i) or uncross-linked MLD capsid II (CII in Figure 5, panel i), after Coomassie staining. In addition, a single band was observed in Figure 5 for both uncross-linked capsid I (CI band in Figure 5) and cross-linked capsid I (CI-g band in Figure 5). A single CI-g and CII-g band was also seen when the other particles were not present and the gel was stained with Coomassie blue (data not shown).

Nonetheless, band splitting was observed after 2d-AGE of glutaraldehyde cross-linked rapid ipDNA-capsid II particles analyzed in a separate quadrant embedded within the same Coomassie-stained gel frame (R-CII in Figure 5, panel ii). Also present in these samples was bovine serum albumen (indicated by BSA in Figure 5), added as a carrier just before gel loading and, therefore, of variable σ , because of non-saturating, variable levels of reaction with glutaraldehyde. In Figure 5, panel ii, slow ipDNA-capsid II was also observed and underwent band splitting, when the more sensitive GelStar staining was used (not shown); dashed circles indicate the positions of the two slow ipDNA-capsid II bands in Figure 5,

panel ii, based on the GelStar staining. Thus, the first test indicates that band splitting is not a feature of the procedure, i.e., is not artifactual for either slow or rapid ipDNA-capsid II.

A second test was to determine the effect of changing the concentration of the second-dimension gel, while adjusting the time of the second electrophoresis so that the distance migrated in the second dimension remained constant within 10%. For each agarose percentage used in a second dimension gel, particles in fractions with densities of both 1.343 g/ml and 1.419 g/ml were analyzed in two different quadrants of one composite gel. The result was that the R_E -component of the splitting (i.e., the component that caused formation of two size lines) decreased as the concentration of the second dimension gel decreased from 2.0% to 1.6% and 1.4%, for both slow and rapid ipDNA-capsid II (Figure 6; gel percentage is indicated at the top; fraction density in g/ml is indicated at the right; only the ipDNA-capsid region is shown). That is to say, the formation of two size lines is caused by gel sieving, not by an artifact of electrophoresis, and one source of the splitting is confirmed to be a difference in R_E .

Nonetheless, a second source of the splitting is confirmed to be σ , as shown by the following. When sieving was reduced, two size lines approached each other, as illustrated in Figure 3b for two split bands similar to those in Figure 6. In addition, the gap between outermost bands fills, as illustrated by the insert of Figure 3b and discussed in the Legend to Figure 3. This change did, indeed, empirically occur in Figure 6, as seen via by the filling of the gap between outermost bands along the single size line, when resolution by R_E was lost at the lower second dimension gel concentrations in Figure 6. But, four bands were no longer resolved because the loss of fractionation by R_E caused the overall separation to decrease. The particles that form the observed bands cannot be identified after loss of fractionation by R_E . The occurrence of the above gap filling also confirms the slow and rapid capsid II designations that have been assigned these particles above.

The profiles of Figure 6 also indicate that the size difference revealed by the band splitting was greater at 1.419 g/ml than it was at 1.343 g/ml. This conclusion is drawn from the observation that, as the gel percentage was reduced in Figure 6, the split bands first moved to the same size line (and were no longer well resolved from each other) for the 1.6% gel at 1.343 g/ml and for the 1.4% gel for at 1.419 g/ml. Thus, at the time that cross-links were introduced, the shell of ipDNA-capsid II was structurally fluid enough to have a detectably variable R_E .

3.5 The R_E values of the glutaraldehyde cross-linked ipDNA-capsids

To determine the R_E values for the various cross-linked ipDNA-capsids II, we can, in theory, compare migration during 2d-AGE with the migration of standards of known R_E . The standards would be in a separate quadrant of the same composite gel during 2d-AGE. The basic foundation for comparative sieving-based determination of R_E has been previously established for spherical particles (see [21]) with confirmation via studies of rod-shaped particles [24]. The radii used for calibration in [21] were obtained primarily by small-angle x-ray scattering. All data obtained by small-angle x-ray scattering has background that is subtracted ([22], for example). Some of this background is potentially caused by fluidity of radius.

Recently, some results of cryo-electron microscopy (cryo-EM) produced radii that raise further questions about (1) whether the shells of even the mature versions of the related phages, T3 and T7, are fluid in structure in solution and, therefore, (2) what radii should be used for these particles when they are used as standards for 2d-AGE. Specifically, the emptied, DNA-free shell of the T3-related phage, T7, was found by cryo-EM to be identical in radius to the DNA-filled shell of phage T7, except for a 0.08 nm outward bowing of faces

of the outer, empty, icosahedral shell. The same observation was made for phage HK97 [25]. Yet, the DNA-free shell of mature T7 has been found by small angle x-ray scattering to have the radius of capsid II [22], i.e., a radius 6–7% smaller than the radius of phage T7. We (W. Jiang and P. Serwer, unpublished observations) have confirmed that MLD capsid II is no smaller than mature phage by cryo-EM, in the case of both T3 and T7. No indication exists, to our knowledge, that either the results from x-ray scattering or the results from cryo-EM are incorrect.

One possible (not proven) explanation is that (1) more than one radius exists for capsid II-like capsids in solution, (2) thermally driven fluidity of radius occurs and (3) most particles of empty capsid II-like capsids, including the emptied capsid of the phage, are selected for relatively large R_E during preparation for cryo-EM, either during pre-freezing suspension of particles in a thin film (i.e., via increase of the percentage of particles at the liquid-air interface) or subsequent freezing. The small-angle x-ray scattering-determined values of radius (Materials and Methods Section) will be used for standards here because, like the R_E values of 2d-AGE, these radii were determined in (unfrozen) solution.

The R_E of the larger versions of both slow and rapid T3 ipDNA-capsid II was found to be indistinguishable from the R_E of MLD capsid II, by use of purified T3 capsid I and T3 MLD capsid II as standards for radius during 2d-AGE. This conclusion was drawn from comparison of the migration of the standards in Figure 5, panel i with the migration of the glutaraldehyde cross-linked ipDNA-capsids from a fraction with a density of 1.372 g/ml (Figure 5, panel ii). Figures 5, panels i and ii, show separate quadrants of the same gel. The standards include uncross-linked T3 MLD capsid II (CII in Figure 5, panel i), glutaraldehyde cross-linked T3 MLD capsid II (CII-g), uncross-linked T3 capsid I (CI) and glutaraldehyde cross-linked T3 capsid I (CI-g). Also, in Figure 5, panel i, a dashed size line has been drawn for the CI-g band (indicated by CI-g at the lower right). Inspection of Figure 5, panel i reveals no significant change in θ (and, consequently, no significant change in R_E) caused by the cross-linking of either capsid I or MLD capsid II.

The following was done to determine the radii of the slow and rapid ipDNA-capsid II in Figure 5b. First, the CI size line from Figure 5, panel i was reproduced by horizontal translation in Figure 5, panel ii. Then, inspection of Figure 5, panel ii showed directly that the R_E of the larger versions of both slow and rapid ipDNA-capsid II is larger than the R_E of capsid I. Finally, the same analysis was performed with the CII size line (not shown), which revealed that the larger versions of both slow and rapid ipDNA-capsid II have a R_E indistinguishable from the R_E of MLD capsid II (28.2 ± 0.3 nm [22]), with an accuracy of ± 0.1 nm. We note that the accuracy of the comparative 2d-AGE-based determination of differences of R_E is higher than the accuracy of the absolute determination of radius by small-angle x-ray scattering.

Furthermore, Figure 5, panel ii shows that the smaller versions of both slow and rapid ipDNA-capsid II have θ larger and, therefore, R_E smaller than the R_E of either capsid I or MLD capsid II. Quantitative analysis, based on [21], indicates an effective radius of 25.3 ± 0.3 nm for the smaller version of both slow and rapid ipDNA-capsid II. That is to say, the smaller version is contracted by ~11% relative to MLD capsid II and ~4% relative to capsid I.

3.6 Cross-linking of HE ipDNA-capsids

Although the particle analyzed in the current investigation is cross-linked ipDNA-capsid II, cross-linked HE ipDNA-capsids were also observed in the same samples after 2d-AGE. The HE arc was not observed in the 2.0 % second dimension gel of Figure 4a, panels i and ii, but was observed when the concentration of the second dimension gel was decreased to 1.4%

during 2d-AGE (data not shown). Thus, cross-linking appears to have increased the size of the HE ipDNA-capsids. In support, some HE ipDNA-capsids in Figure 4a, panels iii and iv and in Figure 4b, panel iv (labeled HE) underwent an apparent move to a position of higher θ after cross-linking (HE in Figure 4a, panels i and ii and Figure 4b, panel ii). The corresponding value of R_E is above 60 nm, but cannot be determined precisely with the current size standards.

For completeness, we note the following evidence in Figure 4 of additional effects of glutaraldehyde cross-linking. In Figure 4b, panel ii, particles not yet discussed are observed in the profile of cross-linked protein from the fraction with a density of 1.367 g/ml. These particles form two parallel straight lines that extend toward the origin from each of the split slow ipDNA-capsid II bands. Thus, the profile of staining has double-rail appearance, with each rail coincident with a size line and with each rail obscuring one of the slow ipDNA-capsid II bands. This double rail is indicated with a bracket labeled with a question mark in Figure 4b, panel ii and is not observed in the profile of the same sample, uncross-linked (Figure 2b, panel iv). Therefore, the cross-linking has converted other particles to the particles that form the double-rail.

The double rail-forming particles are in the class of capsid II, based on R_E , because they are on the size lines of the larger and smaller versions of slow and rapid ipDNA-capsid II. However, no DNA was detected in any of them (Figure 4a, panel ii), although split versions of both slow and rapid ipDNA-capsid II bands were present in Figure 4a, panel ii. The double rail-forming particles are differentiated from capsid II by σ that is both variable and smaller in magnitude than the σ of any cross-linked particle previously observed with a capsid II shell.

The double rail-forming capsids in Figure 4b, panel ii are apparently produced by the following means. (1) During cross-linking, some HE ipDNA-capsid particles expel DNA and change to a more capsid II-like capsid. (2) While this occurs, the access of glutaraldehyde to some surface amino groups is blocked so that the glutaraldehyde-induced increase in the magnitude of σ is decreased.

3.7 Band splitting as a function of the density of ipDNA-capsid II

Given that glutaraldehyde cross-linking does not cause band splitting during 2d-AGE of MLD capsid II ($F = 0.0$), we further investigated the correlation with F of band splitting. Analysis by 2d-AGE of glutaraldehyde cross-linked ipDNA-capsids was performed as a function of density (F increases as density increases), using fractions of a single cesium chloride density gradient. The band splitting was not observed at a density of 1.257 g/ml, although a relatively intense band of rapid ipDNA-capsid II (but not slow ipDNA-capsid II) was present after GelStar staining (Figure 7; images are labeled by density in g/ml; only the capsid II region of a gel is shown). Band splitting was first observed at a density of 1.319 g/ml (Figure 7). The difference in θ between the size lines of the two products of splitting increased slightly (about 5%) as the density increased to 1.343 g/ml and 1.419 g/ml in Figure 7, as expected from the results of Figure 6.

The length of the ipDNA at the onset of band splitting was determined by expulsion of ipDNA from the 1.319 g/ml capsids and fractionating the expelled DNA by agarose gel electrophoresis. The result was that the onset of band splitting occurred at an F of 0.17 ± 0.02 (data not shown). This is an F significantly smaller than the F at the onset of the appearance of HE ipDNA-capsids, 0.28 [16].

3.8 Some additional features of the profile of gradient fractions after 2d- and 1d-AGE

The 2d-AGE profiles of Figure 4a, panels iii and iv, also had a second arc, revealed by GelStar-staining, and formed by unpackaged DNA (indicated by DNA in Figure 4a; see also [16]). Some unpackaged DNA is also seen during 1d-AGE (Figure 2), in the region further from the origin than the HE ipDNA capsids (indicated by a bracket labeled DNA in Figure 2) for fractions with densities between 1.34 and 1.41 g/ml. No bands are seen. Based on the 2d-AGE, some unpackaged, broadly distributed (no bands) DNA also forms a background in the HE region of the 1d-AGE profiles. The source of this unpackaged, band-free, broadly distributed DNA is not known. In contrast, sharp DNA bands were observed in the 1d-AGE profile of fractions that had the highest density content (1.457 g/ml), in case of the fractionation of the ipDNA-capsid-enriched lysates of Figures 2b and 2c.

The DNA that formed these sharp bands originated in ipDNA-capsids, based on the observations that (1) these bands were much weaker for the comparable, 1.455 g/ml fraction of the control fractionation of Figure 2a and (2) similar sharp band-forming ipDNA has previously been observed [16] only after expulsion of DNA from ipDNA-capsids with density that corresponds to $F > 0.28$. The sharp band-forming ipDNAs of the 1.457 g/ml fraction of Figures 2b and 2c were expelled from ipDNA-capsids either before or during buoyant density centrifugation because they were found in a fraction whose contents were denser than the contents of the peak fraction of phage particles (labeled ϕ in Figure 2); the position in the agarose gel of T3 phage is determined from the band in the marker lane, m, for which the sample was T3 phage purified through an additional density gradient. Presumably, the denser-than-phage ipDNA will eventually pellet, because the density of double-stranded DNA is 1.70 g/ml.

Thus, the finding of sharp band-forming DNA in the 1.457 g/ml fractions of Figures 2b and 2c suggests instability of some ipDNA-capsids. Nonetheless, after the buoyant density centrifugation, the remaining ipDNA-capsids did not significantly change their 2d-AGE profile for at least 6 months, the longest time tested (data not shown). Thus, unstable ipDNA-capsids, in addition to the stable ipDNA-capsids investigated here, exist and have, obviously, not yet been characterized. The unstable ipDNA-capsids are likely not to have a tail to stabilize the packaged DNA (i.e., they are likely to be rapid ipDNA-capsid II). The presence of cesium chloride in the storage solution has been previously found essential for the stability of at least ipDNA-capsid II [15]. Thus, for the above experiments, the samples were stored in the cesium chloride solution from the last step of purification.

3.9 Electron microscopy

Electron microscopy confirmed that all capsid-sized, glutaraldehyde cross-linked particles in a sample with a density of 1.356 g/ml were, in fact, capsid-like in morphology. Initially, specimens were negatively stained with sodium phosphotungstate, pH 8.8 (Figure 8a). For comparison, particles of MLD capsid II were also prepared for electron microscopy by use of the technique used for Figure 8a (Figure 8b). Excluding a few vesicles too small to be the particles observed by 2d-AGE, (some are indicated by arrowheads in Figure 8a), most (> 95%) of the particles observed had the morphology and size of either capsid II or ipDNA-capsid II. A few contaminating particles were indistinguishable from capsid I; one is indicated by CI in Figure 8a.

Figure 8a also had an occasional (1 – 3 % of the total) image of a capsid II-like particle that had a hyper-expanded outer shell (some are labeled HE in Figure 8a). These particles are assumed to be HE ipDNA-capsids and were about 1.5x larger than capsid II. These are the first intact HE ipDNA-capsids observed by negative staining, given that only disintegrating HE ipDNA-capsids were observed in the negatively stained preparations of [16]. All of

these HE particles had radii that were less than 2.0x the capsid II radius, i.e., smaller than observed by 2d-AGE for cross-linked HE ipDNA-capsids. Thus, we conclude that HE ipDNA-capsids have become smaller during preparation for electron microscopy, presumably from dehydration.

The specimen of Figure 8a did not differentiate the contracted ipDNA-capsids from the others. This limitation is not surprising because, based on the 2d-AGE, the contracted ipDNA-capsids are only about 11% smaller than capsid II. This difference is too small to reliably find in a negatively stained specimen, because the particles observed are subject to both flattening-induced increase and dehydration-induced decrease in apparent size [26]. Even cryo-EM has potential limitations in this area, given the above differences between results from small-angle x-ray scattering and cryo-EM.

In order to identify contracted ipDNA-capsid II in electron micrographs, we attempted to use criteria other than size. When negatively stained with 1% uranyl acetate (Figure 8c), eccentricity of outer shell structure was observed in some capsid II-like particles; the most dramatically eccentric are indicated by arrowheads in Figure 8c. Most other particles in Figure 8c are capsid II-like with visible packaged DNA; some capsid II-like particles appeared empty (one is labeled by E). Some contaminating particles of capsid I were also seen (one is labeled by CI). The diameter of the non-eccentric region of the partially eccentric particles was, indeed, somewhat (~5%) smaller than the average diameter of the non-eccentric particles, although this difference in size is possibly not significant. The eccentric particles are likely to be ipDNA-capsid II with at least partial contraction of the shell.

4 Discussion

4.1 Agarose gel electrophoresis of intermediates: biophysical characterizations

Both 1d-AGE and 2d-AGE have played essential roles in the detection and characterization of the various T3 ipDNA-capsids. These procedures potentially can play similar roles in the detection and characterization of intermediates of other biotic events. The advantages of techniques in this class include (1) dependence of gel electrophoretic fractionation on σ , a variable that is independent of variables that determine fractionation during ultracentrifugation, (2) capacity for efficient (in time and expense) analysis of samples in large number and (3) capacity for relatively high sensitivity detection, especially when fluorescent stains are used. Thus, when intermediates are pre-fractionated by ultracentrifugation, one rapidly detects and discriminates particles that previously co-fractionated. One can then screen for particles that have new and potentially useful characteristics, even when the particles involved are minor in amount.

As illustrated here, an additional advantage of 2d-AGE is that biophysical information can be obtained. For example, 2d-AGE revealed that the glutaraldehyde cross-linking-dependent band splitting of ipDNA-capsid II is caused by difference in both σ and R_E . The σ -dependence of splitting was roughly the same for slow ipDNA-capsid II as it was for rapid ipDNA-capsid II, which indicates that σ -dependence of band splitting was derived primarily from the shell, not the tail. In addition, all capsids and ipDNA-capsid II's underwent an increase in the magnitude of σ when reacted with glutaraldehyde, presumably because of reaction of glutaraldehyde with surface amino groups that lose one positive charge during cross-linking.

The contracted ipDNA-capsid II was one of two ipDNA-capsid II size variants that existed for both slow and rapid ipDNA-capsid II. The larger variant had the R_E of the traditional capsid II; the smaller variant had a R_E even smaller than the R_E of capsid I. That is to say,

the smaller variant of ipDNA-capsid II is contracted in relation to all forms of T3 capsid investigated in the past and is a new addition to the collection of intermediates. Multiple shell states have also been seen by cryo-EM of other phages, including HK97 [7, 12]. But, none of these states includes either a hyper-expanded or a contracted shell.

4.2 DNA packaging: Source of the DNA cleavage that forms the ipDNA-capsids

The following observations suggested the use of our procedure for increasing the yield of ipDNA-capsids. First, some T3 ipDNA-capsid II particles have the phage tail [15], which suggests that premature cleavage of concatemers was performed by the enzyme that performs the maturation cleavage *in vivo*. This enzyme is gp19 terminase, which is both a packaging-driving ATPase (see Figure 1c) and the endonuclease that conducts the maturation cleavages (reviews: [6, 10, 17]; see Figure 1a,e). Second, *in vitro* T3 DNA packaging is slowed and, as a result, the cleavage activity of terminase is increased, by the elevation of [NaCl] [27]. Therefore, one makes the prediction that increasing of [NaCl] during *in vivo* packaging will increase the yield of ipDNA-capsids via increase in the frequency of premature cleavage of a concatemer-associated genome being packaged.

This prediction is confirmed here, which supports the assumption that premature DNA cleavage by gp19 terminase causes the formation of the ipDNA-capsids. This assumption is further supported by the previous demonstration of sharp bands of ipDNA [15, 16], as also seen in the densest fraction of Figures 2b and 2c. The reason is that no restriction endonuclease-like activity has been found in extracts of T3-infected cells [16]. That is to say, terminase must be the enzyme that produces the ipDNA ends, reasoning by elimination. The previous explanation for the sharpness of the bands was a periodic, terminase cleavage-activation via slowing of packaging that occurred during an expansion/contraction cycle of the DNA packaging motor [16] and accounted for the observation of HE ipDNA-capsids, which were found for F values above 0.28, but not for smaller F values [16].

4.3. DNA packaging: the shell of ipDNA-capsids

The data obtained here show that ipDNA-capsid II has the capacity to enter a contracted state, as suggested by the hypothesis that an expansion/contraction cycle exists. One might argue, nonetheless, that the glutaraldehyde cross-linking has induced the state of contraction, not simply stabilized it. However, both the existence and the R_E of contracted ipDNA-capsid II were sensitive to F . Thus, multiple responses to cross-linking and multiple routes to the contracted state exist. If all capsid II were same, then multiple responses could not exist. The simplest assumption is that the differences in cross-linked ipDNA-capsid II are the differences that existed before cross-linking. This assumption has support from the observation in Figure 5 that the R_E value of both capsid I and MLD capsid II is not changed by cross-linking. In further support, comparison of cryo-EM-derived and small-angle x-ray scattering-derived capsid radii (Results Section) suggests that multiple capsid shell radii do exist in solution before cross-linking, even for MLD capsid II.

4.4 A basic conundrum of gel electrophoretic analysis: Final products of intermediates

The question remains: What do the data suggest about the (devil's advocate generated) possibility that the ipDNA-capsids detected/isolated both here and previously are intermediates of packaging for which the final product is not an infective phage particle? The alternative is that these ipDNA-capsids are intermediates of abortive DNA packaging. For example, our observed ipDNA-capsids might be produced by cleavage of DNA either at sites of previous DNA damage or at sites that were being packaged when damage to the shell occurred. Damage to the shell would most likely be caused by force generated by the DNA packaging motor and transmitted via ipDNA to the shell.

Generation of ipDNA-capsids via DNA damage-induced abortive DNA packaging is unlikely for the following reason. DNA damage is unlikely to cause the formation of sharp ipDNA bands because DNA damage (from ultraviolet light, for example) is basically randomly placed under almost any imaginable scenario, even if packaging causes the damage.

Generation of ipDNA-capsids via shell damage from the force of DNA packaging is also unlikely because the formation of ipDNA-capsids (1) does not monotonically increase as F increases [16] even though the force of packaged DNA on the shell monotonically increases as F increases [28, 29] and (2) is quantized, as revealed by the sharp ipDNA bands. The quantization also makes unlikely generation of ipDNA-capsids via random damage to the shell that occurs either before or during packaging, even if the cause of the damage is not the force of the packaged DNA. Finally, mature phage particles ($F = 1.0$) have never been reported to hyper-expand, even though the force of the packaged DNA is highest in this case. Thus, hyper-expansion and presumably contraction require a source of energy, which implies a system evolved for providing the energy for this purpose. Such a system would not have been retained via natural selection if it did not contribute to productive packaging. In summary, finding an abortive packaging-based scenario that accounts for the data is extremely difficult and may be impossible.

4.5 DNA packaging: Context and rationale

In any case, both the finding made here of a contracted version of ipDNA-capsid II and the proposed expansion/contraction cycle are new and unusual enough to be in need of context and a rationale. These are the following. Although the data from several laboratories indicate that a classical power stroke-based cycle begins phage DNA packaging [6–11], this power stroke-based cycle (called the type 1 cycle) is expected to allow the accumulation of accidentally packaged non-DNA molecules in the cavity of the shell. An *Escherichia coli* cell has a concentration of protein and RNA of 30–40% [30]. In fact, phage T3 shells are known to become porous during DNA packaging because DNA packaging-associated exit of a protein that assists procapsid assembly (scaffolding protein, gp9, in the case of T3/T7; see Figure 1a,b) occurs. Thus, one reasonably proposes that the type 1 cycle will undergo a stall *in vivo* (although not necessarily in a relatively dilute *in vitro* system), because of resistance to packaging that is derived in part from steric/osmotic effects of non-DNA molecules that are accidentally packaged. Continuing along this line of thought, the stall triggers either premature cleavage (at low frequency) or, if the packaging is to be productive, starting of a cycle of another type (i.e., the proposed type 2 cycle; Figure 1d) that removes the accidentally packaged molecules and restarts the type 1 cycle.

In support, a major T3 ipDNA band is found at $F = 0.28$ and possibly is generated by the stall that initiates the type 2 cycle [15, 16]. A mechanism previously proposed for signaling of DNA cleavage is re-channeling of ATP-derived free energy from the process of DNA packaging to the process of exposing the concatemer to the endonuclease domain of terminase [10].

4.6 Possible details of an expansion/contraction cycle

Based on a more detailed proposal in [31], the following are (in brief) some aspects of a possible type 2 cycle. The cycle begins with shell expansion while (1) low permeability of the shell is maintained so that accidentally packaged non-DNA molecules are diluted without comparable entry of additional molecules, thereby partially removing the cause for the stall and (2) the DNA molecule is free to enter the cavity of the capsid's shell. The proposed type 2 cycle continues with shell contraction while (1) increase occurs in the permeability of the shell so that some accidentally packaged non-DNA molecules are

expelled and (2) binding (perhaps clamping) of the DNA molecule also occurs so that the DNA molecule is not expelled.

The possibility exists that the type 2 cycle is the more ancient of the two and that the type 1 cycle began its evolution by making the type 2 cycle more efficient [32]. A type 2 cycle is most efficient if the contraction reduces the shell radius to values that are as small as consistent with physical constraints. Thus, a rationale also exists for the finding made here that ipDNA-capsid II has a contracted shell.

5. Conclusions

5.1 Some fundamentals of analysis

We have obtained and analyzed intermediates that, by their basic nature, are no longer active in packaging because of the loss of the unpackaged DNA segment. Further studies are needed to directly test hypotheses via “native” intermediates, i.e., the intermediates that underwent DNA cleavage *in vivo* to produce the ipDNA-capsids observed here. Solid arrows connect proposed native intermediates in Figure 1. Although useful for initial studies, the cross-linking used here to identify contracted ipDNA-capsids will obviously have to be omitted in a study of the biochemical activities of native intermediates.

Nonetheless, the importance of the strategy used here is emphasized by that fact that we are aware of no success with a more direct strategy dependent on native intermediates, despite half a century of studies of phage DNA packaging. Presumably, investigators need additional input from a more indirect, ipDNA-capsid-based strategy, before a direct strategy can be successfully implemented. To obtain needed additional input, therefore, we both began and continued here with a more indirect strategy that includes analysis of several intermediates (ipDNA-capsids in this case) that are from the pathway of DNA packaging, have properties advantageous for analysis, but are no longer active. Based on discussion in Section 4.4, above, we assume here that the final product of these intermediates *in vivo* is an infective phage particle, not a defective particle such as a phage with damaged DNA or capsid.

5.2 Future strategies of experimentation

The current study provides a foundation for the rigorous future analysis of shell states, possibly via cryo-EM, and the role of shell state changes in DNA packaging. When shell states are known, then, optical (fluorescent, for example), state-specific capsid-tagging and optical monitoring of the various states can be used to perform a real-time analysis, perhaps via single-molecule fluorescence microscopy (review: [10]).

In addition, testing hypotheses can be performed via testing the accuracy of predictions of changes in the level of the ipDNA-capsids already identified. An example is testing the effect of raising [NaCl] on ipDNA-capsid amount, as done in Figure 2, and as discussed in Section 4.2. In the future, a second example will be determining whether some mutants selected for propagation in high [NaCl]-containing media have increased levels of contracted and HE ipDNA-capsids, as though the type 2 cycle has become triggered more easily to overcome the elevated [NaCl]-induced inhibition of gp19 terminase. In unpublished experiments, we have already selected T3 mutants that propagate at NaCl concentrations that prevent propagation of wild type T3. This selection was possible because host propagation is less sensitive to elevated [NaCl] than phage T3 propagation.

To achieve the important, but difficult, isolation of native DNA packaging intermediates, our current ipDNA-capsids will be used either to screen T3 mutations or to find conditions of T3 infection that cause arrest at intermediate stages of DNA packaging. A major

advantage of using a phage system is that either genetics or addition of a packaging-inhibiting compound is relatively efficiently used to help increase the amount of native intermediates.

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Abbreviations

1d-AGE	one-dimensional agarose gel electrophoresis
2d-AGE	two-dimensional agarose gel electrophoresis
ipDNA	incompletely packaged DNA
ipDNA-capsid	capsid with ipDNA
<i>F</i>	ipDNA length/mature DNA length
cryo-EM	cryo-electron microscopy
μ	electrophoretic mobility
R_E	effective radius
σ	average electrical surface charge density
HE	hyper-expanded

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Figure 1.

Phage T3/T7 *in vivo* DNA packaging. Solid arrows connect intermediates of T3/T7 DNA packaging (called native intermediates) that are thought to be present in an infected cell during productive DNA packaging, based on isolated/characterized intermediates. Each dashed arrow connects a native intermediate to an intermediate that has been derived from the native intermediate (sometimes via premature DNA cleavage by terminase) and then isolated, characterized and used to deduce the presence of the native intermediate. Capsid I is the procapsid. MLD (Metrizamide low density) capsid II is an early intermediate isolated by its unusually low density during buoyant density centrifugation in a Metrizamide density gradient; the low density is caused by impermeability to Metrizamide [20]. The ipDNA-capsids have ipDNA in the internal cavity of the shell of one of the following capsids: capsid II (ipDNA-capsid II), a hyper-expanded capsid (HE ipDNA-capsid) and capsid II variants found to be contracted after cross-linking (contracted ipDNA-capsid II). Slow ipDNA-capsid II has the phage injection organelle (tail); rapid ipDNA-capsid II does not. Slow ipDNA-capsid II migrates more slowly than rapid ipDNA-capsid II during agarose gel electrophoresis [15, 16]. The contribution from the current study is contracted ipDNA-capsid II. The figure indicates the following proposed stages of *in vivo* packaging: (a) binding of capsid I to a concatemeric DNA molecule and the initiating DNA cleavage, (b) entry of DNA and conversion of capsid I to capsid II, (c) packaging via the type 1 cycle with the type 2 cycle not yet initiated (also indicated by "Type 1 only"), (d) initiation/continuation of the type 2 (back-up) cycle in response to a stall of the type 1 cycle and

restarting/continuation of the type 1 cycle (also indicated by “Type 1 and Type 2), (e) terminal DNA cleavage and assembly of the tail. If the type 1 cycle does not undergo a stall, the proposed type 2 cycle does not activate. Duplication of capsids in the early stages [i.e., (a) and (b)] represents cooperativity detected by single-molecule fluorescence microscopy (review: [10]). The term, x-link, indicates glutaraldehyde cross-linking. dsDNA signifies double-stranded DNA. The proteins indicated are connector protein, gp8, scaffolding protein, gp9, major shell protein, gp10 and three proteins that form an internal cylinder assembled on the connector, gp14, gp15 and gp16. The proteins of the tail are gp11, gp12 and gp17; gp17 forms the tail fiber (reviews: [6, 10, 17]).

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Figure 2.

The effects of adding NaCl to T3-infected cells during DNA packaging. Three 6-liter cultures were infected with T3 and then processed, as described in the Materials and Methods Section, until the buoyant density centrifugation. After buoyant density centrifugation, each fraction of each density gradient was analyzed by use of 1d-AGE with GelStar staining. The cultures were (a) a control culture that received growth medium at 21.5 min after infection, (b) a culture that received growth medium and 0.2 M (final concentration) of additional NaCl at 21.5 min after infection and (c) a culture that received growth medium and 0.4 M (final concentration) of additional NaCl at 21.5 min after infection. The cultures were made with the same bacterial inoculum and the same preparation of medium within 1.5–2.5 hours of each other. A bold arrow at the left indicates the direction of electrophoresis. Arrowheads indicate the origin surfaces of electrophoresis. Densities (g/ml) are at the top of lanes. The samples for the lanes marked “ ϕ ” are the fractions of the density gradients that have phage particles in peak amount. The samples for the lanes marked “m” have T3 phage, which has further purified, as described in the Methods Section, and added as a marker. S-CII indicates slow ipDNA-capsid II (with a tail); R-CII indicates rapid ipDNA-capsid II (without a tail); HE indicates HE (hyper-expanded) ipDNA-capsids; DNA indicates capsid-free DNA. Trace amounts of GelStar-staining material were also detected co-migrating with capsid I (marked CI) and outer membrane vesicles (OMV). OMV-associated nucleic acid was previously reported in [16]. A vertical arrow indicates the position of ipDNA-capsids that have $F = 0.28$.

Figure 3.

The dependence of fractionation by 2d-AGE on R_E and σ . (a) Illustration is presented of the following two features: First, the value of R_E increases as the angle (θ) between the direction of the first dimension electrophoresis and a size line decreases. A size line passes through the effective origin (O) and the center of a band; all particles on a single size line have the same R_E , but differ in average σ . Second, the magnitude of σ increases as the distance migrated in the first dimension increases. (b) Illustration is presented of four bands similar to the bands that were observed in Figure 4 for cross-linked ipDNA-capsid II after 2d-AGE with a second dimension gel that produces relatively high resolution by R_E . The particles fall on two different size lines, and the particles also differ in σ . The insert shows the four bands at higher magnification. The arrows in the inset indicate what happens to the banding pattern when the concentration of the second dimension gel is lowered enough so that resolution by R_E no longer occurs. The arrows in the inset are not horizontal to account for the small amount of sieving of the first dimension gel.

Figure 4.

The effect of glutaraldehyde cross-linking on the profile of ipDNA-capsids during 2d-AGE. The following were subjected to 2d-AGE (time of first and second dimensions, 3.0 and 8.5 hr, respectively) and then staining with (a) GelStar (nucleic acid-specific) and (b) Coomassie blue (protein-specific). Panel i: a 1.351 g/ml fraction, cross-linked. Panel ii: a 1.367 g/ml fraction, cross-linked. Panel iii: the 1.351 g/ml fraction, not cross-linked. Panel iv: the 1.367 g/ml fraction, not cross-linked. Arrowheads indicate the origin surfaces of sample wells; arrows indicate the directions of the first (I) and second (II) electrophoresis. S-CII indicates the bands formed by slow ipDNA-capsid II (with a tail); R-CII indicates the bands formed by rapid ipDNA-capsid II (without a tail). The ratio of protein to DNA staining intensity is higher for rapid ipDNA-capsid II than it is for slow ipDNA-capsid II. The apparent reason is loss during 2d-AGE of ipDNA from rapid ipDNA-capsid II (see also [15]).

Figure 5.

Comparison of glutaraldehyde cross-linked ipDNA-capsid II with cross-linked and uncross-linked standards. The following samples were subjected to 2d-AGE, followed by staining with Coomassie blue: Panel i: uncross-linked MLD capsid II (CII), glutaraldehyde cross-linked, dialyzed MLD capsid II (CII-g), uncross-linked capsid I (CI) and glutaraldehyde cross-linked, dialyzed capsid I (CI-g). Panel ii: a cross-linked 1.372 g/ml fraction. Arrowheads indicate the origin surfaces of sample wells; arrows indicate the directions of the first (I) and second (II) electrophoresis.

Figure 6.

2d-AGE profile as a function of the concentration of the gel used for the second dimension. Glutaraldehyde cross-linked samples with densities of 1.343 and 1.419 g/ml, as indicated at the right in the figure, were used to obtain 2d-AGE profiles (GelStar staining) at the following concentrations of the gel used for the second dimension [time of the second dimension (hr) is in parentheses]: 2.0% (8.5), 1.6% (6.0) 1.4% (5.0). Agarose percentage is indicated at the top in the figure. The 2d-AGE was performed in three different composite gels. Only the capsid II region of each gel is shown; orientation is the same as in Figures 3–5.

Figure 7. Band splitting vs. density. Portions of gradient fractions of four densities were subjected to 2d-AGE with GelStar staining. Only the capsid II region of each gel is shown; orientation is the same as in Figures 3–5. The panels are labeled by density (g/ml).

Figure 8.

Electron microscopy. Samples were glutaraldehyde cross-linked and then prepared for electron microscopy by use of procedures described in the Materials and Methods Section. One sample was a fraction with a density of 1.356 g/ml ($F = \sim 0.30$). The second sample was MLD capsid II. The sample/negative stain used was (a) 1.356 g/ml fraction/sodium phosphotungstate, pH 8.8, (b) MLD capsid II/sodium phosphotungstate, pH 8.8, (c) 1.356 g/ml fraction/uranyl acetate. CI, capsid I; E, empty. Arrowheads in (a) indicate host-derived vesicles; arrowheads in (c) indicate eccentric capsid shells.