Kinetic Characterization of the Strand Separation ("Helicase") Activity of the DNA Packaging Enzyme from Bacteriophage λ†

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ABSTRACT: Bacteriophage λ is assembled from preformed viral capsids (proheads), tails, and genomes that are excised from a concatemeric DNA precursor. The enzyme responsible for insertion of the genome into the precapsid is known as terminase. This enzyme possesses site-specific endonuclease, ATPase, and DNA strand separation ("helicase") catalytic activities, which work in concert to excise and package a single viral genome during phage assembly. We have previously characterized the endonuclease [Tomka, M. A., & Catalano, C. E. (1993) *J. Biol. Chem. 268*, 3056−3065] and ATPase [Tomka, M. A., & Catalano, C. E. (1993) *Biochemistry 32*, 11992−11997] catalytic activities of λ terminase and present here similar studies on the strand separation activity of the enzyme. Strand separation requires terminase, divalent metal, and adenosine nucleotides with a hydrolyzable β,γ-phosphate bond. Two apparent binding sites for ATP-mediated strand separation were identified, one of which appears to be distinct from the high- and low-affinity sites previously observed for ATP hydrolysis [Hwang, Y., Catalano, C. E., & Feiss, M. (1995) *Biochemistry 35*, 2796−2803]. Salt stimulates the reaction at low concentrations but is strongly inhibitory at elevated concentrations, presumably due to impaired DNA binding. The above results are identical with either a complex DNA mixture (a nicked, annealed DNA duplex in the presence of excess nonspecific DNA) or a purified DNA substrate; however, a kinetic analysis of the reaction revealed that the observed rate was ≈5-fold greater with the purified DNA substrate. Moreover, while *Escherichia coli* integration host factor (IHF) stimulates terminase-mediated strand separation with both substrates, the observed stimulation is more pronounced with the complex DNA mixture (10-fold rate increase) than the purified DNA substrate (5-fold rate increase). Our data are consistent with a model where IHF binding to the terminase assembly site forms a binary protein·DNA complex readily distinguishable from bulk DNA. The implications of these results to the process of DNA packaging in bacteriophage λ are discussed.

The assembly of an infectious virus proceeds through an ordered series of nucleoprotein complexes utilizing a variety of proteins of both viral and host origin (Fields & Knipe, 1990; Hendrix et al., 1971). In bacteriophage λ, the mature viral genome consists of a 48.5 kb[1] double-stranded DNA duplex with complementary single-stranded "sticky" ends which is packaged into a preformed capsid, or head (Daniels et al., 1983, Feiss & Becker, 1983). The enzyme responsible for genome packaging is known as terminase, an enzyme common to all of the double-stranded DNA bacteriophages and the eucaryotic herpesvirus groups (Black, 1988; Casjens, 1985; Roizman & Sears, 1991). The preferred packaging substrate in phage λ assembly is a concatemeric DNA duplex composed of multiple genomes linked in a head-to-tail fashion (immature DNA), and terminase functions to excise a single genome from the concatemer and insert the duplex within the confines of the viral capsid (Feiss, 1986; Murialdo, 1991).

A model for genome packaging in phage λ has been proposed (Becker & Murialdo, 1990; Catalano et al., 1995; Feiss, 1986) that proceeds as follows (see Figure 1): The terminase subunits (gpA, 72 kDa, and gpNu1, 20.4 kDa) and the *Escherichia coli* protein integration host factor (IHF) assemble onto a DNA sequence known as *cos* (cohesive end site), which represents the junction between left and right ends of individual (mature) λ genomes in the concatemer. This nucleoprotein prenicking complex ($T{\cdot}D_{cos}$) next introduces symmetric nicks at the *cos* subsite *cosN* (endonuclease activity), yielding the nicked, annealed intermediate ($T{\cdot}D_L{\cdot}D_R$). This reaction is referred to as *cos* cleavage or the TER reaction. Terminase-mediated strand separation, presumably driven by ATP hydrolysis, affords the first 12-base-pair single-stranded "sticky" end of the mature λ genome, which remains bound to the enzyme ($T{\cdot}D_L$). This strand separation activity has historically been referred to as a "helicase" activity (Catalano et al., 1995, Rubinchik et al., 1994). The stable binary enzyme·DNA intermediate (complex I) next captures an empty viral prohead, forming complex II, and the enzyme unidirectionally translocates across the duplex, thus packaging the viral genome into the head.[2] Upon encountering the next downstream *cos* sequence, terminase again nicks the duplex at *cosN*, separates the strands, and

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[1] Abbreviations: β-ME, 2-mercaptoethanol; *cos*, cohesive end site, the junction between individual genomes in immature concatemeric λ DNA; gpA, the large subunit of phage λ terminase; gpNu1, the small subunit of phage λ terminase; IHF, *Escherichia coli* integration host factor; kb, kilobase; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; TER reaction, the endonuclease activity of the terminase enzyme.

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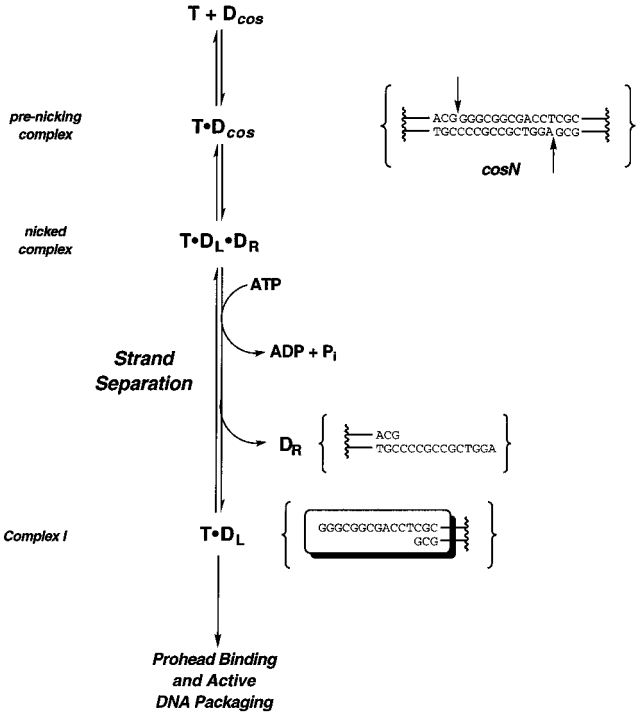

FIGURE 1: Scheme for λ terminase-mediated genome packaging. Each of the putative biochemical packaging intermediates is indicated at center, with their names listed to the left. T = terminase holoenzyme, which is composed of the phage λ gpA and gpNu1 proteins. Also part of these nucleoprotein complexes but not explicitly shown is *E. coli* IHF. The sequence of *cosN* is shown in braces at the top right of the figure with the sites of terminase-mediated duplex nicking indicated with arrows. The sequences of the 12-base-pair single-stranded "sticky" ends D_R and D_L are also indicated on the right of the figure. Complex I ($T{\cdot}D_L$), the first stable intermediate in the packaging pathway, is shown with terminase bound and represented as an oval. Details are described in the text.

releases the DNA-filled capsid, which is further processed into a fully infectious viral particle. Similar packaging pathways are used by all of the double-stranded DNA phages as well as the eucaryotic herpesvirus groups (Black, 1988; Roizman & Sears, 1991).

λ terminase possesses site-specific endonuclease, ATPase, and DNA duplex strand separation catalytic activities, all of which must work in concert to excise a single viral genome from the concatemeric DNA precursor and package it within a preformed prohead (Becker & Murialdo, 1990; Catalano et al., 1995, Feiss, 1986). Coordination of these multiple catalytic activities is critical to efficient assembly of the virus, and understanding how these activities interact is prerequisite to a clear understanding of the biochemistry of the process. We have previously examined the nuclease activity of the enzyme and optimized the reaction with respect to reaction requirements and conditions (Tomka & Catalano, 1993b). Kinetic analysis of the *cos* cleavage reaction demonstrated that (1) the reaction corresponded to a single enzyme turnover, presumably due to the formation of the stable intermediate complex I (Figure 1) and that (2) the reaction time course was complex and varied with the concentration of enzyme included in the reaction mixture (Tomka & Catalano, 1993b). We have similarly optimized the ATPase activity of the enzyme and have further demonstrated that two sites for ATP hydrolysis exist within the enzyme, a high-affinity site located within gpA, and a DNA-stimulated low-affinity site located within the smaller gpNu1 subunit (Hwang et al., 1995; Tomka & Catalano, 1993a). We present here a detailed examination of the strand separation activity of phage λ terminase that optimizes the reaction with respect to reaction conditions and requirements. We further present a kinetic analysis of the reaction and the influence of IHF on the reaction kinetics. The results are compared to those obtained for the endonuclease and ATPase activities of the enzyme to take a first step toward a detailed understanding of the coordination of these catalytic activities critical to the replication of this virus.

EXPERIMENTAL PROCEDURES

Materials and Methods. Tryptone, yeast extract, and agar were purchased from Difco. Bacteriophage λ DNA and *Acc*I restriction endonuclease were purchased from BRL. β,γ-methylene-ATP, divalent metals (as the chloride salts), spermidine, putrescine, and ampicillin were purchased from Sigma Chemical Co. ATP-γ-S, β,γ-imino-ATP, and the natural nucleoside triphosphates were purchased from Boehringer Mannheim Biochemicals. All other materials were of the highest quality commercially available.

Protein Purification. Phage λ terminase was purified from AZ1930 (generously provided by H. Murialdo, University of Toronto, Ontario, Canada) as described by Tomka and Catalano (1993b). The concentration of catalytically active enzyme was determined by active-site titration experiments (Tomka & Catalano, 1993b). *E. coli* integration host factor was purified from HN880 (a kind gift of H. Nash, National Institutes of Health, Bethesda, MD) by the method of Nash *et al.* (1987). All of our purified proteins were homogeneous as determined by SDS−PAGE and densitometric analysis using a Molecular Dynamics laser densitometer and the ImageQuant data analysis package. Unless otherwise indicated, protein concentrations were determined by BCA assay (Dunn, 1994).

Preparation of DNA Substrates. The "complex" DNA substrate used in these studies consisted of mature phage λ DNA that was digested to completion with *Acc* I restriction endonuclease according to the manufacturer's recommendations. This mixture contained 10 fragments, including the annealed mature genome right end (D_R, 5.58 kb) and the mature genome left end (D_L, 2.19 kb) forming the prenicked 7.77 kb strand separation DNA substrate. The complex mixture was used without further purification. The "purified" DNA substrate used in these studies was derived from the complex mixture by separation of the fragments on an 0.8% agarose gel and isolation of the nicked, annealed 7.77 bp DNA fragment by electroelution from the gel. The eluted DNA was extracted with phenol−chloroform and precipitated with ethanol, and the pellet was taken into TE buffer, pH 8.0, using standard methods (Maniatis et al., 1982). To ensure complete annealing of the two fragments, the DNA was diluted into 50 mM Tris·HCl buffer, pH 8.0, containing 10 mM $MgCl_2$ and incubated at 50 °C for 2 h. In all cases, the concentration of the DNA substrates was determined from the absorbance at 260 nm and a calculated formula weight

[2] Currently accepted models for terminase-mediated DNA packaging presume that the terminase subunit(s) is(are) part of the translocating complex and likely forms the DNA packaging "machine". There is, however, no direct evidence for participation of either of the terminase subunits in this process.

of 3.2×10^7 and 5.1×10^6 g/mol for the complex DNA mixture and the purified DNA substrate, respectively (Maniatis et al., 1982).

Strand Separation Activity Assay. Unless otherwise indicated, the reaction mixtures (10 μL) contained 20 mM Tris·HCl buffer, pH 8.0, 11 mM $MgCl_2$, 1 mM ATP, 7 mM β-ME, 2 mM spermidine, 1 mM EDTA, and 5 nM of the indicated DNA substrate. The reaction was initiated with the addition of terminase holoenzyme to a final concentration of 100 nM and allowed to proceed at 37 °C. The reaction was stopped at the indicated times with the addition of 3.4 μL of quench buffer (200 mM EDTA, 20% glycerol, 0.16% bromphenol blue, and 0.16% xylene cyanol). The DNA was fractionated on a 0.8% agarose gel, the ethidium bromide-stained products were analyzed by video densitometry, and the raw data were quantitated using the Molecular Dynamics ImageQuant software package as previously described (Tomka & Catalano, 1993b).

Kinetic Analysis. The assay mixtures contained enzyme in excess of the DNA substrate and thus represent single-turnover kinetic conditions. Each data set was therefore analyzed according to a monophasic exponential time course according to

$$\text{DNA products} = A[1 - \exp(-k_{obs}\tau)] \qquad (1)$$

where DNA products represents the amount of DNA separated at time τ, A is the extent of the reaction at infinite time, and k_{obs} is the observed rate of the reaction. The variables A and k_{obs} were determined by nonlinear regression analysis of the experimental data using the Igor data analysis program (Wave Metrics, Lake Oswego, OR) as described previously (Tomka & Catalano, 1993b).

RESULTS

Optimization of the Strand Separation Assay Conditions. The initial steps in phage λ genome packaging require assembly of the terminase subunits at *cos* and gpA-mediated nicking of the duplex at *cosN*, yielding the nicked, annealed packaging intermediate ($T{\cdot}D_L{\cdot}D_R$, Figure 1) (Becker & Murialdo, 1990; Catalano et al., 1995; Feiss, 1986). Terminase-mediated strand separation, presumably powered by ATP hydrolysis, then yields complex I ($T{\cdot}D_L$), the first stable intermediate in the packaging pathway. We have previously examined the endonuclease and ATPase activities of λ terminase and optimized these reactions with respect to salt and polyamine concentration and the requirement for divalent metals (Tomka & Catalano, 1993a,b). We present here similar studies on the strand separation activity of the enzyme. Early studies that examined this catalytic activity utilized DNA substrates with an intact *cosN* sequence (Higgins et al., 1988), conditions where a kinetic analysis of the reaction is complicated by the requirement for duplex nicking prior to strand separation (see Figure 1). More recent studies have utilized a nicked, annealed substrate, thus obviating a prerequisite nicking step (Rubinchik et al., 1994). The substrate used in these studies was λ Charon 8 DNA digested with *Bam*HI, which was used without further purification. The reaction mixtures thus contained a prenicked strand separation substrate in the presence of multiple "nonspecific" DNA fragments, a situation that may similarly complicate a kinetic analysis of the reaction. We have therefore optimized the strand separation activity of λ terminase using a similar DNA mixture ("complex" DNA substrate) and compared this to reactions using a "purified" *cos*-containing DNA substrate to determine the effect of excess nonspecific DNA on the reaction kinetics. In each case, the DNA substrate contained a nicked, annealed *cosN* site so that kinetic analysis of strand separation would not be complicated by a prerequisite nuclease step.[3] A complete description of these substrates is presented in Experimental Procedures.

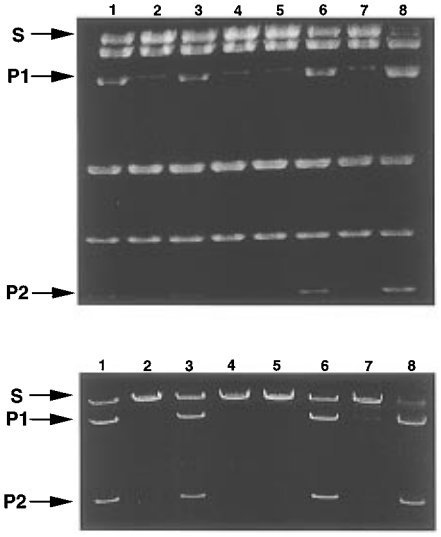

FIGURE 2: Strand separation activity of λ terminase: Agarose gel (0.8%) demonstrating terminase-dependent strand separation activity for the complex (upper panel) and purified (lower panel) DNA substrates. The reaction conditions were as described in Experimental Procedures with the following modifications: (1) Enzyme was omitted and the sample was heated to 70 °C prior to loading to separate the nicked, annealed DNA strands; (2) enzyme was replaced with bovine serum albumin (100 μg/mL); (3) assay was performed as described without modification; (4) ATP was omitted from the assay mixture; (5) $MgCl_2$ was omitted from the assay mixture; (6) spermidine was omitted from the assay mixture; (7) 100 mM NaCl was added to the assay mixture; (8) 50 nM IHF was added to the assay mixture. The position of the 7.77 kb substrate (S) and the 5.58 and 2.19 kb product bands (P1 and P2, respectively) are indicated in each panel. Additional "nonspecific" phage λ bands in the complex mixture (6.96, 3.57, and 2.7 kb, top to bottom) are clearly evident in the upper panel.

Figure 2 demonstrates the strand separation activity of λ terminase using both the complex (upper panel) and purified (lower panel) DNA substrates. In each case, the reaction was dependent upon the presence of enzyme, magnesium, and ATP (lanes 2, 4, and 5), inhibited by NaCl (lane 7), and strongly stimulated by *E. coli* integration host factor (IHF, lane 8). A detailed investigation of each of the reaction components was next performed and these data are presented below. In each case, the results for the complex and the purified DNA substrates were essentially identical.

While divalent metal was strictly required for both the nuclease and ATPase activities of the enzyme, *cos* cleavage activity was dependent upon Mg^{2+} or Mn^{2+} (Tomka & Catalano, 1993b), while ATP hydrolysis was supported by a variety of divalent metals (Tomka & Catalano, 1993a). Table 1 shows that the metal requirement for strand separation activity paralleled that observed for the ATPase activity

[3] Assembly of the terminase subunits onto the nicked, annealed *cos* sequence still precedes strand separation, and protein assembly steps must be considered in the kinetic analysis of the reaction.

Table 1: Divalent Metal is Required for Strand Separation[a]

divalent metal	% relative activity
none	ND[b]
Mg^{2+}	100
Ca^{2+}	25
Sr^{2+}	22
Ba^{2+}	12
Mn^{2+}	30
Cd^{2+}	ND
Co^{2+}	ND
Cu^{2+}	ND
Zn^{2+}	ND

[a] The reaction conditions were as described in Experimental Procedures except that the indicated divalent metals (as the chloride salts) were included at a concentration of 11 mM. The purified DNA fragment was used as the substrate and the amount of product formed in 10 min was determined by gel assay as described. The values represent the average of experiments done in duplicate. One hundred percent activity corresponds to separation of ≈50% of the annealed DNA substrate. Virtually identical results were obtained in assays using the complex DNA mixture (data not shown). [b] ND, strand separation activity not detected.

of the enzyme, with a variety of divalent metals promoting strand separation. Moreover, the magnesium concentration dependence is maximal at ≈8 mM (Figure 3A), which more closely resembles that observed for ATP hydrolysis (Tomka & Catalano, 1993a). Energy for separation of the 12 base pairs in the nicked DNA duplex is presumably provided by the hydrolysis of ATP (Becker & Murialdo, 1990; Catalano et al., 1995; Feiss, 1986). Table 2 demonstrates that while this reaction is also supported by dATP, none of the other nucleoside triphosphates is effective in this capacity. While this result is consistent with our prior demonstration that only the adenine nucleotides were efficiently hydrolyzed by the enzyme (Tomka & Catalano, 1993a), it conflicts with results obtained by Rubinchik et al. (1994), who observed GTP-mediated strand separation activity.[4] Finally, Table 2 shows that the poorly hydrolyzable ATP analogs do not support strand separation by λ terminase, a result consistent with previously published work (Higgins et al., 1988; Rubinchik et al., 1994).

ATP stimulated strand separation in a concentration-dependent manner (Figure 3B) and analysis of these data were consistent with two apparent binding sites for ATP. The first was a lower affinity site with a $K_{m,app} = 49 \pm 9.6$ μM, a value quite similar to that reported previously by Rubinchik et al. (1994). Additionally, however, our analysis revealed strong stimulation at ATP concentrations significantly lower than those used in the earlier study, which exposed an additional, higher affinity site on the enzyme (Figure 3B, inset; $K_{m,app} = 0.24 \pm 0.03$ μM).

Consistent with our previous experiments on the endonuclease activity of the enzyme (Tomka & Catalano, 1993b), polyamines inhibited terminase-mediated strand separation in a concentration-dependent manner with only 20% residual activity observed in the presence of 10 mM spermidine (data not shown). Similar, though not as striking, effects were observed with putrescine (not shown). The effect of salt on

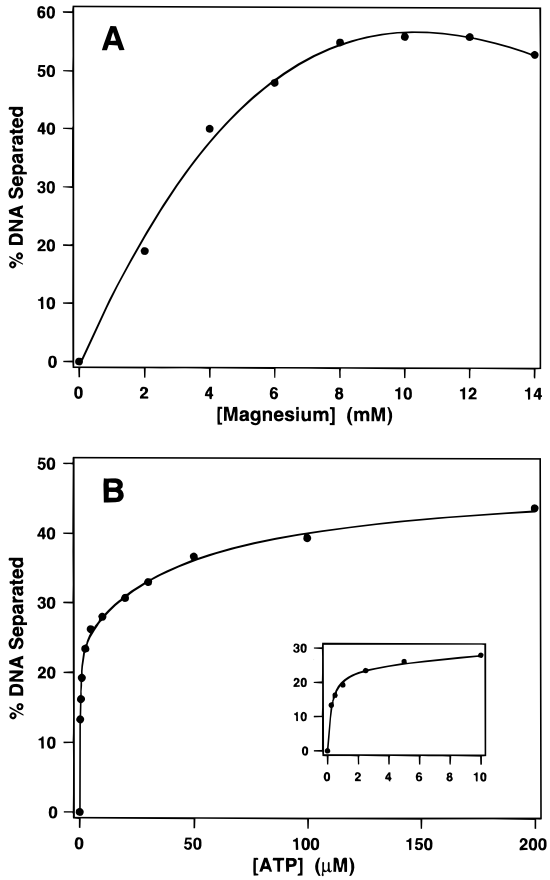

FIGURE 3: Strand separation requires divalent metal and ATP. The reaction conditions were as described in Experimental Procedures except that $MgCl_2$ (panel A) or ATP (panel B) was included at the indicated concentrations. The purified DNA was used as the substrate and the amount of strand separation at 10 min was determined by gel assay as described. Each data point represents the average of three separate experiments. The solid line in panel B represents the best fit of the data to the double hyperbolic curve function: DNA products $= \boldsymbol{A}_1[\mathbf{ATP}]/(\boldsymbol{K}_{m,app1} + [ATP]) + A_2[ATP]/(K_{m,app2} + [ATP])$, where $K_{m,app1}$ and $K_{m,app2}$ represent the apparent binding constants for the two ATP binding sites and A_1 and A_2 represent the extent of stimulation at saturation. The inset in panel B is an expansion of the data between 0 and 10 μM ATP. We note that attempts to fit the data to a single hyperbolic curve function yielded significant error in the regression lines ($\Psi^2 = 189$ vs 3.3 for single vs double hyperbolic curve plot, respectively).

strand separation activity was of particular interest. We have shown previously that while NaCl strongly inhibited the *cos* cleavage reaction (Tomka & Catalano, 1993b), salt stimulated the ATPase activity of the enzyme (Tomka & Catalano, 1993a). Table 3 demonstrates that terminase-mediated strand separation was modestly but consistently stimulated by low concentrations of NaCl and was considerably inhibited as the concentration was increased. Virtually identical results were obtained with KCl (not shown) and potassium glutamate (Table 3), although the stimulation observed at low salt concentrations was more pronounced with the latter.

Effect of IHF and Kinetic Analysis of Strand Separation. Figure 4 demonstrates that strand separation efficiency increases in an enzyme concentration dependent manner for both the complex and purified DNA substrates used in these studies. *E. coli* integration host factor (IHF) modestly stimulates phage replication *in vivo* (Granston et al., 1988; Mendelson et al., 1991) and promotes DNA packaging *in vitro* (Gold & Parris, 1986); and we have previously demonstrated that the endonuclease activity of λ terminase

[4] Consistent with the results presented here, previous studies performed in our laboratory could not demonstrate terminase-mediated GTP hydrolysis (Tomka & Catalano, 1993a). Interestingly, however, both GTP and dGTP stimulated ATP hydrolysis, which suggests that while the guanosine nucleotides are not substrates, they do, in fact, interact with the enzyme and influence catalytic activity.

Table 2: Nucleotide Requirement for Strand Separation[a]

nucleotide	% relative activity
none	ND[b]
ATP	100
GTP	9
CTP	3
UTP	7
dATP	125
dGTP	3
dCTP	7
dTTP	1
ATP-γ-S	9
β,γ-imino-ATP	6
β-γ-methylene-ATP	3

[a] The reaction conditions were as described in Experimental Procedures except that terminase was added to 30 nM and the indicated nucleotide was included at a concentration of 1 mM. The complex DNA mixture was used as the substrate and the amount of product formed in 20 min was determined by gel assay as described. The values represent the average of experiments done in duplicate with one hundred percent activity corresponding to the separation of ≈40% of the annealed DNA substrate. Virtually identical results were obtained in assays using the isolated DNA fragment. [b] ND, strand separation activity not detected.

Table 3: Salt Affects the Strand separation Activity of λ Terminase[a]

salt	concn (mM)	% relative activity
none		100
NaCl	25	104
	50	61
	75	39
	100	30
	150	19
	200	11
potassium glutamate	25	116
	50	104
	75	67
	100	35
	150	18
	200	12

[a] The reaction conditions were as described in Experimental Procedures except that salt was included as indicated in the table. The isolated fragment was used as the DNA substrate and the amount of product formed in 25 min was then determined by gel assay as described. The values represent the average of experiments done in duplicate with 100% activity corresponding to the separation of ≈50% of the annealed DNA substrate. Virtually identical results were obtained with the complex DNA mixture as a substrate.

was stimulated 3−4-fold by this protein, but only under conditions of limiting enzyme (Tomka & Catalano, 1993b). The strand separation activity of λ terminase is similarly affected by IHF with a 3−4-fold stimulation observed at low enzyme concentrations (Figure 4). Interestingly, the effects were more striking with the complex DNA substrate. Moreover, while the complex DNA substrate remained responsive to increasing concentrations of terminase in the presence of IHF (Figure 4A), strand separation of the purified DNA substrate was optimal even at the lowest enzyme concentration examined (Figure 4B).

The single time point assay discussed above demonstrates that IHF has a dramatic effect on the strand separation activity of λ terminase. This phenomenon was examined in more detail and the time courses for strand separation for both the complex and purified DNA substrates are displayed in Figure 5. Each of these data sets was best described by

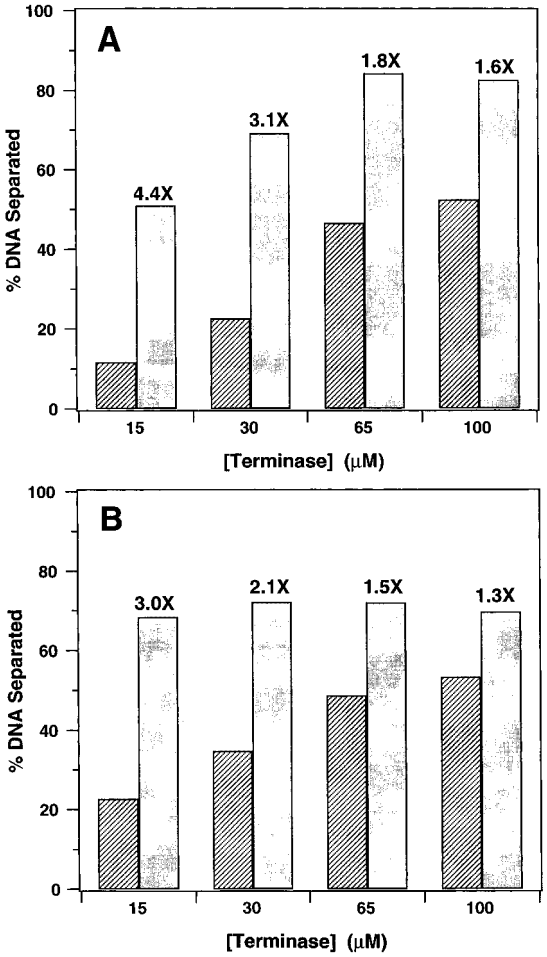

FIGURE 4: IHF stimulates the strand separation activity. The reaction conditions were as described in Experimental Procedures except that enzyme was included at the indicated concentration. The amount of complex DNA substrate (panel A) or purified DNA substrate (panel B) separated at 10 min in the absence (hatched bar) or presence (solid bar) of 50 nM IHF was determined by gel assay as described. The numbers at the top of the solid bars represent *x*-fold stimulation by IHF. Each data set represents the average of two separate experiments.

a single-exponential time course, and kinetic analysis of the data yielded the rate constants presented in Table 4. Of note is that the rate constant for the reaction in the absence of IHF was significantly greater for the purified DNA substrate than that obtained for the complex mixture (5.5× and 4.5× at 30 and 100 nM enzyme, respectively). Addition of IHF to the reaction mixture dramatically affected the observed rate with ≈10- and ≈6-fold increase in the rate constants for the complex and purified DNA substrates, respectively. As above, the effects of IHF were not as striking when elevated concentrations of enzyme were used in the reaction mixture (see Table 4).

DISCUSSION

Terminase, the DNA packaging enzyme of bacteriophage λ, possesses site-specific endonuclease, ATPase, and duplex strand separation catalytic activities, which must work in concert to insert a single viral genome into an empty precapsid, a critical step in the viral assembly pathway (Becker & Murialdo, 1990; Catalano et al., 1995; Feiss, 1986). We have optimized the strand separation activity of the enzyme with respect to polyamine and salt concentration, divalent metal and nucleoside triphosphate requirement, and

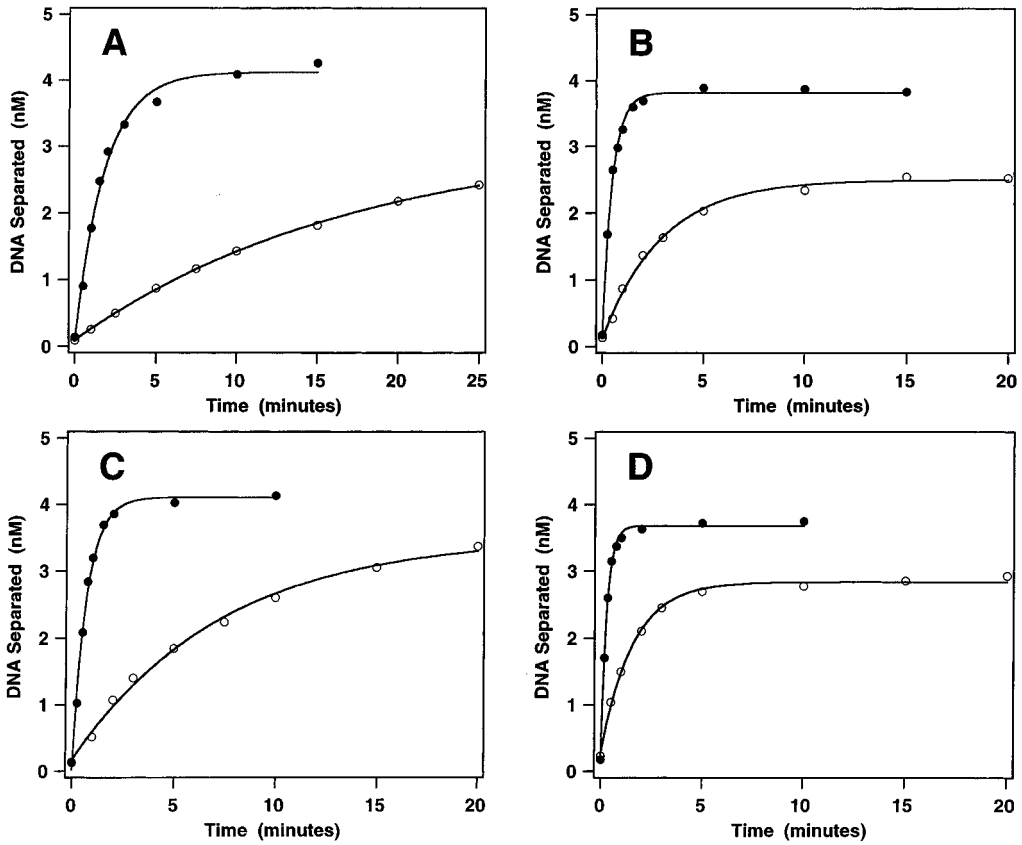

FIGURE 5: Kinetic analysis of strand separation. The reaction conditions were as described in Experimental Procedures except that the concentration of enzyme was either 30 nM (panels A and B) or 100 nM (panels C and D). Aliquots (10 μL) were removed from the reaction mixture at the indicated times, and the amount of complex DNA substrate (panels A and C) or purified DNA substrate (panels B and D) separated was determined by gel assay as described. Reactions were performed in the absence (○) or presence (●) of 50 nM IHF. Each data point is the average of three separate experiments and the solid line is the best fit of the data to a monoexponential curve function as described. The kinetic constants derived from this analysis are presented in Table 4.

Table 4: Rate Constants for the Strand Separation Activity of λ Terminase[a]

[terminase] (nM)	[IHF] (nM)	DNA substrate	k_{obs} (min^{-1})	IHF stimulation
30	0	complex mix	0.0019 ± 0.0001	-
30	50	complex mix	0.018 ± 0.001	9.5
30	0	isolated fragment	0.011 ± 0.001	-
30	50	isolated fragment	0.068 ± 0.003	6.2
100	0	complex mix	0.0014 ± 0.0001	-
100	50	complex mix	0.014 ± 0.001	10
100	0	isolated fragment	0.0064 ± 0.0003	-
100	50	isolated fragment	0.035 ± 0.001	5.5

[a] The data presented in Figure 5 were analyzed as described in Experimental Procedures. Details of the experiment are presented in the legend to Figure 5.

enzyme concentration-dependent stimulation of the reaction by the *E. coli* assembly protein IHF. We have found that the optimized reaction conditions represent a fusion of those required for ATP hydrolysis and strand nicking reactions. Similar to ATP hydrolysis (Tomka & Catalano, 1993a), terminase-mediated strand separation was supported by a variety of divalent metals and required ATP (or dATP) for efficient catalysis. Spermidine, which inhibited nuclease activity but did not affect ATP hydrolysis (Tomka & Catalano, 1993a,b), was inhibitory to strand separation activity. This may reflect polyamine-induced conformational changes in DNA structure (Lohman, 1986; Record et al., 1985) that affect protein binding but have little effect on ATP binding to the enzyme. The effect of salt on the strand separation activity of the enzyme was most interesting in that it has been found to have disparate effects on the nuclease and ATPase activities. While NaCl stimulated ATP hydrolysis with maximal stimulation observed at 200 mM (Tomka & Catalano, 1993a), all salts examined strongly inhibited the *cos* cleavage reaction at concentrations greater than 100 mM (Tomka & Catalano, 1993b). The latter effect results from attenuated binding of the terminase subunits to DNA at high ionic strength (Yang et al., 1997). While low concentrations of salt stimulated strand separation activity, elevated concentrations were strongly inhibitory suggesting competition between two required steps in strand separation. Given that ATP hydrolysis is required for separation of the nicked, annealed strands of the duplex, it is reasonable that stimulation of ATPase activity would have a positive effect. As the concentration of salt is increased, however, assembly of the terminase subunits at *cos* becomes inefficient and the observed activity decreases.

We have demonstrated that ATP-mediated strand separation activity exhibits a $K_{m,app} \approx 50$ μM, which is similar to that obtained by Rubinchik et al. (1994). We note that this $K_{m,app}$ is comparable to that obtained for the high-affinity ATPase site located in gpA ($K_m \approx 5$ μM) (Hwang et al., 1995; Tomka & Catalano, 1993a) and is consistent with the observation that the isolated gpA subunit possesses substantial strand separation activity (Rubinchik et al., 1994). It is unlikely that the ATPase site located within the gpNu1 subunit ($K_m \approx 500$ μM in the presence of DNA) plays a role in the strand separation activity of the enzyme as there is no additional stimulation up to 5 mM ATP; however,

closer inspection of the ATP concentration dependence revealed an additional apparent ATP binding site with a much greater affinity for ATP ($K_{m,app} \approx 0.25\ \mu M$), which strongly stimulated strand separation activity. Higgins et al. (1988) have suggested that DNA packaging by λ terminase may require the action of a NTP reactive center that is distinct from and possesses a nucleotide specificity different from those presently identified in the enzyme. This suggestion is consistent with the fact that (1) the rate of ATP hydrolysis by terminase holoenzyme is insufficient to account for that required for active DNA packaging (Hwang et al., 1995; Rubinchik et al., 1994; Tomka & Catalano, 1993a) and (2) our prior demonstration that GTP stimulated ATP hydrolysis and yet was not hydrolyzed by the enzyme (Tomka & Catalano, 1993a) (see footnote 4). The kinetic data presented here suggest that a third, very high affinity NTP binding site may reside in the holoenzyme. Within this context, a third region of homology to ATPase catalytic centers has been identified in the primary sequence of the phage λ gpA protein (Hwang and Feiss, personal communication). That two ATPase catalytic sites may exist in gpA is consistent with the observation that two putative ATPase reactive centers have similarly been identified within the primary sequence of the large terminase subunits of bacteriophage T3 (Kimura & Fujisawa, 1991).

Our results are, for the most part, harmonious with those obtained previously by Gold and co-workers using *Bam*HI-digested Charon 8 λ DNA (analogous to our complex mixture) as a strand separation substrate (Rubinchik et al., 1994). Moreover, the majority of results presented above were virtually identical regardless of whether the complex DNA mixture or the purified DNA substrate were used in the reaction mixture. Major differences were observed between the substrates, however, with respect to the effect of IHF on the reaction kinetics. While strand separation was stimulated by IHF with both DNA substrates, especially at the lower enzyme concentrations, the magnitude of the stimulation was greater with the complex DNA substrate. Moreover, an enzyme concentration-dependent increase in product formation was observed with the complex DNA mixture under all conditions examined, while this effect was abolished when IHF was included with the purified DNA substrate. We have previously suggested that IHF-mediated stimulation of the *cos* cleavage reaction was due to an increase in the assembly rate of the terminase subunits at *cos*, the rate-limiting step at low enzyme concentrations (Tomka & Catalano, 1993b; Woods et al., 1997). Assembly of the terminase subunits at *cos* is similarly required for strand separation (see footnote 3) and the observed stimulation by IHF likely reflects these slow, protein concentration-dependent assembly steps. This suggestion is fully consistent with our results as terminase assembly at *cos* may be impaired in the complex DNA mixture due to binding to nonspecific DNA. In all cases examined, IHF promotes the assembly of nucleoprotein complexes by forming a DNA structure conducive to the binding of additional proteins (Nash, 1996). It is thus reasonable to suggest that the IHF effects are more striking in the complex mixture due to the formation of an IHF·DNA_{cos} binary complex, which is readily distinguished from bulk DNA and which may promote terminase assembly. This is consistent with our demonstration that (1) the observed rate constant for strand separation is 4−6-fold greater for the purified DNA substrate compared to the complex DNA mixture and that (2) the observed rate of the reaction increases 10- and 5-fold for the complex mixture and purified DNA substrate, respectively, in the presence of IHF.

The strand separation activity of phage λ terminase is required to separate the nicked, annealed strands created by the endonuclease activity of the enzyme, thus yielding the 12-base-pair "sticky" ends of the mature λ genome (Becker & Murialdo, 1990; Catalano et al., 1995; Feiss, 1986). ATP (but not ATP hydrolysis) stimulates nuclease activity and promotes fidelity in the DNA nicking reaction (Higgins & Becker, 1994; Higgins et al., 1988). ATP hydrolysis is required for strand separation and ultimately to provide the driving force for insertion of DNA into the viral capsid. The three activities of λ terminase are thus closely linked, functionally if not biochemically, and coordination of the catalytic activities is critical to efficient assembly of the virus. The present study is our first step toward an understanding of strand separation activity of λ terminase and its interaction with the ATPase and endonuclease activities of the enzyme. Perhaps not unexpectedly, the strand separation activity of the enzyme reflects the requirements for ATP hydrolysis and DNA binding, both of which must occur efficiently for efficient strand separation. Many aspects related to the mechanism of strand separation remain unanswered, however. While historically referred to as a "helicase" activity (Catalano et al., 1995; Rubinchik et al., 1994), we suggest that the observed ATP-dependent strand separation activity of λ terminase is not, strictly defined, a helicase activity. Rather, ATP hydrolysis may drive a complex conformation that binds tightly and specifically to the single-stranded end formed by duplex nicking (D_L, Figure 1). In this model, ATP and ADP stabilize different protein conformations that bind duplex and single-stranded DNA, respectively. This reaction, which is formally a single turnover of the active rolling strand separation model proposed by Lohman and co-workers (Lohman, 1993; Wong & Lohman, 1992), results in the complete separation of the nicked, annealed duplex, forming a nucleoprotein complex tightly bound to DNA. In this model, the unusual stability of complex I derives from specific interactions of the terminase subunits with the 12-base single-stranded left end of mature λ DNA. Ongoing work in our laboratory is directed toward a detailed, molecular understanding of the strand separation activity of the enzyme including questions of substrate specificity, the energetics of strand separation, and a definition of the roles of protein and nucleotide in the process of DNA packaging.

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