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The Enzymology of a Viral Genome Packaging Motor Is Influenced by the Assembly State of the Motor Subunits

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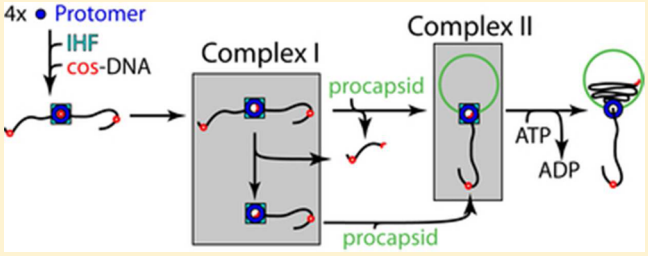
Supporting Information

ABSTRACT: Terminase enzymes are responsible for the excision of a single genome from a concatemeric precursor (genome maturation) and concomitant packaging of DNA into the capsid shell. Here, we demonstrate that lambda terminase can be purified as a homogeneous "protomer" species, and we present a kinetic analysis of the genome maturation and packaging activities of the protomeric enzyme. The protomer assembles into a distinct maturation complex at the *cos* sequence of a concatemer. This complex rapidly nicks the duplex to form the mature left end of the viral genome, which is followed by procapsid binding, activation of the packaging ATPase, and translocation of the duplex into the capsid interior by the terminase motor complex. Genome packaging by the protomer shows high fidelity with only the mature left end of the duplex inserted into the capsid shell. In sum, the data show that the terminase protomer exhibits catalytic activity commensurate with that expected of a bone fide genome maturation and packaging complex in vivo and that both catalytically competent complexes are composed of four terminase protomers assembled into a ringlike structure that encircles duplex DNA. This work provides mechanistic insight into the coordinated catalytic activities of terminase enzymes in virus assembly that can be generalized to all of the double-stranded DNA viruses.



The developmental pathway for the double-stranded DNA (dsDNA) viruses represents an ordered assembly process in which many aspects are conserved across adenovirus, herpesvirus, and bacteriophage groups.[1−3] A key step in virus assembly involves the packaging of a viral genome into a preformed procapsid shell.[4−8] The preferred packaging substrate is generally a linear concatemer of genomes that is the product of viral DNA replication (immature DNA).[9,10] Genome packaging requires excision of a single genome from the concatemer (genome maturation) and concomitant translocation of the duplex into the capsid shell powered by ATP hydrolysis (DNA packaging). The terminase enzymes perform both of these functions, and the motors package DNA to near-liquid crystalline density, generating up to 50 atm of pressure within the capsid shell.[4,7,8] The reactions are strongly conserved among all of these viruses, both prokaryotic and eukaryotic, and terminase enzymes may provide a novel target for antiviral therapeutics. Bacteriophage lambda is prototypical of these viruses and has been extensively characterized by genetic, biochemical, and biophysical approaches. Our lab has harnessed the lambda system as a model with which to interrogate the mechanistic features of the packaging process.

Lambda terminase is composed of two gene products: gpA, the large terminase subunit (TerL), and gpNu1, the small terminase subunit (TerS), in a $TerL_1{\cdot}TerS_2$ heterotrimer complex (Figure 1A).[11,12] This "protomer" possesses several catalytic activities related to genome maturation and packaging,[13,14] and current models describing virion assembly are summarized as follows (see Figure 1C).

(i) Multiple terminase protomers assemble at a cohesive end site (*cos*), which represents the junction between successive genomes in the concatemer (Figure 1B). The assembly process is mediated by the terminase TerS subunit and *Escherichia coli* integration host factor (IHF), which cooperatively bind and bend the duplex at the *cosB* subsite.[15] This provides a duplex architecture to which the protomer binds with high affinity; however, the stoichiometry of IHF and terminase protomers bound in this maturation complex remains unclear.

(ii) The endonuclease activity of TerL introduces symmetric nicks into the duplex at the *cosN* subsite (Figure 1B). Subsequent separation of the nicked duplex, catalyzed by the so-called helicase activity of TerL, affords the mature, 12-base single-stranded mature left end of the genome (D_L) tightly bound by the enzyme. This stable intermediate is commonly termed Complex I (Figure 1C).[14]

(iii) The maturation complex next binds to the portal ring of an empty procapsid to yield the packaging motor complex [Complex II (Figure 1C)]; the structural features of the packaging motor also remain unclear. Whatever the case, assembly of the motor triggers the release of the terminase from

Received: July 2, 2012
Revised: September 25, 2012
Published: October 25, 2012

   dx.doi.org/10.1021/bi300890y | *Biochemistry* 2012, 51, 9342−9353

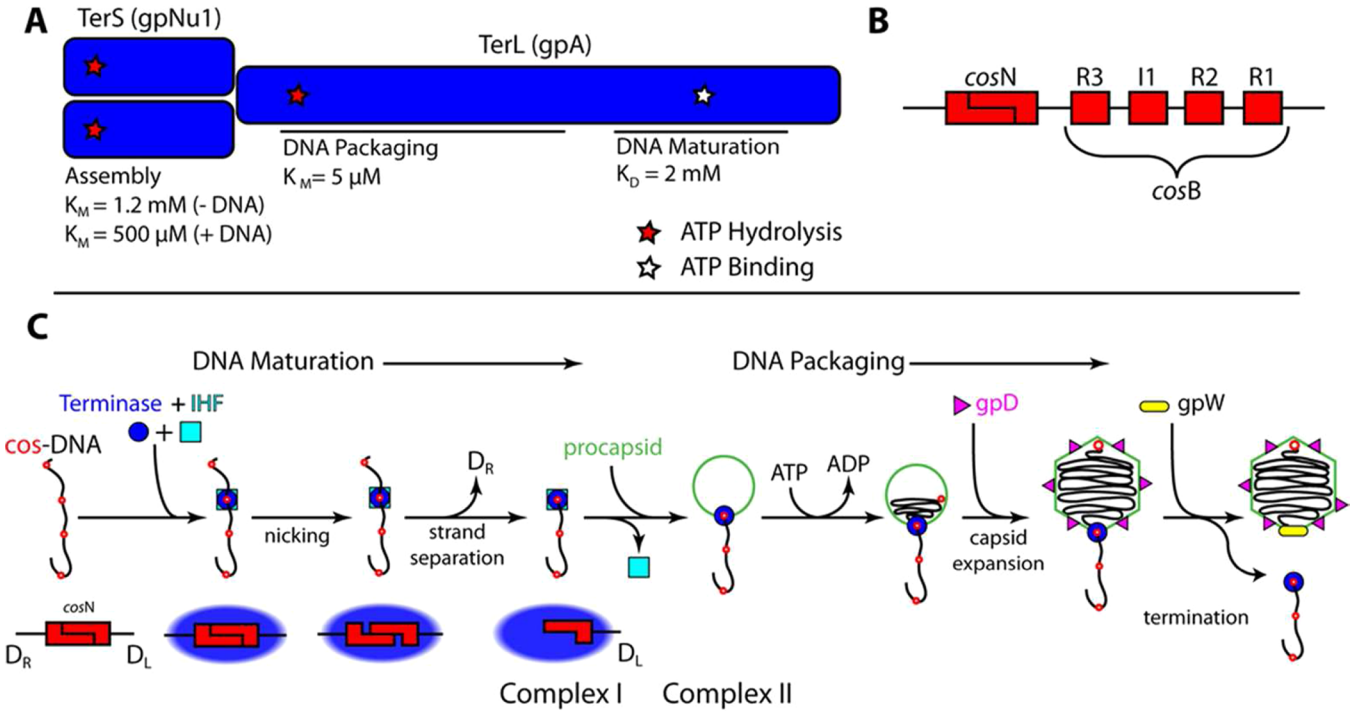


Figure 1. Terminase has multiple catalytic activities required for the maturation and packaging of the viral genome. (A) The terminase protomer is composed of one TerL subunit and two TerS subunits. The TerL subunit provides all of the catalytic activities of the enzyme in two functional domains as indicated (maturation and packaging domains). The TerS subunits are required for site-specific assembly of the maturation complex at *cos*. Three ATP binding sites have been identified in the protomer (the assembly site, the packaging ATPase site, and DNA maturation site) and are denoted with stars. Experimentally determined binding constants are presented below each indicated site. (B) The *cos* sequence of the lambda genome is multipartite. The terminase TerL subunit introduces symmetric nicks within the *cosN* subsite to generate the 12 base "sticky" ends of the mature lambda genome. Cooperative assembly of the TerS subunit and IHF at the *cosB* subsite mediates specific assembly of terminase at *cos*. IHF binds to the I1 consensus sequence, while TerS binds to the three "R" elements. (C) Current model for genome maturation and packaging by lambda terminase. Concatemeric (immature) DNA is presented as a line with multiple *cos* sites depicted as red dots. The bottom panel shows details of terminase-catalyzed duplex nicking and strand separation reactions. Details are provided in the text.

the *cos* site and translocation of DNA into the procapsid, powered by ATP hydrolysis (*cos* clearance).

(iv) DNA packaging triggers expansion of the procapsid shell to the mature capsid conformation, which then binds the gpD decoration protein.[16,17] This provides structural integrity to the shell so that it can withstand the internal force generated by the tightly packaged DNA.[17,18] Upon reaching the downstream *cos* site in the concatemer, the terminase motor engages the terminal *cos* sequence (*cos* capture). This terminates translocation activity and again activates the end maturation activities of the enzyme. Duplex nicking and strand separation afford the mature D_R end of the packaged genome, and the gpW adaptor protein replaces the terminase at the portal vertex. Subsequent addition of the gpFII protein and a preassembled tail affords an infectious virus, while the terminase·D_L-DNA complex (regenerated Complex I) binds a second procapsid to initiate a second round of processive packaging.[14,19]

The enzymology of lambda terminase has been extensively studied, and this work has yielded significant insight into the mechanism of genome maturation and packaging. Most recent studies have utilized highly purified terminase preparations; however, we recently demonstrated that the purified enzyme exists as a mixture of the 5.1 S protomer in slow equilibrium with a heterogeneous "13.3 S species".[a,11,12] We refer to this purified preparation as the terminase "mix". Undoubtedly, the structural heterogeneity of the terminase mix is responsible, at least in part, for the complex behavior previously observed in kinetic and biophysical interrogation of the enzyme, and this has complicated mechanistic interpretation of the data. Here, we show that a homogeneous preparation of the lambda terminase protomer may be isolated in high yield, and we have defined conditions for the long-term storage of the enzyme. We further describe a kinetic interrogation of the genome maturation and packaging activities of the protomer and contrast this with the pure but heterogeneous terminase mix used in published studies. While many activities are similar, important differences between the protomer and the terminase mix that are relevant to the function of the enzyme are observed. In summary, these studies suggest that the terminase protomer is the biologically relevant species during a productive viral infection in vivo, and this work provides mechanistic insight into the coordinated activities of terminase enzymes in virus assembly.

EXPERIMENTAL PROCEDURES

Materials and Protein Constructs. Tryptone, yeast extract, agar, and ampicillin were purchased from Fisher Scientific. Terrific broth was purchased from Difco. All nucleoside triphosphates were purchased from Sigma-Aldrich. Chromatography medium was purchased from GE Healthcare Life Sciences. Mature lambda DNA was purchased from Invitrogen. All other materials were of the highest quality available. The plasmid pCT-*λ*, a 12 kb plasmid that contains a unique wild-type *cos* sequence, was purified by a published procedure.[20] Cell lysis utilized a Thermo Scientific IEC "French" laboratory press. All protein purifications utilized

dx.doi.org/10.1021/bi300890y | *Biochemistry* 2012, 51, 9342−9353

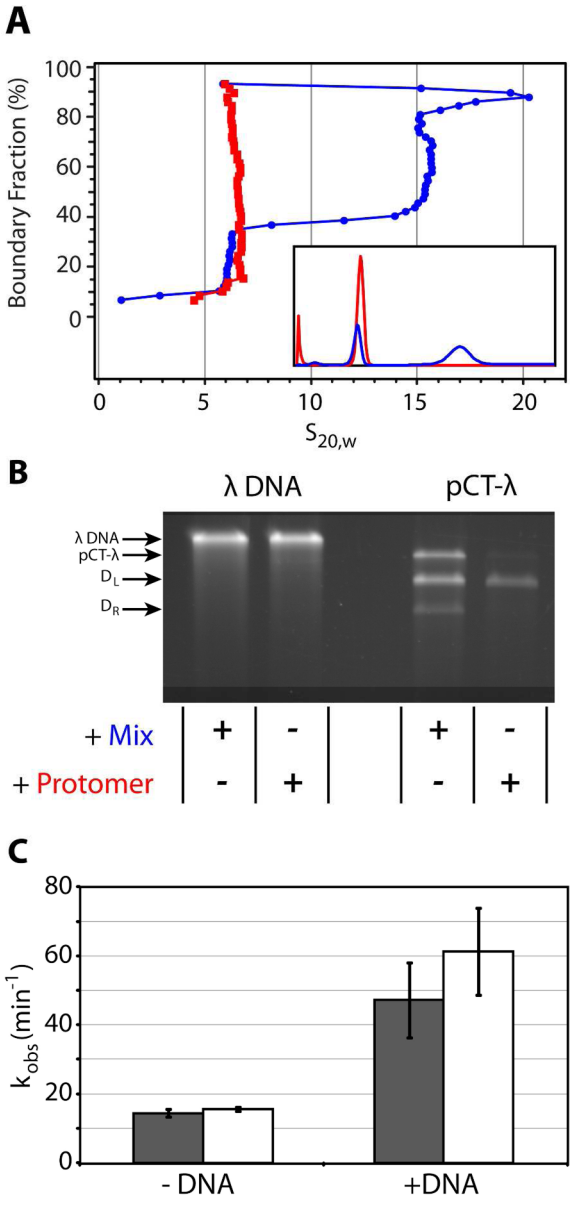


Figure 2. Purification and packaging activity of the lambda terminase protomer. (A) Sedimentation velocity data for the terminase mix (blue) and isolated protomer (red) were analyzed using the van Holde–Weischet method as described in Experimental Procedures. The mix clearly contains both the protomer and assembled species, while the isolated protomer is homogeneous. The inset shows the $c(s)$ analysis of the same data using Sedfit. (B) Fidelity of DNA packaging by the mix and by the isolated terminase protomer. The DNase protection assay was performed as described in Experimental Procedures using 100 nM terminase protomer or terminase mix and 5 nM mature lambda DNA (λ-DNA) or pCT-λ, as indicated. The positions of mature λ-DNA, uncleaved pCT-λ, and the D_L and D_R products resulting from *cos* cleavage of pCT-λ are indicated at the left. There is no difference between the protomer and the mix when packaging a mature lambda genome (left). The mix packages full-length pCT-λ and both nuclease products in a linked maturation–packaging reaction. In contrast, the protomer packages the mature D_L genome end with high fidelity. (C) The observed rate of ATP hydrolysis by the terminase protomer was determined in the presence of 50 μM (gray bars) and 1 mM (white bars) ATP. Duplex DNA (pCT-λ) was added to the reaction mixture as indicated. Each bar represents the average of at least three separate experiments with standard deviations indicated.

the Amersham Biosciences ÄKTApurifier core 10 System from GE Healthcare. Full-length, native sequence *E. coli* integration host factor (IHF) was purified from HN880 cells as previously described.[21]

Expression and Purification of Lambda Terminase. The terminase enzyme used in this study was expressed from OR1265[pQH101] cells as previously described.[22] This vector expresses full-length, native sequence gpNu1 and full-length, native sequence gpA with six histidines directly appended to the C-terminal Glu residue of the large gpA subunit. Expression and purification of the terminase mix were conducted as previously described,[22] with modification to optimize the yield of the protomer. Briefly, purified terminase eluted from the HisTrap FF column was dialyzed overnight at 4 °C against buffer Q [20 mM phosphate buffer (pH 6.8) containing 100 mM NaCl, 1 mM EDTA, 7 mM β-ME, and 10% glycerol (v/v)]. The dialysate was loaded onto a 1 mL HiTrap Q column, and bound proteins were eluted with a 20 column volume gradient to buffer Q containing 1 M NaCl. The terminase-containing fractions (~300 mM NaCl) were pooled, and aliquots were stored at −80 °C. These samples contained both the homogeneous protomer and the heterogeneous "assembled" species (Figure 2A) and are termed the "terminase mix". We have previously demonstrated that the self-association behavior and the catalytic activities of the H6 terminase mix are indistinguishable from those of the native, untagged enzyme.[22]

To isolate the terminase protomer, a 1 mL aliquot of purified terminase mix was applied to a HiPrep S-300 HR gel filtration column (120 mL) equilibrated and developed with buffer Q. The terminase protomer eluted at ~65 mL, and the protomer-containing fractions were pooled, aliquoted, and stored at −80 °C. The $TerL_1{\cdot}TerS_2$ protomer concentration was determined spectrally (ε_{280} = 15000 M^{-1} cm^{-1}).

Sedimentation Velocity Analytical Ultracentrifugation Analysis. Sedimentation velocity (SV) experiments were performed using terminase that had been purified as described above. All experiments were performed in buffer Q containing 350 or 100 mM NaCl for the terminase mix or the protomer, respectively. Data were collected using a Beckman XL-A analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA) using 12 mm Epon charcoal two-sector centerpieces at 42000 rpm. Absorbance data were collected at 280 nm, using a spacing of 0.001 cm, with four averages in the continuous scan mode; scans were collected every 15 min. Samples were run at 7 °C. The raw data were analyzed using both the UltraScan comprehensive data analysis suite[23,24] and the SedFit/SedPhat data analysis packages.[25,26]

Terminase Activity Assays and Kinetic Analysis. The *cos* cleavage endonuclease and strand separation assays were performed by a published procedure.[21] The single-turnover reaction time courses were fit to an exponential rate equation of the form

$$\text{products} = A_\infty - \sum_{i=1}^{n} A_i e^{-k_i t}$$

where products represents the fraction of substrate converted to product at time t and A_∞ is the extent of the reaction at infinite time. A_i represents the fraction of the rate associated with the ith phase of the reaction when fitted for multiple rates, and k_i is the associated observed rate constant. The kinetic data were fit to both single-exponential (n = 1) and double-exponential (n = 2) rate equations. A double-exponential time

dx.doi.org/10.1021/bi300890y | *Biochemistry* 2012, 51, 9342–9353

course was deemed appropriate only if (i) the quality of the fit (ψ^2) improved by an order of magnitude and (ii) the derived rate constants differed by at least 10-fold. The Microsoft Excel solver function was used with error minimization to achieve the reported fits.

The synchronous DNA packaging assay was performed as described previously,[27,28] with modification as described in the Supporting Information. The ATPase assay was conducted as described above for the DNA packaging reaction except that [α-^{32}P]ATP (1000−3000 cpm/pmol) was added to the reaction mixture and the reaction was allowed to proceed at 37 °C. ATP hydrolysis was quantified by thin layer chromatography as previously described.[13,29]

RESULTS

Isolation of the Homogenous Terminase Protomer. The goal of this study is to characterize the biochemical properties and catalytic activity of the terminase protomer. This requires an efficient purification protocol that affords a homogeneous enzyme preparation in high yield. We previously demonstrated that purified lambda terminase is composed of a homogeneous $TerL_1{\cdot}TerS_2$ heterotrimer complex (the protomer) and a heterogeneous higher-order assembly of approximately four protomers (the assembled species).[11] The assembled species slowly dissociates, ultimately yielding ∼30% homogeneous protomer after incubation for 2 weeks in Tris buffer (pH 8) containing 150 mM NaCl.[12] We further demonstrated that the association state of the enzyme is influenced by NaCl concentration and temperature;[11] however, preparative isolation of the protomer was time-consuming and inefficient. In an effort to purify the homogeneous protomer in high yield, the effect of salt and pH on the association state of the enzyme was investigated in greater detail and the purification protocol was optimized. Lowering the pH to 6.8 and decreasing the NaCl concentration to 100 mM result in rapid dissociation of the assembled species to afford ∼50% homogeneous protomer after incubation for only 2 h (data not shown). Further decreases in pH or salt concentration hasten the process but result in a significant loss of protein due to aggregation (not shown). The optimized purification protocol described in Experimental Procedures affords 4 mg of protomer/L of cells that is >95% pure as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and >95% homogeneous as determined by sedimentation velocity analytical ultracentrifugation analysis (Figure 2A). The purified protomer can be stored at −80 °C for >8 months without evidence of self-association or loss of catalytic activity.

Fidelity of Genome Packaging by the Terminase Protomer. Genome maturation in vivo affords the D_R and D_L genome ends, but only the latter is utilized as a packaging substrate (see Figure 1C).[14] In contrast, in vitro packaging assays indicate that both D_R and D_L ends as well as the uncleaved substrate can be packaged into the capsid depending on experimental conditions.[27] These published studies used the purified terminase mix, which contains both protomer and heterogeneous assembled species as described above. We proposed that the protomer represents the biologically relevant species during a productive viral infection,[11,12] and here we directly compare the fidelity of DNA packaging by the terminase protomer and the terminase mix. As shown previously, both the protomer and the terminase mix efficiently package a full-length lambda genome in the presence of IHF (Figure 2B).[11] While the data demonstrate that both preparations exhibit high processivity (the entire genome is packaged), this experiment does not interrogate fidelity because packaging could start at either genome end. We next examined packaging coupled to genome maturation using pCT-λ, a 12 kb linearized plasmid that contains an intact *cos* sequence.[20] In this assay, the terminase mix efficiently matures the duplex at *cos* and then packages the mature D_L end; however, the mix also packages the D_R end and in addition the uncleaved, full-length substrate (Figure 2B). In other words, the terminase mix packages DNA nondiscriminately. In contrast, the protomer also matures the duplex at *cos* but shows a strong preference for packaging only the D_L-containing duplex with little to no packaging of either the mature D_R end or the substrate duplex observed (Figure 2B). Hence, coupled maturation and packaging with the terminase protomer establishes fidelity in the packaging reaction.

ATP Binding and Hydrolysis by the Terminase Protomer. DNA packaging is fueled by a high-affinity ATPase catalytic site in the N-terminus of the TerL subunit (the packaging ATPase site).[14] In addition, lambda terminase possesses a second ATP binding site in the C-terminus of TerL that regulates the genome maturation activities of the enzyme and a third ATP binding site in the TerS subunit that regulates the binding of terminase to DNA [the maturation and assembly ATP binding sites, respectively (Figure 1A)]. Kinetic and mutagenesis studies of the terminase mix identified two catalytically active ATPase sites in the enzyme: the high-affinity packaging ATPase in TerL and a low-affinity site in TerS;[28−33] the genome maturation site is catalytically silent.[34] In most proteins where nucleotides regulate biological activity, NTP and NDP[b] stabilize alternate conformations of the protein and catalytic turnover at the site is extremely slow. For instance, G-proteins involved in signal transduction pathways are activated by GTP but are inactive with GDP bound; slow hydrolysis of the bound GTP ($k_{cat} \sim 10^{-2}$−10^{-3} min^{-1}) acts as a "switch mechanism" that allows regulation of biological activity.[35] Within this context, ATP hydrolysis by the assembly site in the terminase mix is unusual. We hypothesized that the observed low-affinity ATPase activity is aberrant and the result of improper protomer associations in the assembled species contained in the terminase mix. To directly test this hypothesis, we performed a kinetic analysis of ATP hydrolysis by the isolated protomer to define catalytically active ATPase sites in the enzyme.

We have historically used 50 μM ATP to isolate ATP hydrolysis to the high-affinity packaging ATPase site (K_m = 5 μM).[13,30,32] The nucleotide concentration is then increased to 1 mM to interrogate additional ATP hydrolysis by the maturation site in the terminase mix ($K_m \sim 500$ μM).[13,29,30,32] Consistently, an increased level of ATP hydrolysis by the terminase mix is observed at elevated ATP concentrations (data not shown); however, when a similar approach is taken with the terminase protomer, no significant increase in ATP hydrolysis is observed with an increasing ATP concentration (Figure 2C). We interpret these data to indicate that the low-affinity ATP binding site in TerS is catalytically silent in the terminase protomer, even in the presence of DNA; this is exactly what is expected of a regulatory nucleotide-binding site. In summary, the highly purified terminase protomer possesses (i) an ATP binding site in TerS that regulates DNA binding activity and (ii) an ATP binding site in the C-terminus of TerL that regulates genome maturation activity; catalytic turnover at these sites is undetectable. In addition, the motor possesses a high-

Table 1. Kinetic Analysis of the Genome Maturation Reactions

[protomer] (nM)	*cos* cleavage reaction rate	strand separation reaction rate	cleavage:separation reaction extent ratio
100	k_{obs} = 2.1 min^{-1}	k_{obs} = 0.25 min^{-1}	1.4
80	k_{obs} = 1.4 min^{-1}	k_{obs} = 0.19 min^{-1}	1.4
40	k_{obs} = 0.95 min^{-1}	k_{obs} = 0.045 min^{-1}	1.4
20	k_{fast} = 0.69 min^{-1} (60% total amplitude) k_{slow} = 0.086 min^{-1}	k_{obs} = 0.033 min^{-1}	1.4

affinity packaging ATPase catalytic site in the N-terminus of TerL that fuels the translocation of DNA into the capsid (see Figure 1A).

Kinetic Analysis of Genome Maturation by the Terminase Protomer. As depicted in Figure 1C, maturation of the genome end by lambda terminase in vitro involves nicking of the duplex at *cosN* and ejection of the D_R end to afford Complex I, a stable intermediate in the absence of procapsids ($t_{1/2}$ ~ 8 h).[36] The reaction time course thus represents a single catalytic turnover by the enzyme, and the data are analyzed according to an exponential increase in the level of product formation. Previous studies in our lab have demonstrated multiple exponential phases in the time course for the *cos* cleavage reaction.[20,37] We suggested that terminase assembly at *cos* is the rate-limiting step in genome maturation; however, this interpretation is complicated by the presence of structural heterogeneity in the purified preparation, as discussed previously[37] and above.

Here, we describe a detailed kinetic analysis of the *cos* cleavage and subsequent strand separation reactions by the isolated terminase protomer using pCT-λ at a concentration of 5 nM. At low protomer concentrations (20 nM), the *cos* cleavage reaction time course is poorly described by a single-exponential function and a double-exponential rate equation is required to adequately describe the data (Figure S1 of the Supporting Information, dashed line and solid line, respectively). This analysis affords rate constants for the fast and slow phases of the reaction that are similar to those previously obtained in our lab (Table 1).[20,37] Presumably, the slow rate constant reflects slow assembly of a catalytically competent nuclease complex at *cos* at limiting protomer concentrations. In contrast, at protomer concentrations of >20 nM (4-fold excess enzyme), the slow phase disappears and the reaction time course is well described by a single-exponential rate equation (blue data, Figure 3A). Interestingly, the observed rate constant is linearly dependent on protomer concentration (Figure 3B and Table 1). The catalytically competent maturation complex is composed of multiple protomers (vide infra), and these data indicate that terminase assembly is not the rate-limiting step in the kinetic time course under these conditions. If this were the case, a higher-order concentration dependence would be observed. Regardless, these data indicate that the rate of *cos* cleavage, and by extension the rate of protomer assembly at *cos*, is fast under conditions that mimic those observed during a productive virus infection in vivo (~100 nM terminase).[38,39]

In contrast to the *cos* cleavage reaction, the kinetic time course for the strand separation reaction is well described by a single-exponential rate equation at all protomer concentrations examined. Two observations are of interest. (i) The observed rate of strand separation is an order of magnitude slower than the rate of duplex nicking, and (ii) the extent of the strand separation reaction is significantly smaller than that observed for *cos* cleavage under all reaction conditions (Figure 3A,B and Table 1). These observations have implications for the nature of Complex I in the packaging pathway in vivo and are discussed further below.

Stoichiometry of the Maturation and Packaging Motor Complexes. Early models hypothesized that a terminase dimer assembles at the *cosN* subsite to introduce symmetric nicks into the duplex (see Figure 1).[40,41] This model is based on the symmetric nature of *cosN*,[42] based on the identification of a putative bZIP protein dimerization motif in the TerL subunit,[43] and in analogy to the "orthodox" type II restriction endonuclease enzymes.[44] In contrast, it has been presumed that the packaging motor is composed of an oligomeric terminase ring, in analogy to the translocating ring helicases.[45−47] These models predict that additional terminase protomers are recruited to the maturation complex to complete a higher-order packaging motor complex. We have rigorously demonstrated that the protomer can assemble into a stable tetrameric ring complex in solution ($[TerL_1{\cdot}TerS_2]_4$) and proposed a simplified model in which the ring tetramer is responsible for both the maturation and packaging reactions.[11,12] As a first step in addressing this hypothesis, we examined the extent of genome maturation as a function of protomer concentration; several features of this analysis are of interest. (i) The extent of strand separation is always less than the extent of *cos* cleavage at all protomer concentrations, and (ii) the extents of both reactions reach an apparent maximum at approximately four to six protomers per DNA molecule (Figure 3C). We interpret these data to indicate that there are no major differences in the protomer stoichiometry between the two catalytic complexes during nicking and separation reactions. The data further place an upper limit on the number of protomers assembled into the maturation complex. This analysis presumes that the catalytic activity of the preparation is 100%, which is rarely the case. If we assume a more reasonable ~90% active enzyme preparation, this places an upper limit of four to five protomers in the maturation complex.

We next examined the effect of protomer concentration on the DNA packaging activity of terminase, and the data suggest a more complex relationship. In contrast to the end maturation reactions, little to no packaging activity can be detected below a protomer:DNA stoichiometry of 4 (Figure 3C). The extent of the reaction increases with a further increase in protomer concentration until essentially 100% of the input DNA has been packaged in the presence of 20-fold excess protomer. It is noteworthy that under these conditions, the extent of packaging (~100%) exceeds the extent of strand separation by the enzyme [~50% (Figure 3C)]. Control studies indicate that procapsids affect neither the rate nor the extent of strand separation by the enzyme (data not shown). This observation indicates that the packaging motor can utilize the nicked, annealed duplex bound by terminase in addition to the strand-separated duplex in Complex I (see Figure 1).

Motor Assembly and Regulation of the Packaging ATPase Site. The protomer possesses a weak basal ATPase

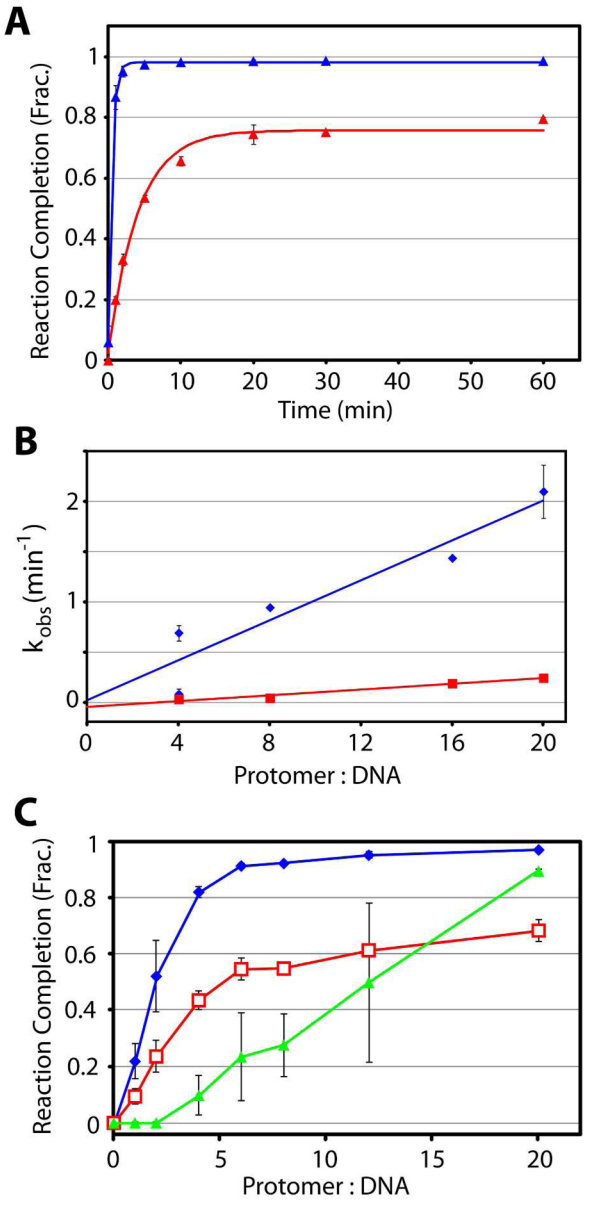


Figure 3. Genome end maturation in vitro is limited by a slow strand separation rate. (A) Time courses for the *cos* cleavage (blue) and strand separation (red) reactions in the presence of 100 nM terminase protomer. Each data point represents the average of at least three separate experiments with standard devations indicated (in some cases, the error is smaller than the data point and is obscured). The solid line is the best fit of the data to a single-exponential time course; derived kinetic parameters are listed in Table 1. (B) Observed rates of the *cos* cleavage (blue) and strand separation (red) reactions as a function of terminase protomer concentration. The data points at 4- and 20-fold excess terminase represent the average of three separate experiments with error bars indicated; the data points for 8- and 16-fold excess terminase are the result of a single experiment. Analysis of the data affords the following bimolecular rate constants: k_{on} = (2.8 ± 0.5) × 10^5 M^{-1} s^{-1}, and k_{on} = (0.43 ± 0.05) × 10^5 M^{-1} s^{-1} for *cos* cleavage and strand separation, respectively. (C) Terminase stoichiometry in the maturation and packaging complexes. The extents of the *cos* cleavage (blue), strand separation (red), and DNA packaging (green) reactions are shown as a function of an increasing protomer concentration.

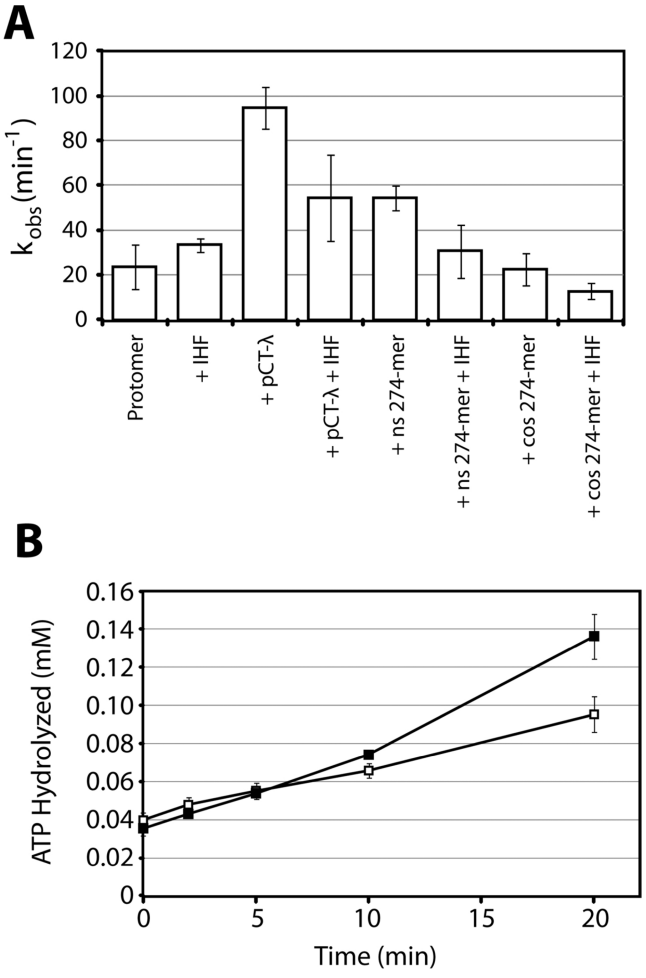


Figure 4. Activation of ATPase activity in the packaging motor complex. (A) The ATPase activity assay was conducted as described in Experimental Procedures using 200 nM terminase protomer, 1 mM [α-^{32}P]ATP, and IHF and/or DNA as indicated. The observed rate is reported as an average of at least three separate experiments with the standard deviation indicated. (B) The ATPase–*cos* cleavage reaction was conducted as described in Experimental Procedures using 200 nM protomer and allowed to proceed for 5 min. Buffer (□) or 40 nM procapsids (■) were then added to initiate the DNA packaging reaction. Aliquots were removed at the indicated times, and ATP hydrolysis was quantified by the TLC assay. Each data point represents the average of at least three separate experiments with the standard deviation indicated. The addition of procapsids increases ATPase activity.

activity, while the isolated ring tetramer efficiently hydrolyzes ATP.[11] Figure 4A demonstrates that ATP hydrolysis by the protomer is also stimulated by duplex DNA and at biologically relevant enzyme concentrations (200 nM). Interestingly, addition of IHF to the reaction mixture attenuates DNA-stimulated ATPase activity. The substrate used in this experiment (pCT-λ) is a 12 kb duplex that contains the *cos* sequence (200 bp) and thus the I1 recognition element (Figure 1B).[20] On the basis of these and published observations, we hypothesized that nonspecific DNA promotes ring tetramer assembly on the duplex and thus stimulates ATP hydrolysis; in contrast, site-specific, cooperative assembly of IHF and the protomer at a *cos* site engender a maturation complex in which the packaging ATPase is downregulated (Figure 1A).[21,37] We reasoned that the vast excess of nonspecific DNA in pCT-λ (12 kb) relative to the 200 bp *cos* sequence precludes complete sequestration of all the terminase complexes and incomplete

dx.doi.org/10.1021/bi300890y | *Biochemistry* 2012, 51, 9342–9353

abrogation of the packaging ATPase activity. To directly test this hypothesis, we utilized short (274 bp) duplexes that contained the entire *cos* sequence (*cos*-274) or that was of random sequence (ns-274). Consistent with our hypothesis, the short nonspecific duplex stimulates ATP hydrolysis, though not as strongly as the longer pCT-λ duplex (Figure 4A). In contrast and as predicted, basal ATP hydrolysis by the protomer is unaffected by the *cos*-274 duplex and may in fact be attenuated in the presence of both *cos*-274 and IHF.

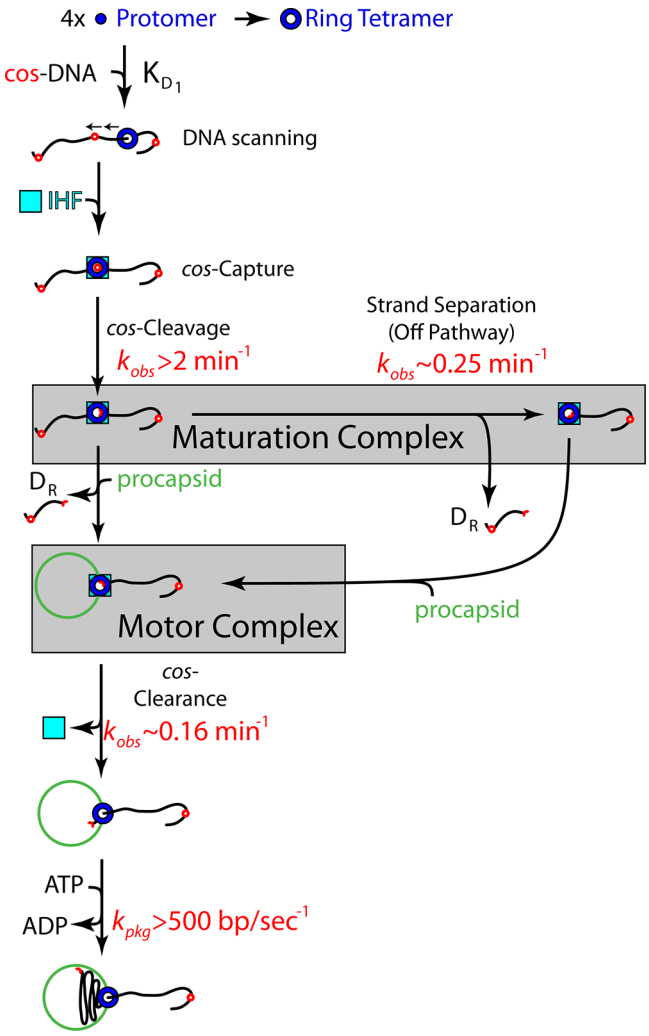
The next step in the packaging pathway is the binding of procapsids to Complex I (Figure 1). This triggers "*cos* clearance", which includes release of the motor from the *cos* site and activation of the packaging ATPase to power translocation. We next examined the effect of procapsids on the ATPase activity of Complex I. The data presented in Figure 4B show that the protomer possesses modest ATPase activity during maturation of the genome ends and in Complex I, but that ATP hydrolysis is stimulated upon the subsequent addition of procapsids. Given that turnover at the maturation and assembly ATP binding sites is not observed (vide supra), this increase in activity must reflect ATP hydrolysis by the packaging ATPase in TerL.

DISCUSSION

The packaging of a viral genome into a preformed procapsid shell is a highly conserved process in the complex double-stranded DNA viruses. Terminase enzymes catalyze both the genome maturation and DNA packaging reactions and are essential to virus assembly and infectivity. Given their central role, terminases may serve as specific targets for antiviral therapeutics. Unfortunately, purification of these enzymes in a soluble, well-behaved, and homogeneous state has been problematic in all viral systems, and this has been a major impediment to their biochemical characterization. The isolation of lambda terminase in a soluble, homogeneous, and highly active form has allowed us to interrogate the catalytic properties of the enzyme and to probe the packaging pathway in detail, without the ambiguity associated with a heterogeneous enzyme preparation.

What Is the Nature of "Complex I"? Complex I was first described as a stable terminase·DNA intermediate isolated from infected cells in the absence of procapsids.[48] This intermediate could be chased into a packaging complex (Complex II) with the addition of procapsids. We previously demonstrated that the product of the maturation reaction in vitro is composed of terminase tightly bound to the mature D_L end of the genome but that has ejected the D_R end.[36] We proposed that this accurately reflected Complex I isolated in vivo, and current models incorporate this concept, as depicted in Figure 1C; however, the data presented here clearly demonstrate that separation of the nicked strands is quite slow and in fact unnecessary for utilization of the duplex by the packaging motor. On the basis of these data, we propose that while ejection of the D_R end from the maturation complex occurs in vitro, this reaction does not accurately reflect the natural packaging pathway in vivo. Rather, terminase protomers assemble into a maturation complex at *cos* and rapidly nick the duplex strands. Separation of the nicked, annealed strands is slow, and procapsids quickly capture this intermediate. Either procapsid binding or activation of the packaging motor triggers ejection of the D_R end, which leads to *cos* clearance and translocation of DNA into the capsid shell (Scheme 1). This revised model is harmonious with published data and further addresses a

Scheme 1. Kinetic Model for Maturation and Packaging by the Lambda Terminase Protomer



conundrum that has been present in the literature for more than 30 years; although *cos* cleavage and strand separation reactions are quite efficient in vitro, mature ends are not observed in the absence of procapsids in vivo. We suggest that while the reaction can easily be observed in vitro, strand separation from the maturation complex is sufficiently slow that it precludes the generation of free, mature ends within the cell. For the remainder of this work, we use the terms genome maturation complex and packaging motor complex rather than the vague Complex I and II terminology.

Stoichiometry of the Genome Maturation and Packaging Motor Complexes. We have proposed that the terminase ring tetramer observed in solution is representative of the complex utilized for both genome maturation and packaging activities,[12] and the data presented here are consistent with this hypothesis. With respect to duplex nicking, this model is analogous to the type IIE and IIF restriction endonuclease enzymes in which the catalytically competent enzyme complex is a tetramer within which two subunits each bind to one copy of the recognition sequence.[44,49] This interaction induces looping of the DNA between the sites and nicking of the duplex at either or both of the recognition elements. We propose that the terminase ring tetramer adopts a similar strategy, bending and wrapping DNA at the *cos* site and

dx.doi.org/10.1021/bi300890y | *Biochemistry* 2012, 51, 9342−9353

introducing site-specific nicks into the duplex at *cosN* using two symmetrically disposed TerL subunits. The other two subunits similarly bind DNA but are catalytically silent. This model is consistent with the observation that binding of terminase to *cos*, especially in the presence of IHF, bends and wraps the duplex occluding >250 bp of DNA.[50]

The maturation complex next binds to the procapsid to complete the packaging motor complex. On the surface, our data suggest that additional protomers must be recruited to assemble a functional motor. It is feasible that additional subunits are recruited to the putative ring tetramer to engender a complex of higher-order stoichiometry; however, we disfavor this model based on the stability of the ring once formed.[11,12] Another possibility is that a second terminase ring could be recruited to power translocation, as is observed in the eukaryotic MCM and viral SV40 helicases where two hexameric rings form the active motor complex.[51,52] We suggest a third possibility. Strong cooperative interactions between subunits are presumed, if not directly demonstrated, in all known biological motors. Preliminary data in our lab indicate that this is true of the terminase packaging motor as well and that incorporation of a single defective protomer into the motor has profound effects on DNA packaging activity (B. T. Andrews and C. E. Catalano, manuscript in preparation). If we assume that our purified protomer preparation is ∼90% active (vide supra), the defective subunits could significantly alter the observed dependence of protomer concentration on packaging activity. Unfortunately, the present data does not allow discrimination between these possibilities, and biochemical, biophysical, and structural studies are being conducted to provide a detailed description of the packaging motor complex. At present, we prefer a simple and unified model in which both the maturation complex and the packaging motor complex are composed of a single terminase ring tetramer encircling viral DNA.

The Terminase Protomer Establishes Fidelity in Genome Packaging. In vitro packaging using the purified but heterogeneous terminase mix exhibits low packaging fidelity, and varying amounts of the D_R strand and even the uncleaved substrate are packaged with reasonable efficiency.[27,28] This is similarly observed in other viral systems such as bacteriophage T4 and SPP1 where in vitro packaging using purified terminase is essentially nonspecific.[53−55] In contrast, packaging by the lambda terminase protomer exhibits high fidelity, and only the mature D_L end is packaged into the capsid shell, as expected of a biologically relevant species in vivo.

The mechanism for high fidelity of the protomer can be described on the basis of a variety of biochemical data. The terminase protomer assembles into a ring tetramer in solution, and the preassembled complex possesses site-specific (*cos*) maturation activity. The complex also efficiently packages duplex DNA, but in a nonspecific manner. This reaction is independent of IHF, and we presume that the ring tetramer can bind to any duplex end and then to a capsid to initiate DNA packaging. Terminase remains predominantly in the protomeric state at in vivo concentrations (100 nM), and the maturation and packaging activities of the protomer, while it is kinetically identical to the ring tetramer, have a strict requirement for IHF. In this scenario, the protomer is devoid of maturation and packaging activities and assembly of the catalytically competent complexes occurs only at the *cos* site, mediated by IHF. Duplex nicking at *cosN* affords an intermediate in which terminase is tightly and specifically bound to the mature D_L end and which only then binds to a procapsid to initiate packaging. This sequence of events bestows fidelity in the genome packaging reaction, as depicted in Scheme 1.

Regulation of the Packaging ATPase and a Model for Genome Packaging. The terminase protomer has a low basal ATPase activity, while that of the isolated ring tetramer is robust.[11,12] We have proposed that this reflects assembly of the packaging ATPase catalytic site at the subunit interface of two protomers in the complex, in analogy to many hexameric ring helicases.[11,56,57] We show here that nonspecific DNA duplexes also stimulate ATPase activity, but at much lower and physiologically relevant terminase concentrations (100 nM). In contrast, duplexes that contain the *cos* sequence do not stimulate ATPase activity and may in fact attenuate ATP hydrolysis in the presence of IHF. We interpret the ensemble of data presented here and in published work in the following model for genome packaging.

Nonspecific DNA promotes the assembly of four protomers into the ring tetramer, similar to that we have observed in solution studies. We speculate that the ring encircles the DNA and that the activated ATPase activity reflects a translocating complex that has engaged in a "one-dimensional" search for a *cos* site sequence, a feature common to many site-specific DNA binding proteins.[58,59] IHF binds to a *cos* site in the concatemer and introduces a strong bend in the duplex; capture of the translocating complex by the bent duplex architecture engenders a stable, site-specifically bound maturation complex in which the packaging ATPase is downregulated and in which *cos* cleavage activity has been activated. Within this context, we have shown that terminase and IHF cooperatively bind to *cos*-DNA to afford a distinct and stable complex, while binding to nonspecific duplexes yields multiple, diffuse bands in electrophoretic mobility shift studies (R. Sanyal and C. E. Catalano, manuscript in preparation).[15,36] The activated maturation complex nicks the duplex, which is followed by procapsid binding. This affords the fully assembled motor complex that triggers strand separation and reactivates the packaging ATPase to fuel translocation of the DNA into the capsid shell.[c]

The model presented above is harmonious with published work and with the data presented here. It invokes coordinated interactions among the terminase subunits, *cos*-DNA, IHF, and the portal vertex of the capsid that regulate the assembly of a stable maturation complex and its transition to a dynamic packaging motor complex. A further complication is the observation that binding of ATP to the assembly ATP binding site in TerS (i) regulates DNA binding interactions,[13] (ii) downregulates genome maturation activities,[21] and (iii) activates the packaging ATPase site in TerL.[29] Thus, complex allosteric regulation of the catalytic activities of terminase fine-tunes each complex to its specific role: excision of a single genome from the concatemer and subsequent packaging of the duplex into the capsid shell.

The Protomer Is the Biologically Relevant Species. Taken together, the biochemical features of the purified protomer closely match those expected of a biologically relevant species, as follows. First, the protomer is the prevalent species at in vivo concentrations. Second, the kinetics of protomer assembly at *cos* and the subsequent DNA nicking reaction is much faster than previously reported. Third, the procapsid binds to terminase only after it has promoted maturation of the D_L end to afford a motor with high packaging fidelity. Finally, ATP hydrolysis by the isolated protomer and in

the maturation complex is weak but is strongly stimulated in the packaging motor complex.[11] Thus, nonproductive ATP hydrolysis is averted in the unassembled protomer and in the maturation complex that must remain bound to *cos*, but the packaging ATPase is activated when required to power translocation. In summary, the isolated protomer is devoid of catalytic activity and the nuclease, strand separation, ATPase, and DNA translocation activities are sequentially activated as appropriate to each nucleoprotein complex along the packaging pathway.

Viral Genome Packaging Motors. Biological motors are essential to cellular vitality and serve a variety of roles. They are multimeric complexes that transduce the chemical energy of ATP hydrolysis to mechanical work. The terminase motors perform an equally important role in virus assembly and are among the most powerful biological motors characterized to date. Recent structural and single-molecule studies have yielded significant insight into the physical nature of the motors and have led to viable though conflicting models for the mechanism of DNA translocation by the complexes. Validation of these models requires solution-based, biochemical interrogation of the catalytic properties of the enzymes. This work thus complements the structural studies by providing a biochemical framework that describes the enzymology of the packaging motors.

It is clear that the functional terminase motors act as higher-order multimers of TerL and TerS subunits, but the nature of these complexes remains uncertain. Structural studies demonstrate that isolated TerS subunits assemble into ring structures composed of 8−12 subunits, depending on the virus studied.[60−62] In contrast, the DNA binding domain of the lambda TerS subunit assembles into a stable dimer,[63−65] which is consistent with the observed stoichiometry of the terminase protomer ($TerL_1{\cdot}TerS_2$). Assembly of four lambda protomers affords a ring tetramer containing eight TerS subunits, which we presume are radially disposed in the packaging motor complex. Crystal structures of the isolated TerL subunit of phage T4[66] and the nuclease domains of phage P2,[67] phage SPP1,[68] and cytomegalovirus TerL subunits[69] have also been published. These studies reveal that the TerL subunits are composed of two structural domains, an N-terminal translocation domain and a C-terminal maturation domain. Although there is no structural information available for the lambda TerL subunit, genetic and biochemical data indicate a similar structural and functional domain organization.[14,34]

Cryo-electron microscopy studies in the bacteriophage ϕ29 system suggest that the packaging motor is composed of five "ATPase" subunits.[70] This virus represents a distinct class of dsDNA viruses that package monomeric genomes, that uniquely utilize a packaging RNA (pRNA) as part of the functional motor complex, and do not utilize a TerS subunit, per se.[d,7,8,71] Adenoviruses utilize analogous genome replication and perhaps DNA packaging strategies.[72] In contrast, lambda is representative of viruses such as herpesviruses and many bacteriophages that package genomes from a concatemeric DNA precursor.[4,8,10,14] In these cases, the terminase enzymes serve dual functions: excision of a single genome from the concatemer and concomitant packaging of the duplex into the capsid. These enzymes uniformly utilize a TerS subunit that is responsible for recognition of viral DNA and a TerL subunit that performs all of the maturation and packaging functions. Bacteriophage T4 terminase requires a small subunit for specific packaging of viral DNA in vivo, but it is dispensable for packaging nonspecific duplexes in vitro.[73] Cryo-electron microscopy structural studies of the T4 packaging motor suggest that the complex assembled from isolated TerL subunits is pentameric, similar to the ϕ29 ATPase complex described above.

Unfortunately, there are no high-resolution data for a fully assembled hetero-oligomeric terminase enzyme from any source. The isolation of a homogeneous, well-behaved, and functional lambda terminase protomer composed of both TerS and TerL subunits with a well-defined stoichiometry has allowed detailed biochemical and biophysical characterization of the enzyme. The ensemble of biochemical data suggest that the catalytically competent maturation and motor complexes are composed of four terminase protomers assembled into a ringlike structure that encircles duplex DNA; this differs from the pentameric motors proposed in ϕ29 and T4. Regardless, the essential features of the motors, including mechanochemical coupling of ATP hydrolysis to motor movement, cooperative interactions between the motor subunits during translocation, and the capacity to generate significant packaging forces, will certainly be recapitulated among all of the virus classes, and the results presented here provide mechanistic insight into the enzymology of these fascinating biological motors.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Protocol for the synchronous DNA packaging assay, statistical analysis of the packaging ATPase data, and kinetic time course data for the maturation activities of the terminase protomer. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by National Institutes of Health Grants GM088186 (C.E.C.) and F32GM-905652 (B.T.A.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the members of the Catalano laboratory for helpful discussions of this work. We further thank Rishi Sanyal for providing the IHF and Shannon Kruse for providing procapsids used in these studies.

■ ABBREVIATIONS

β-ME, β-mercaptoethanol; *cos*, cohesive end site of the bacteriophage lambda genome; immature DNA, concatemeric lambda DNA; mature DNA, genome length lambda DNA found within the viral capsid that contains the 12 kb complementary single-stranded ends; TerL, large terminase subunit, also known as gpA; TerS, small terminase subunit, also known as gpNu1; terminase protomer, homogeneous holoenzyme composed of TerL and TerS subunits in a $TerL_1{\cdot}TerS_2$ heterotrimer complex; ring tetramer, catalytically competent terminase complex composed of four protomers in a ringlike structure $(TerL_1{\cdot}TerS_2)_4$; terminase mix, pure but

structurally heterogeneous mixture of terminase protomers observed in purified protein preparations.

■ ADDITIONAL NOTES

[a]Purified lambda terminase is composed of a homogeneous 5.1 S species and a heterogeneous 13.3 S species that can be separated by gel filtration chromatography. The latter likely represents a tetramer of protomers with additional gpNu1 subunits nonspecifically associated with the complex. The 13.3 S species dissociates to the protomer, which at elevated concentrations can be reassembled to a homogeneous 14 S ring tetramer species in vitro.

[b]We use the terms NTP and NDP to denote triphosphate and diphosphate nucleotides, respectively, in a generic sense.

[c]We note that the proposed translocating complex that engages in a one-dimensional search for *cos* is physically distinct from the packaging motor complex. In the latter case, the presumed ring tetramer is associated with the portal ring of a procapsid, which likely affects the catalytic activity and mechanochemical properties of the motor.

[d]Genome replication in the ϕ29-like viruses utilizes a protein-primed replication strategy, and the product is a genome monomer that contains the initiator protein covalently attached at the 5′ end. This protein plays a role in DNA recognition by the packaging motor and may serve as a *de facto* TerS subunit.

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