Kinetic and Mutational Dissection of the Two ATPase Activities of Terminase, the DNA Packaging Enzyme of Bacteriophage λ†

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ABSTRACT: Terminase, the DNA packaging enzyme of bacteriophage λ, is a heteromultimer of gpNu1 (21 kDa) and gpA (74 kDa) subunits, encoded by the λ *Nu1* and *A* genes, respectively. Sequence comparisons indicate that both gpNu1 and gpA have a match to the P-loop motif of ATPase centers, which is a glycine-rich segment followed by a lysine. By site-specific mutagenesis, we changed the lysines of the putative P-loops of gpNu1 (K_{35}) and gpA (K_{497}) to arginine, alanine, or aspartic acid, and studied the mutant enzymes by kinetic analysis and photochemical cross-linking with 8-azido-ATP. Both the gpNu1 and gpA subunits of wild-type terminase were covalently modified with 8-N_3-[^{32}P]ATP in the presence of UV light. Saturation occurred with apparent dissociation constants of 508 and 3.5 μM for gpNu1 and gpA, respectively. ATPase assays showed two activities: a low-affinity activity (K_m = 469 μM), and a high-affinity activity (K_m = 4.6 μM). The gpNu1 K_{35}A and gpNu1 K_{35}D mutant terminases showed decreased activity in the low-affinity ATPase activity. The reduced activities of these enzymes were recovered when 10 times more DNA was added, suggesting that the primary defect of the enzymes is alteration of the nonspecific, double-stranded DNA binding activity of terminase. ATPase assays and photolabeling of the gpA K_{497}A and gpA K_{497}D mutant terminases showed reduced affinity for ATP at the high-affinity site which was not restored by increased DNA. In summary, the results indicate the presence of a low-affinity, DNA-stimulated ATPase center in gpNu1, and a high-affinity site in gpA.

Terminase is the enzyme that mediates packaging of λ DNA into the prohead, the protein shell that is the capsid precursor. Terminase is a heteromultimer composed of 21 kDa gpNu1[1] (the product of gene *Nu1*) and 74 kDa gpA (the product of gene *A*) subunits. Terminase binds and cuts λ DNA at *cos*, the site that contains recognition signals for packaging, thus generating the cohesive ends of virion DNA. Terminase also separates the cohesive ends, and interacts with the prohead, the empty protein shell into which DNA is packaged [reviewed in Becker and Murialdo (1990), Murialdo (1991), and Catalano *et al.* (1995)].

Functional domains of terminase have been defined by genetic studies (see Figure 1), as follows. The first 91 amino acids of gpNu1 contain a domain for DNA binding (Frackman *et al.*, 1985); this segment contains a putative helix−turn−helix DNA binding motif [residues 5−24; Andrew Becker, cited in Feiss (1986) and Kypr and Mrazek (1986)], followed by a putative ATP reactive center (starting at about residue 29; Becker & Gold, 1988). The carboxyl terminus of gpNu1 is a domain which interacts with the amino terminus of gpA (Frackman *et al.*, 1985; Wu *et al.*, 1988). Other motifs within gpA include a putative ATP binding center (starting near residue 491; Guo *et al.*, 1988) and a putative basic leucine zipper (residues 573−616; Davidson & Gold, 1992). Mutations affecting the basic leucine zipper

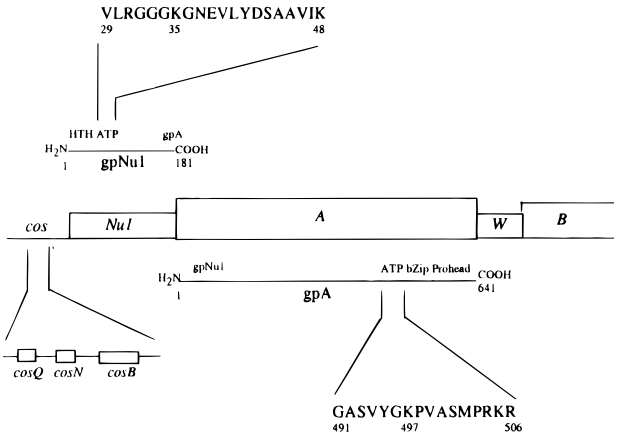

FIGURE 1: The left end of the bacteriophage λ chromosome. Genes *Nu1* and *A* encode the small subunit (gpNu1, 181 residues) and the large subunit (gpA, 641 residues) of terminase. Gene *B* encodes the portal protein (533 residues). (Top) Approximate locations of domains in the gpNu1 polypeptide. HTH represents the putative helix−turn−helix motif for binding *cosB*, ATP represents the putative ATP binding site in gpNu1, and gpA represents a specificity domain for interaction with gpA. The amino terminus of the polypeptide is at the left as indicated. (Bottom right) Approximate locations of domains in the gpA polypeptide. gpNu1 represents a specificity domain for gpNu1 binding, and ATP represents the putative ATP binding site in gpA. bZip is the putative basic leucine zipper, and Prohead represents the specificity domain for interaction with the prohead. The amino terminus of the polypeptide is at the left as indicated. (Bottom left) *cos* structure. *cos* is the site at which terminase acts, *cosN* is the site at which nicks are introduced, *cosB* is the terminase binding site, and *cosQ* is a site required for termination of DNA packaging.

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[1] Abbreviations: bp, base pair(s); *cos*, cohesive end site; 8-N_3-ATP, 8-azido-ATP; gpNu1, gene product of *Nu1*; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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or residues 401 and 403 specifically inactivate the endonuclease activity of terminase (Davidson & Gold, 1992). The carboxyl-terminal 32 amino acids of gpA define a domain required for prohead interaction (Frackman *et al*., 1984; Wu *et al*., 1988; Yeo & Feiss, 1995a,b).

cos consists of three subsites: *cosQ*, *cosN*, and *cosB* [Figure 1; reviewed in Becker and Murialdo (1990), Cue and Feiss (1993b), and Catalano *et al*. (1995)]. Terminase binds λ DNA at *cosB* and introduces staggered nicks at an adjacent site, *cosN*, to generate the cohesive ends found on virion DNA (Higgins *et al*., 1988). Sequence-specific binding of terminase to *cosB* occurs via the gpNu1 subunit. Purified gpNu1 specifically binds three sites in *cosB*: R3, R2, and R1 (Shinder & Gold, 1988). It is assumed that the helix−turn−helix motif is directly involved in the binding of terminase to *cosB* (Feiss, 1986; Becker & Murialdo, 1990) although how gpNu1 specifically interacts with *cosB* is not known (see Figure 1). Following nicking of *cosN* and strand separation, terminase remains tightly bound at the left end of the chromosome, presumably at *cosB* and the left cohesive end, in a complex called complex I (Becker *et al*., 1977; Sippy & Feiss, 1992; Cue & Feiss 1993a). This complex then binds a prohead to form complex II, a step that is facilitated by the viral assembly catalyst, gpFI, and DNA packaging ensues (Becker *et al*., 1977, 1988; Davidson *et al*., 1987). Terminase remains bound to the DNA packaging complex during translocation (Feiss & Widner, 1982; Feiss *et al*., 1985). Finally, terminase of the packaging complex recognizes the next downstream *cos* and introduces staggered nicks at *cosN* to terminate DNA packaging; *cosQ* is required for termination (Cue & Feiss, 1993b).

ATP plays multiple roles in λ DNA packaging. The rate and fidelity of *cosN* cleavage are stimulated by ATP and certain other nucleotides including poorly hydrolyzed ATP analogs (Higgins *et al*., 1988; Cue & Feiss, 1993a; Higgins & Becker, 1994a,b), indicating that ATP *hydrolysis* is not required for the endonucleolytic reaction. However, ATP hydrolysis *is* required to separate the nicked, annealed strands produced by *cos* cleavage (Higgins *et al*., 1988). Since ATP hydrolysis is required for complex I formation, this intermediate is thought to be assembled following *cos* cleavage and strand separation (Becker *et al*., 1977; Sippy & Feiss, 1992; Kuzminov *et al*., 1994). Finally, ATP hydrolysis is required and in fact powers DNA packaging, and it has been suggested that ATP hydrolysis by λ terminase is required for translocation (reviewed in Earnshaw and Casjens (1980), Feiss and Becker (1983), and Becker and Murialdo (1990)].

Terminase holoenzyme has been predicted to have two ATP reactive centers, based on the deduced primary amino acid sequences of the gpNu1 and gpA subunits (Becker & Gold, 1988; Guo *et al*., 1987). Kinetic studies, however, have given conflicting results. Gold and Becker (1983) observed a holoterminase ATPase activity with a K_m of about 300 μM, but also found that the half-maximal concentration of ATP for stimulation of the endonuclease reaction was 5 μM, implying the existence of a second center. Tomka and Catalano (1993b) reported that terminase possesses two ATPase activities: one with high ATP affinity ($K_m = 5$ μM) and one, stimulated by dsDNA, of low affinity ($K_m = 1.3$ mM). In contrast, Rubinchik *et al*. (1994) reported that gpA and holoterminase each possess a single ATPase activity of intermediate binding affinity ($K_m = 40$ μM). In this report, we confirm that terminase holoenzyme has two kinetically-distinct ATP reactive centers and have assigned one center to each of the terminase polypeptides.

EXPERIMENTAL PROCEDURES

Media. Luria broth (LB), Luria agar (LA), 2×YT broth, and SOB were prepared as described in Sambrook *et al*. (1989). When required, kanamycin and ampicillin were used at final concentrations of 50 and 100 μg/mL, respectively. Final concentrations of isopropyl β-D-thiogalactopyranoside (IPTG) and X-gal used in LA were 0.1 mM and 0.02% (w/v), respectively.

General Recombinant Techniques. Restriction enzymes, bacteriophage T4 DNA ligase, Klenow fragment of DNA polymerase I, calf intestinal alkaline phosphatase, and T4 polynucleotide kinase were purchased from New England Biolabs, International Biotechnologies Inc., Promega, and Boehringer-Mannheim, and were used according to the manufacturer's recommendations. DNA sequencing (Sanger *et al*., 1977) was performed with reagents purchased from Promega and Pharmacia. Nucleoside triphosphates were from Boehringer-Mannheim. 8-N_3-ATP and 8-N_3-[α-^{32}P]-ATP were obtained from ICN Biochemicals. [α-^{32}P]ATP and [α-^{32}P]dATP were from Amersham. λ DNA was purchased from New England Biolabs. Cellulose PEI-F thin-layer chromatography plates were obtained from J. T. Baker. All other materials were of the highest quality commercially available. Plasmid DNA and M13 replicative-form DNA were prepared as described by Birnboim and Doly (1979). Single-stranded DNA from pIBI31 (International Biotechnologies, Inc.) was prepared as described (Vieira & Messing, 1987). DNA fragments were purified from agarose gels as described by Vogelstein and Gillespie (1979). Preparation of competent cells and transformation were performed as described by Hanahan (1983).

Sequence Designations and Nomenclature. All references to λ sequences are based on the numbering convention described in Daniels *et al*. (1983). Numbering of the λ sequence begins with the first base of the left cohesive end and continues along the l strand (the top strand) in a 5′ to 3′ direction. The position of each restriction cut site is given as the first nucleotide of the recognition sequence. The single-letter designation for amino acids is used.

Mutagenesis. GpNu1 and gpA contain amino acid segments that show sequence homology to a motif, the P-loop, that is found in a number of known ATPases (Becker & Gold, 1988; Guo *et al*., 1987). The P-loop, which consists of a glycine-rich segment followed by a lysine, is thought to form a flexible loop with the lysine involved in ATP hydrolysis (Saraste *et al*., 1990). The putative ATP binding motifs of gpNu1 and gpA have each been identified by the P-loop sequence (Fry *et al*., 1986; Walker *et al*., 1982; Saraste *et al*., 1990). The proposed sequences of the P-loops of gpNu1 and gpA are amino acid residues 29−35 (VLRGGGK) for gpNu1 and amino acid residues 491−497 (GASVYGK) for gpA.

Six mutations affecting terminase were constructed; gpNu1 K_{35}R, gpNu1 K_{35}A, and gpNu1 K_{35}D, mutations altering the invariant lysine residue of putative ATP binding sites in the gpNu1 subunit; and gpA K_{497}R, gpA K_{497}A, and gpA K_{497}D, mutations altering the invariant lysine residue of putative ATP binding sites in the gpA subunit. Mutagenesis was performed using the method of Kunkel (1985). For intro-

ducing mutations into *Nu1*, the 456 bp *Eco*RI (λ bp 194) to *Eco*RV (λ bp 650) fragment from pRV1 (Cue, 1991) was inserted into the *Eco*RI and *Eco*RV sites of M13mp19. Phage was amplified in BW313, an *Escherichia coli dut*$^-$*ung*$^-$ strain, to obtain uridylated single-stranded template DNA (Kunkel, 1985). To construct mutations in *A*, the 128 bp *Eco*RV (λ bp 2084) to *Sph*I (λ bp 2212) fragment from pCM101 (Chow *et al*., 1987) was inserted into pIBI31 that had been prepared by successive treatment with *Eco*RV, *Sph*I, and calf intestinal alkaline phosphatase. BW313 was transformed with the resulting plasmid, and uridylated single-stranded template DNA corresponding to the plasmid was generated by infection with the helper phage M13KO7 (Vieira & Messing, 1987). Mutagenic primers were used to make the following changes: for changing K_{35} of gpNu1, the 35th codon of *Nu1* was changed from AAG to CGC for K_{35}R; GCC for K_{35}A, and GAC for K_{35}D. The K_{35}R and K_{35}A changes created *Sac*II and *Nar*I restriction enzyme sites, respectively. For changing K_{497} of gpA, the 497th codon of *A* was changed from AAG to CGT for K_{497}R, GCT for K_{497}A, and GAT for K_{497}D. The K_{497}R, K_{497}A, and K_{497}D changes created *Aat*II, *Sac*I and *Xho*II sites, respectively.

After *in vitro* synthesis of the complementary strand using the Klenow fragment of DNA polymerase I, the double-stranded circular DNAs were transformed into *E. coli* JM107 (*dut*$^+$*ung*$^+$) (Yanisch-Perron *et al*., 1985), the mutant phages or plasmids were isolated, and the presence of the desired mutations was confirmed by restriction digestion and direct nucleotide sequencing (Sanger *et al*., 1977). To introduce terminase mutations into pCM101, the terminase expression vector, standard recombinant DNA manipulations were used.

Overproduction and Purification of Wild-Type and Mutant Terminases. *E. coli* OR1265 cells (Reyes *et al*., 1979) harboring plasmids for preparation of wild-type and six mutant terminases were grown, induced, and harvested as described in Chow *et. al.* (1987). The purification was carried out using the following modifications of the method of Tomka and Catalano (1993a). Cells resuspended in buffer A (20 mM Tris-HCl, pH 9.0, 1 mM EDTA, 7 mM β-mercaptoethanol, and 10% glycerol) were disrupted by passage through a French Press at 15 000 psi. Following precipitation with 45% ammonium sulfate, the pellet (fraction I) was resuspended in buffer C (20 mM sodium citrate, pH 6.8, 1 mM EDTA, 7 mM β-mercaptoethanol, and 10% glycerol) and dialyzed against buffer C. The dialyzed protein (fraction I) was applied to a Q-Sepharose FF column (2.6 $\times$ 15 cm) equilibrated with the same buffer. Protein was eluted with a 400 mL linear gradient to 1.0 M NaCl. Fractions were collected and examined by SDS−PAGE. Fractions containing terminase were pooled, dialyzed against buffer C (fraction II), and applied to an S-Sepharose FF column (1 $\times$ 15 cm) equilibrated with buffer C. Protein was eluted with a 20 mL linear gradient to 200 mM NaCl followed by an 80 mL linear gradient to 400 mM NaCl. Fractions containing terminase were pooled, dialyzed against buffer C (fraction III), and applied to a Blue-Sepharose FF column (1 $\times$ 10 cm) equilibrated with buffer C. Proteins were eluted with a 20 mL linear gradient to 200 mM NaCl followed by an 80 mL linear gradient to 400 mM NaCl. Fractions containing terminase were pooled and dialyzed against buffer A containing 50% glycerol and concentrated to 1 mg/mL using a Centricon 30 concentrator (Amicon Inc.), and the preparations were stored at −70 °C. The protein concentration of terminase was determined by the Bradford method (Bradford, 1976). The yield was 10−15%. The purified enzymes were >95% pure, as determined by Coomassie Blue staining of 10 μg of protein separated by SDS−PAGE.

ATPase Activity of Terminase. The standard assay was performed in a 20 μL reaction containing 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 6 mM spermidine, 7 mM β-mercaptoethanol, 1 mM EDTA, 0.15 nM λ DNA, and various concentrations of [α-^{32}P]ATP. The concentrations of terminase used are indicated in the figure legends; and the values in the figures are normalized to 50 nM terminase. The reactions were initiated with the addition of enzyme and incubated for 20 min at 25 °C. Aliquots (2 μL) were removed from the reaction mixture every 5 min and the reaction stopped by the addition of one-half volume of 500 mM EDTA. Aliquots (2 μL) of the stopped reaction mixtures were spotted onto a cellulose PEI-F TLC plate, and the plate was developed with 300 mM sodium phosphate buffer (pH 8.0). The ATP and ADP spots were visualized with autoradiography. The radiolabeled nucleotides were excised and quantitated by scintillation counting and/or the Ambis radioanalytic image system (AMBIS Systems, Inc., San Diego, CA). The initial velocity of ATP hydrolysis was determined within the linear range of the each reaction.

Kinetic Analysis of ATP Hydrolysis. The data were analyzed using a model which assumes two independent, noninteracting ATP binding sites within the holoenzyme:

$$\mathrm{E} + \mathrm{ATP} \overset{K_{m1}}{\rightleftharpoons} \mathrm{E{-}ATP_1} \xrightarrow{k_1} \mathrm{E} + \mathrm{ADP} + \mathrm{P_i}$$

$$\mathrm{E} + \mathrm{ATP} \overset{K_{m2}}{\rightleftharpoons} \mathrm{E{-}ATP_2} \xrightarrow{k_2} \mathrm{E} + \mathrm{ADP} + \mathrm{P_i}$$

where $E-ATP_1$ and $E-ATP_2$ represent ATP bound at two distinct catalytic sites on the protein [see Tomka and Catalano (1993b)]. The kinetic expression for this model is

$$\frac{k_{obs}}{[E]} = \frac{k_1[ATP]}{K_{m1} + [ATP]} + \frac{k_2[ATP]}{K_{m2} + [ATP]} \qquad (1)$$

where [E] is the total enzyme concentration, k_{obs} is the observed rate of ATP hydrolysis at a given concentration of ATP, and k_1 and k_2 and K_{m1} and K_{m2} represent the maximal velocities and Michaelis constants, respectively, for the two binding sites of the holoenzyme. The kinetic constants for ATP hydrolysis 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).

8-N_3-ATP Photoaffinity Cross-Linking. Unless otherwise indicated, the standard reaction condition for the 8-N_3-ATP photolabeling was 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 6 mM spermidine, 7 mM β-mercaptoethanol, 1 mM EDTA, 500 nM λ terminase, 0.15 nM λ DNA, and various concentrations of 8-N_3-[α-^{32}P]ATP. The reaction was started by adding the enzyme and the mixture incubated on ice for 1 min. UV irradiation was then carried out using the short-wavelength lamp (Spectroline, Model ENF-24, 254 nm) at a distance of 4 cm from the bottom of the reaction tube (approximately 6000 μW/cm^2). Reactions were stopped by the addition of EDTA to 100 mM.

Analysis of Photolabeling Reactions by SDS−PAGE. Trichloroacetic acid was added to 10% to stop photolabeling

reactions followed by 15 min on ice, followed by centrifugation at 4 °C (12000*g* for 15 min). The pelleted proteins were washed with cold acetone, dried, and suspended in 10 μL of 50 mM Tris-HCl (pH 6.8), 0.25% SDS, 0.025% bromophenol blue, and 25% glycerol. Samples were subjected to 12.5% (w/v) SDS−PAGE (Laemmli, 1970). After electrophoresis, the gels were stained for 10 min in Coomassie Blue R-250 (Bio-Rad) and destained for several hours. Autoradiography of dried gels was done at −70 °C using XAR X-ray film (Kodak), and the extent of photolabeling was quantitated by scintillation counting and/or by an Ambis radioanalytic image system (AMBIS Systems, Inc., San Diego, CA).

RESULTS

Photoaffinity Labeling of Wild-Type Terminase with 8-N_3-ATP. Photolabeling of the wild-type λ terminase at increasing concentrations of 8-N_3-ATP was done to determine the extent of photolabeling *versus* 8-N_3-ATP concentration. As shown in Figure 2a, labeling of the gpA subunit was efficient at 8-N_3-ATP concentrations as low as 5 μM, while labeling of the gpNu1 subunit was inefficient even at 2 mM 8-N_3-ATP. The data were fit to a rectangular hyperbola using nonlinear regression analysis which yielded apparent binding constants of 508 μM and 3.5 μM for binding to the gpNu1 and gpA subunits, respectively (Figure 2b). This confirms that terminase has two distinct ATP binding sites, one in gpNu1 and one in gpA, and strongly suggests that the gpNu1 subunit has a low-affinity ATP binding site and that the gpA has a high-affinity ATP binding site.

The extent of photolabeling of both subunits increased linearly with time up to 5 min of UV irradiation at both 5 and 500 μM 8-N_3-ATP (data not shown). While photolabeling was saturable, and thus suggested active-site-specific labeling (Figure 2), competition studies with natural nucleoside triphosphates were performed to verify that the photolabeling was indeed active site-directed. Addition of a 20-fold excess of unlabeled ATP or dATP resulted in strong inhibition of photolabeling of both the gpA and gpNu1 subunits. Partial inhibition by other nucleoside triphosphates was also observed (Table 1); however, the extent of inhibition was substantially less than that observed with ATP or dATP, indicating that the binding sites in the gpNu1 and gpA subunits are specific for adenine nucleotides. These competition studies are consistent with our previous work showing inhibition of ATP hydrolysis by dATP but limited competition by other nucleotides (Tomka & Catalano, 1993b).

Kinetic Analysis of Wild-Type and gpNu1 Mutant Terminases. The observed rate of ATP hydrolysis by wild-type terminase as a function of ATP concentration is shown in Figure 3. Nonlinear regression analysis of the data revealed two sites for ATP hydrolysis with K_m and k_{cat} values of 4.6 μM and 38 min^{-1}, respectively, for the high-affinity site and K_m and k_{cat} values of 469 μM and 84 min^{-1}, respectively, for the low-affinity site. These data are in reasonable agreement with our data published previously (Tomka & Catalano, 1993b). Importantly, the χ^2 value for regression analysis using equation 1 (see Experimental Procedures) was highly significant ($\Psi^2 = 0.00101$) and greater than an order of magnitude smaller than that obtained when the data were fit to a single hyperbolic curve ($\Psi^2 = 0.0261$), characteristic of ATP hydrolysis at a single catalytic site.

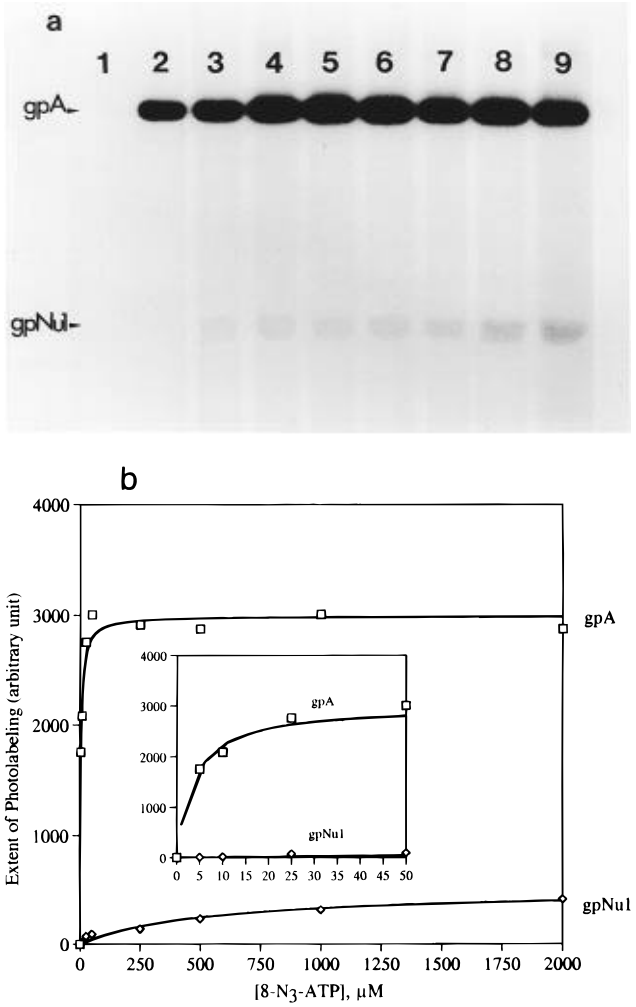

FIGURE 2: (a) Photolabeling of terminase in the presence of varying concentrations of 8-N_3-[α-^{32}P]-ATP. Photolabeling was performed under standard conditions in a 20 μL reaction with 1 min of UV irradiation. The 8-N_3-[α-^{32}P]ATP (10.6 μCi/μmol) concentrations added were: (lane 1) 0 μM, (lane 2) 5 μM, (lane 3) 10 μM, (lane 4) 25 μM, (lane 5) 50 μM, (lane 6) 250 μM, (lane 7) 500 μM, (lane 8) 1 mM, and (lane 9) 2 mM. (b) Extent of photolabeling of terminase by 8-N_3-[α-^{32}P]ATP. The intensities of bands corresponding to gpNu1 and gpA in the autoradiogram shown in panel a were quantitated by the AMBIS radioanalytic image system. The inset shows an expansion of the data between 0 and 50 μM 8-N_3-[α-^{32}P]ATP.

Figure 3 and Table 2 demonstrate that ATP hydrolysis by the gpNu1 $K_{35}R$ mutant terminase was essentially identical to that of wild-type terminase. Changing $lysine_{35}$ to alanine or aspartic acid, however, yielded enzymes with significantly altered kinetic properties (Figure 3; Table 2). Under standard conditions, the $K_{35}D$ enzyme is nearly devoid of catalytic activity at the low-affinity ATP hydrolysis site.

It has been previously demonstrated that DNA-mediated stimulation of ATP hydrolysis by terminase resulted primarily from increased activity at the low-affinity site (Tomka & Catalano, 1993b). We therefore examined the effects of increased concentrations of DNA on the kinetics of ATP hydrolysis by gpNu1 $K_{35}D$ terminase. The data presented in Figure 4 and Table 2 demonstrate that the low-affinity ATPase activity of the gpNu1 $K_{35}D$ enzyme was restored to essentially wild-type levels when the concentration of DNA was increased 10-fold. Similar results were obtained for the gpNu1 $K_{35}A$ enzyme (Table 2). Note that for the DNA

Table 1: Photo-Cross-Linking by 8-Azido-ATP: Competition by Nucleotides[a]

	lane									
	1	2	3	4	5	6	7	8	9	10
8-N_3-ATP, 50 μM	−	+	+	+	+	+	+	+	+	+
competitor nucleotide, 1 mM	−	−	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP
relative binding to gpA (%)	<1	=100%	3	34	48	32	2	33	36	38
relative binding to gpNu1 (%)	<10	=100%	<10	33	42	30	<10	39	39	36

[a] Percent binding of values normalized to levels found in the absence of competitor, i.e., in lane 2. The value for labeling of gpNu1 in lane 2 was 3.3% the value for labeling of gpA in lane 2.

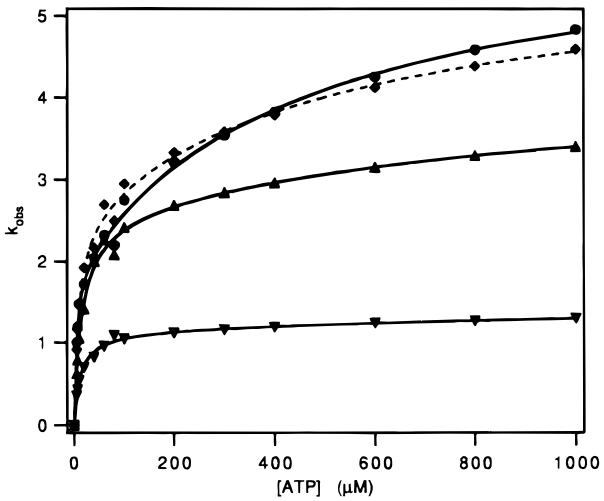

FIGURE 3: Kinetic analysis of the ATPase activity of wild-type and gpNu1 K_{35} mutant terminases. A direct plot of the observed rate of ATP hydrolysis (μM·min^{-1}) by wild-type (●—●), K_{35}R (♦- - -♦), K_{35}A (▲—▲) and K_{35}D (▼—▼) mutant terminase enzymes. The reactions conditions were as described under Experimental Procedures with enzyme included at 10 and 50 nM when low (0−80 μM) and high (100−1000 μM) ATP concentrations were used, respectively. The data displayed were normalized to an enzyme concentration of 50 nM. The curves represent best fits of the experimental data to eq 1 as described under Experimental Procedures.

levels used, terminase is in great molar excess to the *cos* sites of the λ DNA molecules; thus, the DNA stimulation of the ATPase activity is nonspecific, consistent with our previous results (Tomka & Catalano, 1993b).

Kinetic Analysis of Wild-Type and gpA Mutant Terminases. Kinetic analysis of ATP hydrolysis by gpA K_{497}R, gpA K_{497}A, and gpA K_{497}D mutant terminases showed that the conservative gpA K_{497}R mutation had little effect on ATPase activity while the more severe gpA K_{497}A and gpA K_{497}D mutations resulted in significantly altered kinetic parameters. Eadie−Hofstee plots of ATP hydrolysis by these mutant terminases clearly indicate that catalysis at the high-affinity site has been affected (Figure 5). Table 3 further shows that the primary defect in the gpA K_{497}A and K_{497}D mutant terminases is reduced affinity for ATP by the high-affinity ATP binding site. Of note is that while increased concentrations of DNA restored the catalytic activity of the gpNu1 mutant terminases (*vide supra*), increasing λ DNA by 10-fold had no effect on the kinetics of ATP hydrolysis by the gpA mutant terminases (data not shown).

To further confirm that mutations in the gpA ATPase catalytic center resulted in terminases which were deficient in ATP binding, photoaffinity labeling experiments with 8-N_3-ATP were performed. While labeling of wild−type and gpA K_{497}R mutant terminases was quite efficient, mutation of lysine$_{497}$ to alanine and aspartic acid reduced labeling to 27% and 7.5%, respectively, of wild−type terminase (data not shown).

DISCUSSION

The gpNu1 and gpA subunits of λ terminase were photolabeled by UV irradiation in the presence of 8-N_3-ATP. The extent of labeling of terminase holoenzyme as a function of 8-N_3-[α-^{32}P]ATP concentration demonstrated that the gpNu1 and gpA subunits were half-saturated at concentrations of 508 μM and 3.5 μM, respectively. Saturable photolabeling and competition of labeling by natural substrates strongly suggest that 8-N_3-ATP covalently modifies the ATP reactive centers in terminase. Inhibition of photolabeling by other non-adenine-containing ribo- and deoxyribonucleoside triphosphates was weaker, demonstrating that the binding sites of terminase are specific for adenine nucleotide, consistent with previous data showing hydrolysis of only ATP and dATP (Tomka & Catalano, 1993b). These cross-linking results show that each subunit of terminase holoenzyme binds ATP; gpNu1 with low affinity and gpA with high affinity.

Many ATPases contain sequence segments that show homology at the active site, a motif known as the P-loop. The P-loop, which consists of a glycine-rich segment followed by a lysine, is thought to form a flexible loop with the lysine involved in ATP hydrolysis (Fry *et al*., 1986; Walker *et al*., 1982; Saraste *et al*., 1990). Sequence analysis suggests the presence of the P-loop in both the gpNu1and

Table 2: Kinetic Parameters of Wild-Type and gpNu1 Mutant Terminases

	low-affinity ATP binding site		high-affinity ATP binding site		
terminase	k_{cat} (min^{-1})[a]	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	Ψ^2
wild-type	84	469	38	4.6	0.0010
gpNu1 K_{35}R	64	561	50	8.5	0.0011
gpNu1 K_{35}A	38	1217	56	15.5	0.0007
gpNu1 K_{35}D	8	1444	22	11.6	0.00011
gpNu1 K_{35}A (+ λ DNA)[b]	62	528	42	4.8	0.00068
gpNu1 K_{35}D (+ λ DNA)	72	562	28	5.8	0.00055

[a] $k_{cat} = k_{obs}$/[enzyme]. [b] (+ λ DNA) indicates reactions in which the DNA concentration was increased from 0.15 to 1.5 nM.

Table 3: Kinetic Parameters of Wild-Type and gpA Mutant Terminases

	low-affinity ATP binding site		high-affinity ATP binding site		
terminase	k_{cat} (min^{-1})[a]	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	Ψ^2
wild-type	84	469	38	4.6	0.00101
gpA K_{497}R	56	806	38	9.8	0.00037
gpA K_{497}A	70	377	44	24.4	0.00057
gpA K_{497}D	93	1260	38	68	0.000312

[a] $k_{cat} = k_{obs}$/[enzyme].

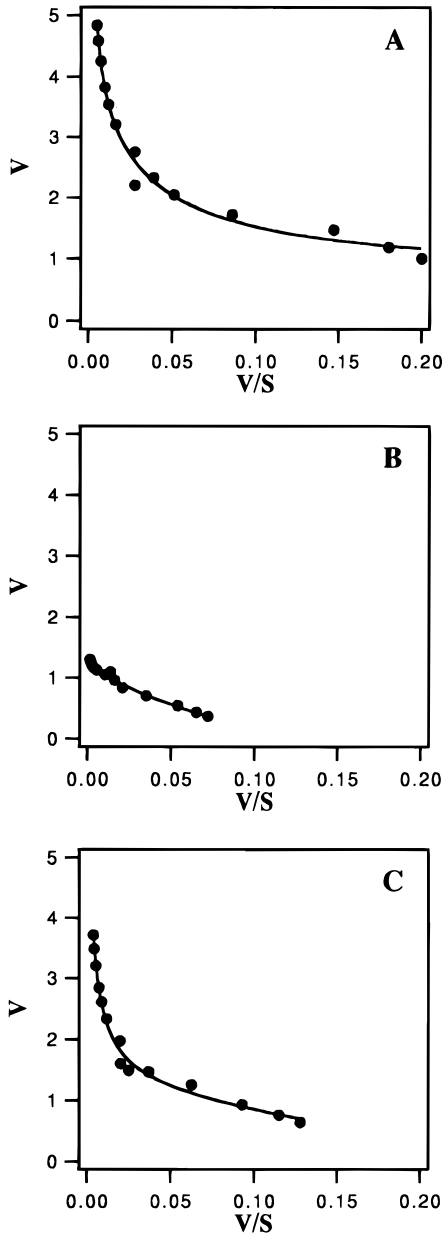

FIGURE 4: Effect of increased concentration of DNA on the ATPase activity of gpNu1 K_{35}D mutant terminase. Eadie−Hofstee plots of the observed rates of ATP hydrolysis (μM·min^{-1}) by wild−type terminase (panel A) and the K_{35}D mutant enzyme in the absence (panel B) and presence (panel C) of elevated DNA concentration. The reaction conditions were as described in Figure 3 except that the concentration of DNA was increased 10-fold in panel C. The data displayed were normalized to an enzyme concentration of 50 nM.

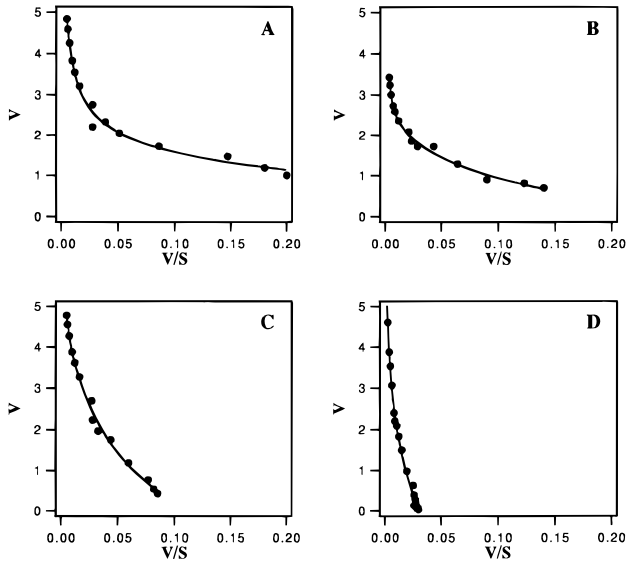

FIGURE 5: Kinetic analysis of the ATPase activity of wild-type and gpA K_{497} mutant enzymes. Eadie−Hofstee plots of the observed rates of ATP hydrolysis (μM·min^{-1}) by wild−type (panel A) and the K_{497}R (panel B), K_{497}A (panel C), and K_{497}D (panel D) mutant enzymes. The reactions conditions were as described in Figure 3.

gpA subunits of terminase (Becker & Gold, 1988; Guo *et al*., 1987), and kinetic analysis of the wild−type terminase showed the presence of two kinetically distinct ATPase sites (Figure 3; Table 2). Because the lysine of the P-loop is highly conserved (Fry *et al*., 1986; Saraste *et al*., 1990), this residue has been changed in a number of mutational studies. These studies have given variable results; for the yeast RAD3 protein, an ATPase−DNA helicase, changing the conserved lysine had no effect on ATP binding, but abolished ATP hydrolysis (Sung *et al*., 1988). For the *Salmonella* MutS repair protein, changing the conserved lysine altered both the affinity for ATP and the rate of hydrolysis (Haber & Walker, 1991). In the present work, we changed the putative conserved P-loop lysines of gpNu1 and gpA, as discussed below.

gpNu1 K_{35} Mutations. Substitution of the K_{35} lysine residue by alanine resulted in a protein with a low-affinity ATPase center that had both a reduced affinity for ATP and a reduced hydrolysis rate (Figure 3 and Table 2). The K_{35}D substitution generated an enzyme nearly devoid of ATPase activity at the low-affinity site; however, this reduced activity cannot be ascribed directly to a defect in ATP binding or hydrolysis. The ATPase activity of the gpNu1 subunit in the gpNu1 K_{35}A and gpNu1 K_{35}D terminases was essentially completely recovered when 2 and 10 times more DNA was added, respectively, suggesting that the gpNu1 K_{35}A and gpNu1 K_{35}D terminases are defective in nonspecific DNA binding, and that ATP binding and hydrolysis in gpNu1 are coupled to DNA binding. Note that for the wild−type enzyme the low-affinity ATPase center is DNA-stimulated; we have previously demonstrated that DNA decreased the K_m by 2.7-fold and increased the k_{cat} by 2.3-fold (Tomka & Catalano, 1993b). In contrast, the gpNu1 K_{35}D enzyme has

a low-affinity ATPase center that is much more strongly stimulated by DNA. The result that alteration of the gpNu1 ATPase center affects DNA binding is consistent with the location of the putative HTH motif approximately 20 residues upstream from the ATPase center. The close proximity of the HTH motif to the ATPase center in the primary structure of gpNu1 could result in close proximity in the tertiary structure, leading to coupling of the two activities (Tomka & Catalano, 1993b). Implicit in this speculation is the proposal that the putative HTH motif is involved in nonspecific DNA binding. We suggest that lysine-35 of gpNu1 may be involved in communication between the DNA and ATP binding domains of gpNu1. Like gpNu1, Rho, the transcriptional termination factor, is also an example of an ATPase in which the terminal lysine of the P-loop is not required for ATP hydrolysis (Dombroski *et al*., 1988). For Rho, inactivation of the ATPase activity required substitution of the conserved P-loop lysine at residue 184 plus substitution of an upstream lysine at residue 181. We note that gpNu1 has an upstream arginine, at residue 31; we have not yet examined the effect of alteration of this residue on the gpNu1 ATPase activity.

gpA K_{497} Mutations. The substitution of alanine or aspartic acid for the lysine at position 497 of the gpA subunit of terminase resulted in enzymes defective in the high-affinity ATPase center due to reduction of ATP binding by factors of 5 and 15, respectively, with little change in k_{cat}. The reduced high-affinity ATPase activities of the gpA subunits in the gpA K_{497}A and gpA K_{497}D terminases were not increased significantly at higher λ DNA concentrations, indicating that the high-affinity ATPase activity of gpA is not directly regulated by DNA binding.

What then are the roles of the two ATP reactive centers in the functioning of λ terminase holoenzyme? Rubinchik *et al*. (1994) found that the endonucleolytic activity of terminase resides in gpA, a finding consistent with the mutational work of Davidson and Gold (1992) and biochemical work performed in the laboratory of one of us (Catalano, unpublished experiments). Cue and Feiss (1993a) demonstrated that ATP concentrations from 1 to 5 μM stimulate sharply *cos* cleavage, and that *cos* cleavage is further stimulated, although less dramatically, at ATP levels of 100 μM to 10 mM. Taken together, our data support a model in which ATP binding and/or hydrolysis within the gpA polypeptide is responsible for stimulating *cos* cleavage at low nucleotide concentrations ($K_m = 5$ μM) while ATP binding by the gpNu1 subunit of the holoenzyme ($K_m = 469$ μM) accounts for the stimulation observed at elevated concentrations. This suggests that ATP binding by gpNu1 stimulates the endonuclease activity which resides in the gpA polypeptide and that gpNu1−gpA interactions may be affected by ATP binding and/or hydrolysis. We note that mutations altering gpNu1 modestly affected the high-affinity ATPase, and *vice versa* (Tables 2 and 3). Our data are thus consistent with a model in which the two ATP reactive centers work in concert to cooperatively assemble a nicking complex with high endonucleolytic fidelity at *cos*.

Subsequent to terminase-mediated duplex nicking at *cosN*, the cohesive ends are separated by the enzyme in a reaction that requires the hydrolysis of ATP (Higgins *et al*., 1988). Rubinchik *et al*. (1994) have found that the strand separation activity of terminase resides in the gpA subunit of the holoenzyme, and it is possible that the high-affinity ATPase site located within gpA is responsible for this reaction.

Finally, ATP hydrolysis is required for DNA translocation into the prohead. In phages T3 and ϕ29, translocation has been shown to require about one ATP hydolyzed per two base pairs packaged (Morita *et al*., 1993; Guo *et al*., 1987). Since translocation occurs at a rate of at least 10 kb/min, the terminase ATP hydrolysis rate of approximately 100−200 ATP min^{-1} $protomer^{-1}$ is inadequate to account for translocation, indicating that one of the known ATPases of terminase may be activated, or that a novel ATPase may be activated by formation of the packaging complex (Tomka & Catalano, 1993b; Catalano *et al*., 1995). Study of the mutant forms of terminase described here should be useful in further elucidating the roles of the ATPase centers in the various functions of terminase in DNA packaging.

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