Isolation and Characterization of T4 Bacteriophage gp17 Terminase, a Large Subunit Multimer with Enhanced ATPase Activity*

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Phage T4 terminase is a two-subunit enzyme that binds to the prohead portal protein and cuts and packages a headful of concatameric DNA. To characterize the T4 terminase large subunit, gp17 (70 kDa), gene 17 was cloned and expressed as a chitin-binding fusion protein. Following cleavage and release of gp17 from chitin, two additional column steps completed purification. The purification yielded (i) homogeneous soluble gp17 highly active in in vitro DNA packaging (~10% efficiency, >10^8 phage/ml of extract); (ii) gp17 lacking endonuclease and contaminating protease activities; and (iii) a DNA-independent ATPase activity stimulated >100-fold by the terminase small subunit, gp16 (18 kDa), and modestly by portal gp20 and single-stranded binding protein gp32 multimers. Analyses revealed a preparation of highly active and slightly active gp17 forms, and the latter could be removed by immunoprecipitation using antiserum raised against a denatured form of the gp17 protein, leaving a terminase with the increased specific activity (~400 ATPs/gp17 monomer/min) required for DNA packaging. Analysis of gp17 complexes separated from gp16 on glycerol gradients showed that a prolonged enhanced ATPase activity persisted after exposure to gp16, suggesting that constant interaction of the two proteins may not be required during packaging.

Many dsDNA bacteriophage require the activity of two "terminase" proteins to cut and package concatameric DNA into assembled phage proheads. In T4, the gp16 (small subunit) and gp17 (large subunit) terminase proteins are essential proteins for DNA packaging. (Similar proteins are gpA and gpNu1 in λ phage, gp18 and gp19 in phage T7/T3, gp2 and gp3 in phage P22, and gp3 and gp16 in phage φ29.) As generally found among these phage terminases, the T4 small subunit confers the specific DNA-binding/association properties (1–3). The large subunit contains the prohead-binding and putative DNA-translocating ATPase activities (3–7). However, because the measured in vitro ATPase activities of isolated terminase proteins (8, 9) have been low in comparison with measured rates of ATP consumption in packaging (10, 11), the identification of terminases as DNA-translocating ATPases has been problematic. What is clear is that packaging is ATP-driven and involves the cooperation of the DNA-associated terminase proteins with the prohead-associated portal vertex protein (7, 12, 13) in phage T4, gp20.

The T4 terminase large subunit protein, gp17, has proven difficult to purify because of the protein’s limited solubility and toxicity to host cells (14). Coexpression with the gp16 small subunit reduces toxicity; thus, gp17 could be purified with gp16 in low amounts from a temperature-induced expression vector (4). A vector that makes gp17 with a 20-amino acid OmpT leader peptide results in low amounts of uninduced, soluble, and active protein, whereas the overexpressed protein is insoluble and inactive and resists renaturation (15). A His-tagged gp17 protein was also overexpressed and showed similar problems in protein solubility as well as amino acid changes, possibly to reduce toxicity. 2 However, Leffers and Rao (5) overcame these problems by modifying the growth conditions and strictly controlling the basal level expression from a His-tagged cloned gene.

Although gp17 terminase is specifically assayed by its ability to make phage by in vitro DNA packaging and to bind and hydrolyze ATP (4, 5, 16), it was unclear whether its ATPase and nuclease activities require interaction with additional nucleic acid or protein factors. To address these issues and to highly purify gp17, we employed three column steps, including the use of dsDNA- and ssDNA-cellulose to separate gp17 from other nucleic acid-interacting proteins.

The purified gp17 terminase ATPase activity is stimulated by interactions with phage T4 proteins gp32 and gp20 and, most strongly, with the terminase small subunit, gp16. We also discovered that the enzyme preparation was composed of at least two forms of the protein, a form that could be activated by gp16 to a high turnover ATPase and a less active form separable by immunoprecipitation with antisera prepared against denatured gp17. After removal of low activity protein, ATPase activity of 400 ATPs/gp17/min remained, the highest activity reported for any large subunit terminase. These experiments suggest that significant structural differences allow separation of the two forms and also show that terminase undergoes a major conformational change to form a gp16-induced multimer whose high activity is not dependent upon continued interaction with gp16. The findings advance our understanding of terminase interactions and conformational changes that lead to the catalytically active packosome.

EXPERIMENTAL PROCEDURES

Chemicals were reagent-grade, and enzymes were used as recommended by the suppliers. Routine cloning was performed as described (24). DNA concentrations were estimated using a spectrophotometric determination at 260 and 280 nm as described (25). Protein determinations were performed in accordance with 18 U.S.C. Section 1734.

Received for publication, August 21, 2002, and in revised form, November 25, 2002

Published, JBC Papers in Press, December 3, 2002, DOI 10.1074/jbc.M208574200

‡ The work was supported by National Institutes of Health Grant AI11676. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; CBD, chitin-binding domain; DTT, dithiothreitol; PEG, polyethylene glycol.

2 R. G. Baumann and L. W. Black, unpublished data.
Gene 17 Cloning and Expression: Construction of Vector pT5—Full-length active gp17 was purified based on the Impact system of cloning (New England Biolabs, Inc.). To clone the wild-type gene 17 sequence with 40 mM DTT, the column was incubated overnight at 4 °C, and the fractions showed that gp17 was cleaved from the moiety-containing vector was electroporated into E. coli HMS174(DE3) and used to perform large-scale purification of active soluble gp17 for these studies.

gp17 Purification—Large-scale cultures were grown at the University of California, San Francisco, Synthesis and Sequencing Facility. (Park College, MD). 100 liters of HMS174(DE3)pT5 were grown to A600 = 0.6, induced for 3 h with 0.5 mM isopropyl-β-D-thiogalactoside, and pelleted (340 g). ~100 g were mixed with a minimum volume (200 ml) of Q buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 5 mM MgCl2, 0.2 mM ZnCl2, and 1 mM ATP containing 10% glycerol), and the cells were gently stirred at 4 °C until completely homogenized. The cells were French-pressed two times at 18,000 p.s.i. and spun at 14,000 × g for 20 min, and the supernatant was removed and recentrifuged overnight at 20,000 × g. The supernatant was removed, applied through Nalgene glass-fiber syringe prefiltrers, and loaded onto a 300-ml column pre-equilibrated in Q buffer. The column was washed with 1–2 volumes of of protein, and protein assemblies were run in 10 mM MgCl2, pH 6.6, 50 ml/liter 1 M sodium citrate, pH 3–4, and equilibrated in Q buffer. A 1 ml column volume of Q buffer with 40 mM dithiothreitol (DTT) was added, and the column was incubated at 4 °C prior to elution by addition of 1-mL protein A-Sepharose slurry was added and the mixture was rocked at 4 °C for 1.5 h. 10–40 µl of 50% protein A-Sepharose slurry were added, and the mixture was rocked for another 1.5 h at 4 °C. The column was centrifuged at low speed at 200 × g, and the supernatant was carefully removed. The pellet was washed repeatedly with dilution buffer and, gell or titer analysis, brought up in a minimum volume (50 or 100 µl) of buffer containing 5.8 g/liter NaCl, 2 g/liter MgSO4·7H2O, 50 µl/liter 1 M Triton-X, pH 7.5, and 5 µl/liter 2% gelatin, and a fraction of the pellet slurry was analyzed.

Antisera and Purification of Anti-gp17 Antibody-containing IgG—The development of polyclonal antisera raised against native and active gp16 (3) and against the denatured form of OmpT leader/gp17 terminase has been described previously by Lin [15], following a protocol of eluting the SDS-PAGE-isolated proteins from gels (28). To purify the gp17 antigens, Fasthrombin was diluted to 5 ml in Q buffer and loaded onto a 1-mL protein A column previously washed with 10 ml of water and 10 ml of 0.1 mM sodium citrate, pH 3–4, and equilibrated in Q buffer. After loading (0.5 ml/min), the column was washed with 10 ml of Q buffer. A large non-binding fraction of serum proteins was saved for further analysis. For IgG elution, 0.1 mM sodium citrate, pH 5.5, was added to the column, and IgG eluting in a single sharp peak was immediately neutralized to pH 7.0 with 2× NaOH (−30–35 µl/1–1.5 ml of sodium citrate/antibody wash). This antibody fraction was not stimulatory for the gp17 ATPase until further dialyzed from sodium citrate against Q buffer (with no ATP) and was concentrated to its original volume of ~1 ml using a Centriprep-50 (Amersham Biosciences) was washed as a 50% slurry in dilution buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.025% sodium azide) and kept at 4 °C prior to use. For coupling, proteins were diluted in 200 µl of buffer; antibody from serum (usually 1–5 µl) was added; and the mixture was rocked for 4 °C for 1.5 h. 10–40 µl of 50% protein A-Sepharose slurry were added, and the mixture was rocked for another 1.5 h at 4 °C. The column was centrifuged at low speed at 200 × g, and the supernatant was carefully removed. The pellet was washed repeatedly with dilution buffer and, gell or titer analysis, brought up in a minimum volume (50 or 100 µl) of buffer containing 5.8 g/liter NaCl, 2 g/liter MgSO4·7H2O, 50 µl/liter 1 M Triton-X, pH 7.5, and 5 µl/liter 2% gelatin, and a fraction of the pellet slurry was analyzed.

In Vitro DNA Packaging—In vitro packaging was performed as previously described (17).

Radioactive ATPase Assays—Purified gp17 and gp16 proteins were preincubated with an equal volume of 8% polyethylene glycol before addition of the same volume of 2× ATPase buffer to start the reaction. The reaction was carefully removed after 10 min, and 1-mL aliquots were removed at various times and processed as described (26).

Nucleotide Assays—DNA cleavage assays were carried out in numerous ways, typically using a reaction mixture that finally contained 20 mm Tris-HCl, pH 8.0, 5 mM MgCl2, 80 mM KCl, 2 mM ATP, 2 mM spermidine, and 2.7% polyethylene glycol (PEG). Mixtures of the terminase proteins (1.5 µg of gp17 + 0.5–1.5 µg of gp16) in 15 µl of buffer were made, and the reactions were started by addition of 4 µl of DNA. Various DNA substrates were analyzed, including linear marker DNAs and plasmid 2.3-GTA2 and pBR322 and pBR32 (4), which contain the gene 16–17 sequence, and pDH72DE3 (29), a kind gift of Dr. E. P. Geduscher. Treatments were incubated at 30 °C for 3 h and heated at 65 °C for 10 min prior to 0.8% agarose gel electrophoresis and ethidium bromide staining following standard methods.

Native Agarose Gel Electrophoresis of Proteins—As in the study of lithium carbonate (32), supernatants were run in TAME buffer (1× = 40 mM Tris base, 20 mM acetic acid, pH 8.1, and 1 mM MgSO4) with 10 mM MgSO4. Gels were run at 70–90 V (20–40 mA) from 19 to 22 °C, and the pH of the buffer was kept constant. Ferguson plot and log-log graph analyses of the gel data were done as described (23), assuming negligible effects of concaving Ferguson plot data due to the changes in agarose concentration from 0.75 to 2.25%.

Glycerol Gradient Separation of Proteins—Glycerol gradients were made inside 5 × 11-mm polyethylene tubes by placing 300 µl of 40% glycerol in Q buffer underneath 300 µl of 10% glycerol in Q buffer and forming the gradient using a Biocomp Gradient Master 105 gradient maker set at 86° and 46 s, run at 22 rpm, and equilibrated to 4 °C. Up to 1 µl of sample could be used, and run at 30,000 rpm in an SW-56 swinging bucket rotor overnight at 4 °C for 15–20 h. Fractions were collected using a modified 25-gauge (5/8) needle pierced through the bottom of the tube to collect drops.
**Purification of Active Soluble gp17:** Expression of a gp17-Intein/CBD Fusion Protein—Purification of the gp17 terminase large subunit was based on the New England Biolabs intein/CBD fusion protein method. Gene 17 was cloned into the pTYP2 fusion vector to make vector pT5, which expresses the gp17-intein/CBD fusion. In the cell lysate, this fusion protein was >75% soluble and produced in large amounts as detected by SDS-PAGE and Western blotting using anti-gp17 antibody (Fig. 1A). Significant accumulation of fusion protein was detected after 3 h of induction with isopropyl-D-thiogalactopyranoside. For protein preparation, cells expressing the gp17 fusion protein were induced for 3 h, centrifuged, and lysed by French press, and the clarified supernatant containing the fusion protein was loaded onto a chitin column (see “Experimental Procedures”). As Fig. 1B shows, many proteins were removed in the insoluble pellet fractions (P1 and P2 lanes) or did not bind and eluted in the flow-through fraction of the column (FT lanes). Many other proteins, bound nonspecifically to chitin or associated weakly with the gp17-intein/CBD fusion protein, could also be removed after washing the column (W1 and W2 lanes). After equilibration of the column overnight in DTT, cleaved gp17 protein eluted over the span of many fractions (fractions 6–36) (Fig. 1B). Further analysis showed that the isolated protein could be concentrated from these dilute fractions using Amicon filtration membranes, but had a solubility limit of ~0.75 mg/ml.

**Slow Proteolysis of gp17 in Chitin Column Fractions—**gp17 from the chitin column is apparently homogeneous as determined by SDS-PAGE (Fig. 1B). Nevertheless, we discovered that gp17 in fractions eluted from the chitin column was slowly converted to 68- and 65-kDa forms if stored at 4 °C without further purification. In Fig. 2, sample A represents the full-length chitin-purified protein, which was slowly converted to sample B. The samples were N-terminally sequenced, and the results of this analysis (Fig. 2, boldface) confirmed that sample A was the full-length gp17 (70 kDa) and that the transition to sample B (65 kDa) resulted from cleavage of gp17 at two distinct positions in its C terminus, both of which were identified within the gp17 sequence by N-terminal sequencing of six amino acid residues of the released peptides.

**Further Chromatographic Purification of gp17**—We also found that gp17 did not bind to ssDNA-cellulose (4) or dsDNA-cellulose (this work), and the use of these media removed not only the proteolytic activity, but also contaminating nucleases present in the preparation that interfered with subsequent analysis. In fact, washing ssDNA/dsDNA-cellulose after gp17 elution with 1 M NaCl and 1% Triton X-100 and concentrating showed that E. coli proteins, some close in size to gp17 and some that cross-reacted with anti-gp17 antibody upon longer storage for 1 week (sample A) or 2 months (sample B) at 4 °C, M lane, molecular mass markers shown in kilodaltons. Below the gels, the boldface regions of the gp17 sequence highlight the results of the N-terminal sequence analysis in confirming the purified gp17 protein by its N-terminal sequence (MEQPIN) in sample A and identifying two additional cleavage fragments of the protein at its C-terminal end present in sample B (SEVFSK, cleavage after Ala571; and EYPVFS, cleavage after Ala586).
**Phage Terminase Multimer with Enhanced ATPase Activity**

In vitro packaging of infected extract concalameric DNAs

| Mutant phage | Defect | No addition | Terminase (gp16 + gp17) |
|--------------|--------|-------------|------------------------|
| 16am17amrII  | Terminase (gp16 + gp17) | <10⁴  | 1 x 10⁸  |
| 2lam         | Prohead (protease)      | <10⁴  | <10⁴  |
| 16am17amrI+2lam |                   | 5 x 10⁷ | 4 x 10⁹  |

* *In vitro* packaging efficiency = 10% of the wild type.

**Nuclease Activity Assay**—Numerous attempts were made to determine whether gp17 alone or with T4 proteins with which it is known to interact can cleave DNA in vitro. Various plasmids and other linear DNAs have been analyzed, some with the gene 16 sequence identified as the location of a *Pac* site (3); and thus far, none have been cleaved significantly by gp17 or by gp17 co- incubated with other T4 proteins (gp20, gp16, and gp32). In numerous experiments, no significant nicking or cutting of various plasmid DNAs was seen for the terminase proteins compared with control buffer with or without co- incubation with the other T4 proteins, even when gp17 and gp16 were added at >10-fold molar excess compared with plasmid DNA.

**ATPase of the Purified gp17 Terminase Large Subunit: Qualitative Effects**—Purified gp17 was found to contain an intrinsic ATPase activity (1 ATP hydrolyzed/gp17/min) that could be significantly stimulated (~100-fold) by addition of the gp16 small subunit terminase protein (120 ATPs hydrolyzed/gp17/min). The gp16 protein itself was previously shown to lack ATPase activity (3). Subjecting gp17 to numerous freeze/thaw cycles did not decrease the ATPase activity of the preparation, although gp17 showed a half-life at 37 °C of ~1 h. Addition of the polymer PEG (~20 kDa) at 8% (w/v) to the terminase proteins increased the activity seen, presumably by promoting protein interactions. The purified terminase subunit lacked detectable nucleic acid, and addition of numerous nucleic acids failed to stimulate the ATPase. These included wild-type T4 DNA cut with restriction enzymes or uncut; *Hind*III DNA fragments; 25-bp, 100-bp, and 1-kb DNA marker fragments; supercoiled plasmid DNA containing genes 16 and 17; relaxed plasmid DNA cut with blunt or sticky end restriction endonucleases; ssDNA; yeast tRNA; and mammalian RNA samples. None showed any substantial effects on the rates of ATP hydrolysis. Mg²⁺ ions were essential for the ATPase, and EDTA abolished activity. The ATPase activity was maximal at ~5 mM MgCl₂. The PEG and MgCl₂ effects on ATPase are in good agreement with the data for optimal T4 *in vitro* packaging by addition of these components (4).

We also tested the effect of gp20 portal addition on the ATPase hydrolysis rate of gp17 plus gp16. A number of different portal protein-containing preparations were analyzed, including purified gp20 dodecamer (18), gp20-green fluorescent protein fusion dodecamer (19), an alternatively purified gp20 protein (20), proheads, and a 24-mer peptide of gp20 identified to interact with gp17 (21). When testing gp20 or gp20-green fluorescent protein fusion dodecamers, consistently higher (up to ~20%) ATPase hydrolysis rates were achieved (data not shown). Proheads or the monomeric gp20 preparation (20) consistently showed no stimulatory effects. Addition of the gp20 peptide inhibited the ATPase slightly, but only at great molar excess. Because interaction of gp17 with the T4 single-stranded binding protein gp32 has been demonstrated (20), we investigated the effects of gp32 on the gp17 ATPase in the presence of various nucleic acid substrates. Under conditions of limiting amounts of gp17 and saturating amounts of gp16, addition of gp32 with ssDNA consistently increased (approximately doubled) the ATPase hydrolysis rate of the gp16-gp17 terminase complex. Addition of RNA with gp32 also increased the rate of ATP hydrolysis, whereas T4 wild-type DNA addition with gp32 had no effect on the hydrolysis rate.

**Stimulation of gp17 ATPase with gp16 and Affinity of Terminase for ATP**—To examine the effect of gp16 addition on the gp17 ATPase, ATPase reactions were assayed with fixed amounts of gp17 and ATP with varying amounts of gp16 added. Addition of gp16 to gp17 at submolar to equimolar amounts dramatically increased the initial ATPase hydrolysis rates observed (Fig. 3A). Above equimolar amounts, the initial ATPase hydrolysis rates increased, but further increases in the amounts of gp16 added in great molar excess (from 10- to 60-fold) had only minor effects on the ATPase hydrolysis rate of gp17 (Fig. 3B), suggesting that the stimulation was saturable. From this analysis, we were able to determine the maximum effect on initial reaction velocity that gp16 would have on gp17 under these reaction conditions and derive a double-reciprocal plot showing a linear correlation for the rate of reaction versus the concentration of gp16 protein (data not shown). From this plot, the *K*_D* for gp16 binding to 0.7 μM gp17 was determined to be 5 μM (relatively weak binding of the two terminase subunits), consistent with previous measurements as well as additional work presented below (4, 20).

To determine the affinity with which the gp17 ATPase binds ATP, we determined the initial rates of ATP hydrolysis at varying concentrations of ATP with fixed concentrations of the terminase large subunit. Two concentrations of the gp16 small subunit were examined, 7- and 21-fold molar excesses of gp16, the latter near saturation. As Fig. 3C shows, increasing the concentration of ATP increased the initial rates of hydrolysis. Incubation of the gp17 ATPase with increasing amounts of gp16 had a positive effect on the initial rates of ATP hydrolysis (5.6 versus 16.7 μM gp16). Both data sets showed increased initial rates, which plateaued beyond addition of 5 mM ATP; and the maximal velocities achieved differed according to the amount of gp16 added to the reaction. The highest initial velocities were achieved at saturating concentrations of gp16 and surpassed 130 ATPs hydrolyzed/gp17/min. Lineweaver-Burk plot (double-reciprocal) analysis of these data showed that the primary effect of the gp16 addition to the ATPase was to increase the *V*_max of the reaction and that the affinity of the enzyme for ATP was not changed due to the increased presence of gp16, as reflected by the same *K*_m (380 μM).

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**TABLE I**

| Mutant phage | Defect | No addition | Terminase (gp16 + gp17) |
|--------------|--------|-------------|------------------------|
| 16am17amrII  | Terminase (gp16 + gp17) | <10⁴  | 1 x 10⁸  |
| 2lam         | Prohead (protease)      | <10⁴  | <10⁴  |
| 16am17amrI+2lam |                   | 5 x 10⁷ | 4 x 10⁹  |

* *In vitro* packaging efficiency = 10% of the wild type.
**Phage Terminase Multimer with Enhanced ATPase Activity**

**Effect of Anti-gp17 Antiserum on the gp16-gp17 Complex ATPase**—In an effort to inhibit the gp17 ATPase with anti-gp17 antibody, anti-gp17 antiserum was mixed with the gp17 protein and incubated for 1 h on ice either before or after addition of gp16. To our surprise, in both cases, addition of anti-gp17 antiserum significantly increased the gp17 ATPase hydrolysis rate (Fig. 4A). Anti-gp17 antiserum had no detectable ATPase activity itself, and preimmune serum or nonspecific serum did not show such stimulation. IgG purified from anti-gp17 antiserum also stimulated the ATPase hydrolysis rate. In contrast, the anti-gp17 antiserum proteins depleted of IgG showed no stimulation of the gp17 ATPase or any detectable ATPase activities when assayed alone (Fig. 4B). The enhanced gp17 ATPase activity assayed in the presence of antiserum was dependent upon addition of gp16. To examine the effect of antibody addition on the gp17 ATPase activity, a kinetic analysis was performed similar to the experiments used to determine the $K_m$ for ATP. The initial rates of ATP hydrolysis for gp17 were determined, with and without anti-gp17 antiserum, for two different subsaturating concentrations of gp16 while varying the concentration of ATP. The concentration of gp17 used for the experiments was kept constant at 0.8 M gp17. The results shown in Fig. 4B resulted in additional increases in the initial ATP hydrolysis rates seen for the increasing concentrations of ATP.

Interestingly, the maximal rate stimulation seen with the two gp16 concentrations was achieved at about the same level (~190 ATPs hydrolyzed/gp17/min) in both experiments, suggesting that antibody addition raised the effective level of gp16 to saturation (comparable $V_{max}$ levels for antibody addition in both cases). The Lineweaver-Burk plot analysis of these data (not shown) showed that, in both cases, the affinity of gp17 for ATP was not changed upon antibody addition and that the main effect of antibody addition (similar to gp16 addition) was to increase the $V_{max}$ of the reaction, with no effect on the $K_m$ for ATP.

**Immunoprecipitation Experiments with Anti-gp17 Antiserum**—We supposed either that the enhancement of gp17 ATPase might result from the direct interaction of antibody with functional gp17 protein, somehow stimulating its activity, or that the antibody might interact primarily with nonfunctional forms of the gp17 protein to prevent their inhibitory interactions with the fraction of active gp17 present in the preparation. Immunoprecipitation experiments on gp17 or gp16-gp17 favored the latter possibility because the bulk of the ATPase activity could not be immunoprecipitated by addition of increasing amounts of anti-gp17 antiserum and protein A-Sepharose. gp17 and gp16-gp17 immunoprecipitation experiments showed that nearly all of the gp17 ATPase activity remained in the supernatant after immunoprecipitation and that the ATPase showed continued dependence on addition of gp16, whether or not anti-gp17 antiserum was added. An analogous experiment was performed using anti-gp16 antibody to immunoprecipitate the gp16 protein alone or just after addition to gp17 on ice prior to performing the ATPase assay. The results showed that the stimulatory activity of gp16 was removed in both cases and that little or no gp17 ATPase-stimulating activity remained in the supernatant after immunoprecipitation (data not shown). This supports previous experimental evidence suggesting that interactions between the two terminase proteins are essential for high gp17 ATPase activity (30). Together, the data favor the hypothesis that anti-gp17 antibody binds primarily the inactive gp17 protein and prevents its inhibitory association with the active fraction of gp17 present.

To investigate this hypothesis, the distribution of both the ATPase activity and gp17 protein in the supernatant and pellet was quantified. The activity remaining in the supernatant could be increased with increasing amounts of antibody, whereas the overall level of gp17 protein left in the supernatant was reduced, effectively increasing the specific activity of the remaining gp17 protein. In this experiment (Fig. 5, A–D), we determined the minimum amount of antibody that could be used to show stimulation of the gp17 ATPase. Addition of

**Fig. 3. Effect of gp16 addition on the ATPase hydrolysis rate of gp17.** In A, conditions were 1.8 μM gp17, 1 mM ATP, and 1.5% PEG. In B, conditions were 0.7 μM gp17, 1 mM ATP, and 1.5% PEG. In C, the effect of varying ATP concentrations on the initial rates of hydrolysis for gp17 is shown. Initial rate data are for two different gp16 concentrations using 0.8 μM gp17. The results shown in C are averages of three independent experiments for each point.
antiserum above 0.2 \mu l/\mu g of gp17 was sufficient to show a significant increase in the gp17 ATPase present in the supernatant after immunoprecipitation. As shown in Fig. 5 (compare A and B), only negligible ATPase activity was found in the pellet after immunoprecipitation. However, analysis of the gp17 protein in the supernatant and pellet fractions showed that addition of increasing amounts of antiserum increased the pellet gp17, reduced the supernatant gp17, and also increased the supernatant ATPase activity. Densitometric quantitation of the gp17 bands seen in the pellets and supernatants in Fig. 5C was performed, and the averages of these results are given below the blot (Fig. 5D). In this experiment, the most active fraction (Fig. 5C, SUPERNATANTS, lane 3) contained only 10% of the original total gp17, thus yielding an ATPase specific activity of 390 mol of ATP/gp17/min. The same results were obtained using anti-gp17 antiserum or IgG purified from this serum. In numerous experiments, increasingly active supernatant fractions had lower levels of gp17 protein.

Analysis of Termination Protein Interactions: Native TAMg Gel Electrophoresis of gp17—In an effort to demonstrate directly the association of gp16 and gp17, native gel electrophoresis was performed at low ionic strength (22). No interaction of the two proteins could be demonstrated. However, the migration of the gp17 protein on the native gels showed that, under low ionic strength conditions, the purified protein migrated as a single, relatively sharp band, consistent with a high molecular mass complex of ~700 kDa determined by Ferguson plot analysis (23). Different commercially available standards and multimeric packasome proteins (gp16 rings and gp20 dodecamer) purified and characterized in the laboratory yielded the expected molecular masses in these plots (data not shown). Although it is intriguing that the portal dodecamer (12 × 61 kDa) and the terminase large subunit multimer showed comparable molecular masses in this procedure (~700 kDa), the ATPase activity of gp17 electrophoresed or dialyzed under the TAMg gel running buffer conditions showed reduced ATPase, making it uncertain whether this complex actually represents the highly active multimeric conformation of the protein.

Developing a Mini-glycerol Gradient Protocol for Small Volume Analysis—As an alternative and less harsh technique for probing the interaction of gp16 and gp17 and to measure the molecular masses of the active complexes, we developed a mini-glycerol gradient protocol for analyzing samples in small volumes. Sedimentation of purified gp17 on these gradients under higher ionic strength (Q buffer) conditions showed that the protein migrated as an apparent monomer as determined in comparison with protein molecular mass standards (data not shown), in contrast to our native TAMg gel results.

Analysis of gp16-gp17 and the gp16-gp17 Immunoprecipitation Supernatant on Glycerol Gradients—Incubation of gp16 with gp16 resulted in diffuse sedimentation of the gp17 protein throughout the glycerol gradients, spanning molecular masses of 70–700 kDa or higher, with the bulk of the protein loaded still resolving at the 70-kDa gp17 monomer position (Fig. 6A). Although the gp16 and gp17 proteins did not co-sediment on the glycerol gradient, the change in the sedimentation profile of gp17 suggests that preincubation of the gp17 protein with gp16 had a significant effect on the gp17 protein’s ability to self-associate and to form multimers that sedimented throughout the gradient; although as shown in Fig. 6C, active forms of gp17 apparently still interacted with inactive forms present in the preparation to disperse the active forms. After immunoprecipitation of the gp16-gp17 complex with anti-gp17 antiserum, which removed a significant fraction of low activity and low molecular mass gp17, the supernatant showed that the remaining high activity gp17 migrated as a well defined high molecular mass complex in the lowest fractions of the glycerol gradient (Fig. 6B). Analysis of the high molecular mass gp17 protein complex showed a significantly ele-
vated ATPase activity (Fig. 6C, compare the fraction activity profiles in A and B). These results agreed with our earlier analyses and suggested that removal of the antibody-binding gp17 fraction from the preparation favored the formation of a high molecular mass complex (700–900 kDa) with high activity.

Effects of Anti-gp17 Antibody Binding on High Molecular Mass gp17 Complex Formation—To test the hypothesis that anti-gp17 antibody-containing IgG binds primarily inactive gp17, we incubated gp17-gp16 or gp17 alone with anti-gp17 antibody-containing IgG prior to performing the glycerol gradient separation of the mixture without immunoprecipitation (Fig. 7, A and B). These results showed that antibody addition resulted in the diffuse sedimentation of gp17 throughout the fractions of the gradient. However, Western blotting and ATPase analysis of the fractions showed that an increasingly active gp17 protein sedimented diffusely through the lower fractions of the glycerol gradient (Fig. 7C), presumably due to the binding (and sequestering from interactions) of primarily inactive gp17 protein. In contrast to the “terminase-alone” gradients (Fig. 6A), these fractions of gp17 sedimented in the presence of antibody showed that the diffusely sedimenting gp17 protein was active, with increasing ATPase specific activities being found in fractions close to the bottom of the gradient. With the bulk of the added IgG sedimenting at the top of the gradients, where the activity was lowest, these results support the notion that two forms of the gp17 protein exist in solution and that the antibody preferentially binds non-active forms of the gp17 protein. Consistent with what was found upon analysis of supernatants in immunoprecipitation experiments, association of active forms of the gp17 protein was favored to yield multimeric complexes with high activity that sedimented toward the bottom of the gradients. The data also suggest that the conversion between the two forms present is a slow one because addition of antibody distinguishes between the two forms that are separated by sedimentation. Interestingly, the ATPase analysis supported this “slow conversion” idea by showing that gp17 preincubated with gp16 had increasing gp16-independent ATPase activity for the fractions at the bottom of the gradient. gp17 incubated with antibody alone showed no such gp16-independent activity (Fig. 7, compare A and B as assayed in C). We infer that preincubation with gp16 resulted in a conformational change in the gp17 protein, activating it into an ATP-hydrolyzing form that persisted for a long time (for hours at 30 °C) even in the absence of gp16.

![Figure 5](image-url)
DISCUSSION

The intein/chitin fusion protein method of preparing phage T4 terminase overexpresses and purifies the gp17 subunit in soluble and active form in essentially a single column step. Nevertheless, we found that adequate terminase purification required two steps beyond intein/chitin affinity chromatography. Without the use of ssDNA/dsDNA-cellulose and blue Sepharose chromatography, strong DNA-binding E. coli proteins and proteases led to confusing results in some assays. Thus, the chitin column-purified protein, even in the presence of protease inhibitors, underwent proteolysis, retaining partial ATPase activity, but releasing 12- and 39-amino acid fragments from the C terminus. This proteolysis is analogous to how certain preparations of the λ phase terminase large subunit, gpA, appear to undergo a slow N-terminal clipping at discrete sites even in the presence of protease inhibitors and at low temperature (31). Parris et al. (31) found that the ATPase and nuclease activities were also retained in the truncated protein and reasoned that the N terminus of the protein itself was labile without the binding of the gpNu1 small subunit protein. The C-terminal regions of the large subunit terminase proteins of λ, T3, and T4 phage appear to be essential for prohead portal interaction (12, 32), although the central region of gp17 is also implicated (21). Thus, gp17 C-terminal proteolysis may similarly reflect protein lability in a region lacking a binding partner.

Our preparations of gp17 failed to bind ssDNA- or dsDNA-cellulose or to hydrolyze numerous plasmid or phage DNAs tested at great molar enzyme excess, readily allowing detection of a single DNA nick or cut per enzyme molecule. Because the terminase large subunits display the specific packaging endonuclease (7), and there is good evidence that the T4 terminase large subunit hydrolyzes transcriptionally active gene 17-containing plasmids in vivo (6, 14), other factors appear to be necessary to achieve DNA-binding and DNA-directed terminase activities in our preparation of gp17. We recently reported that the gp17 terminase large subunit binds to the T4 late σ-factor gp55, so it is possible that the terminase is loaded onto DNA through its interaction with other DNA-bound proteins such as gp55-gp45 and gp55-RNA polymerase (17).

Although gp17 was found to have very low intrinsic ATPase activity (1 ATP hydrolized/gp17/min), several proteins stimulated this activity, the gp16 terminase small subunit most dramatically. Addition of gp32 (single-stranded DNA-binding protein), which binds gp17 (20), doubled the ATP hydrolysis rate, but only in the presence of ssDNA or RNA. The portal protein gp20 also induced modest increases in the ATPase hydrolysis rate. Interestingly, stimulation of gp17 ATPase appears to require that these proteins be multimers because monomeric gp20 and gp32 had no effect. One explanation is that only interaction with the multimers promotes high activity gp17 multimer formation.

The purified gp17 ATPase activity could be stimulated >100-fold by addition of the gp16 terminase small subunit (130 ATPs hydrolized/gp17/min). Analysis showed that the affinity of the enzyme for ATP was not changed with increasing gp16 concentrations; rather, the primary effect on the enzyme was to increase the rate of catalysis (Kcat). The Kcat for ATP was calculated to be 380 μM. These values are in good agreement with those (Kcat = 256 μM and 107 ATPs/gp17/min) reported by Leffers and Rao (5) and with ATPase hydrolysis rates in other bacteriophage systems such as λ phage, whose in vitro gpA ATPase has been estimated at 65–120 ATPs/gpA/min (8, 33).

The T4 terminase gp17 ATPase appears to be DNA-independent, whereas the λ terminase gpA is partially dependent upon DNA (34, 35). Other terminase proteins such as those of the φ29 and T3 systems, whose purifications and partial characterizations have been described, display significant ATPase activity only when in the presence of the prohead, DNA, and other packaging factors (10, 36, 37). The T4 terminase gp17 activity is unique among other large subunit terminase proteins being studied in that it has such a significant activity only when stimulated by the presence of the small subunit terminase. This could relate to the highly soluble and structured...
gp16 ring, which may act to promote specific gp17 multimer formation.

Anti-gp17 antiserum additionally increased the ATPase hydrolysis rate of the gp16-dependent gp17 protein by at least 4-fold. Immunoprecipitation experiments demonstrated that both the total activity and the specific activity of the gp17 ATPase remaining in the supernatant fractions were increased. Calculation of the ATPase activity of gp17 remaining in the supernatant fraction in these experiments showed that gp17 had a catalysis rate of ~400 ATPs hydrolyzed/min/gp17, the highest activity reported for any bacteriophage terminase and one that nears the activity it should possess to account for the highest activity reported for any bacteriophage terminase

The proposal that terminase large subunits can assume different conformational states is not novel one (10, 21, 33, 34, 39). More novel is the possibility that gp17 and other large subunit terminase proteins may belong to a class of recently described intrinsically unstructured proteins (40, 41). Some of the properties of gp17 suggest that it fits criteria for inclusion in this class; thus, gp17 1) is implicated in a critical regulatory stage of phage development (DNA packaging); 2) interacts with multiple proteins, including gp16, gp20, gp32, and gp55 (17) and presumably DNA; 3) binds and hydrolyzes ATP; 4) is highly susceptible to proteolysis; and 5) exists in multiple interconvertible conformational states. The gp16 small subunit interaction is most important for conversion to a highly active and catalytic form, but other protein interactions may be necessary to lock the protein into specific functional conformations for packaging.

Acknowledgments—We thank Drs. Venigalla Rao, Alasdair Steven, Gerard Barcak, Richard Karpel, Richard Thompson, and Kim Collins for critically reading the manuscript and/or for helpful comments. We thank Dong Mei Xie for expert technical assistance.

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