The clamp loader complex (CLC) of bacteriophage T4 is essential for viability and has analogs in both prokaryotes and eukaryotes. The gp44 and gp62 subunits of the T4 CLC, in a 4:1 ratio, tightly associate such that the two proteins co-purify. Using transformed *Escherichia coli*, we were able to demonstrate for the first time purification of the unique protein gp62 in the absence of gp44. We experimentally determined the isoelectric point for the individual subunits. An *in vitro* physical interaction could be observed between the native subunits, which resulted in a reconstituted CLC that displayed the signature pattern of the ATPase functions of native CLC. Thus we demonstrate that the CLC forms via a self-assembly pathway rather than through a translational capture mechanism.

Bacteriophage T4 serves as a common viral model system for DNA replication (reviewed in Refs. 1 and 2). The holoenzyme for this system is comprised of the DNA-dependent DNA polymerase (gp43), the sliding clamp (gp45), and the clamp loader complex (CLC). The phage T4 CLC, gp44/62, is functionally similar to the *Escherichia coli* γ-complex (3, 4) and eukaryotic replication factor C (5, 6). Remarkably, these clamp loaders all consist of five subunits containing structural redundancies (7, 8). The T4 gene 44 protein (35.8 kDa) has sequence homologies with CLC subunits from other organisms, but gp62 (21.4 kDa) is a unique protein, possessing no significant sequence homology to known proteins. The *in vivo* function of the CLC is to chaperone the sliding clamp to the DNA primer-template junction (9, 10). The CLC then initiates a conformational change in the toroidal gp45 that results in deposition of the sliding clamp around double-stranded DNA (11–13). The action of gp44/62 in loading gp45 is catalytic (9, 14, 15). Subsequently, gp45 associates with the T4 DNA polymerase and tethers it to the DNA template, increasing the processivity and altering the fidelity of DNA replication (14, 16–19).

The T4 CLC assembles with a fixed stoichiometry of four gp44 subunits per gp62 subunit (20). The resulting complex has interprotein contacts so tight that, despite the lack of disulfide bonds between subunits, dissociation is only observed under denaturing conditions (21–23). The mechanism by which the stoichiometry is maintained and the nearly covalent subunit interactions develop is unknown. Genes 44 and 62 are adjacent to each other on the T4 genome, are cotranscribed as part of a polycistronic mRNA (24), and are translationally coupled (25, 26). A model of translational capture (Fig. 1, lower path) has been proposed for assembly of the CLC, where gp62 folds on a preformed scaffold composed of gp44 tetramers. This model predicts that assembly of a functional CLC absolutely requires that the subunits co-fold (27–29). The translational capture model was developed considering 1) the tight translational coupling of the 44 and 62 gene expression and 2) the strong intersubunit contacts that develop during complex assembly. Recent studies have shown that the CLC assembles when its subunits are co-expressed in trans, proving that translational coupling does not contribute to the formation of the CLC (34). A second possibility is that the CLC is capable of self-assembly (Fig. 1, upper path). If purified native subunits are combined, the self-assembly model predicts the formation of active CLC, whereas the translational capture model predicts that a denaturing agent followed by refolding of one or all subunits would be necessary for assembly of active CLC. The gp44 protein has been individually purified and found to assemble into a higher order complex with a molecular weight indicating probable tetramer formation (27). It was originally hypothesized that the CLC would assemble poorly from pre-folded subunits (21).

The CLC possesses a low intrinsic ATP hydrolysis activity (30), located in the gp44 subunits (27, 31). The ATPase activity of the CLC is enhanced by the presence of DNA or by the presence of the cognate sliding clamp gp45. In the presence of both gp45 and DNA, the ATP hydrolysis activity of the CLC displays a synergistic stimulation (30, 32). An ATP hydrolysis activity, with a magnitude greater than that of the CLC, was demonstrated for the isolated gp44 tetramers in the presence of DNA. However, stimulation of gp44 tetramers by gp45 was greatly reduced when compared with CLC (27). It was surmised that the gp62 subunits constrains the DNA-stimulated ATPase activity of gp44 and mediates its interaction with gp45.

In this study we demonstrate the purification of gp62 subunits expressed in *E. coli*. The isoelectric points of individual subunits and the CLC were determined. Through chromatographic, electrophoretic, and immunologic techniques, the occurrence of an *in vitro* physical interaction was observed between purified gp44 and gp62 subunits. A CLC was reconstituted that displayed the signature patterns of the ATPase functions of native gp44/62, supporting the self-assembly model of CLC formation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Taq DNA polymerase, Complete™protease inhibitor mixture tablets, and Nonidet P-40 were from Roche Molecular Biochemicals. DE-52 and P-11 bulk chromatographic media were from Whatman. Ampholytes, Econo-Pac High S, CHT-II, and HIC chromatography mixture tablets, and Nonidet P-40 were from Roche Molecular Biochemicals. DE-52 and P-11 bulk chromatographic media were from Whatman. Ampholytes, Econo-Pac High S, CHT-II, and HIC chromatography mixture tablets, and Nonidet P-40 were from Roche Molecular Biochemicals. DE-52 and P-11 bulk chromatographic media were from Whatman.
graphic cartridges and Bio-Rad protein assay solution were from Bio-Rad. Sheep anti-rabbit IgG-coated magnetic beads were from Dynal A. S. Pyruvate kinase/lactic acid dehydrogenase-coupled enzymes were from Sigma. All biochemicals, immunochemicals, and oligonucleotides were obtained from various sources as described previously (26, 34).

Strains, Plasmids, Media, and Growth Conditions—The arabinose-inducible expression vectors as well as E. coli strain LMG194 (F- ΔlacX74 galE galK thi rpsL ΔphoA (PvuII) Δsigma74 leu::Tn10) (33) were donated by the laboratory of J. Beckwith (Harvard University). Construction of plasmids pCLC44 and pCL62S, which express the gene 44 and gene 62 clamp loader subunits, respectively, were described in Janzen et al. (34). Construction of pCLC62H and pCL62S H is described in the next section. LMG194 transformed with either pCLC44 or any one of the pCLC62 constructs was grown in LB broth supplemented with 100 μg/ml ampicillin, with shaking at 200 rpm to an optical density of approximately 1.0. Cells used for pp44 purification were grown at 37 °C, then inducted with 0.2% arabinose for 1 h. Cells used for pp62 purification were grown at 30 °C, induced for 20 min by the addition of 0.2% arabinose, then quenched by pouring the media over ice. Cells were harvested by a 10-min centrifugation in a Sorvall GSA rotor at 10,000 rpm at 4 °C. Cell pellets were stored at −80 °C. Expression of pp62 constructs was measured using the method described in Torgov et al. (26).

Construction of Vectors Expressing gp62—To generate pp62 constructs, two 5'-clamped leader subunits were amplified from the plasmid pTL151WX (27) by the polymerase chain reaction using Taq DNA polymerase according to the manufacturer’s instructions. The forward (sense) primer, which preserved the putative hairpin (H'), had the sequence 5'-GGCGAAATAATCACATATGG-3'. The forward primer used for deletion of the hairpin (H) was 5'-GCAATGCGTGG-3'. The same reverse (antisense) primer was used in both constructs and consisted of the sequence 5'-TTACATTCATAC- GTATTGCGTGG-3'. For the H' and H constructs, gene 62 was amplified with the listed primers. The fragments were gel-purified, 5'-phosphorylated with T4 polynucleotide kinase, and cloned into the Smal site of pBAD18, yielding either pCLC62H or pCL62H.

Purification of the gp62 Subunit—All purification steps were performed at 4 °C. Protein concentrations were determined by the method of Bradford (35). A protease inhibitor mixture tablet was dissolved in 20 ml of lysis buffer (25 mM MOPS pH 7.5, 5 mM DTT, 50 mM NaCl, 0.2% Nonidet P-40, 5% glycerol), and the solution was used to resuspend a frozen cell pellet from 3.2 liters of induced cells. Two mg of lysozyme dissolved in 1 ml of lysis buffer was added, and the suspension was incubated on ice for 1–1.5 h with stirring. Cells were ruptured by 3 cycles of freeze-thaw in a dry ice/ethanol bath, then 300 μl of 2 mg/ml DNase I in 0.3 M CaCl2 and 300 μl of 1.0 M MgCl2 were added. The suspension was incubated on ice with stirring for an additional h. The solution was centrifuged at 16,000 rpm at 4 °C in a Sorvall SS-34 rotor for 20 min, and the supernatant was reserved. The cell pellet was resuspended in 10 ml of lysis buffer and recentrifuged. The supernatants were combined, and ultracentrifuged at 4 °C for 2 h at 35,000 rpm in a Beckman Ti70 rotor. The supernatant was filter-sterilized through a 0.22-μm membrane and termed the cleared lysate.

The cleared lysate was applied to a 20-ml bed volume DEAE column equilibrated in 12.5 mM MOPS, pH 7.5, 1 mM DTT, 0.2% Nonidet P-40, 10% glycerol. The flow-through fraction was collected and applied to a 5-ml packed sulfoic acid column equilibrated in S buffer (12.5 mM MOPS, pH 7.5, 1 mM DTT, 0.1% Nonidet P-40, 10% glycerol). Protein was eluted from the column with a 20-bed-volume linear gradient from 0 to 500 mM NaCl in S buffer. Gp62 eluted at approximately 500 mM NaCl, and fractions containing gp62 were pooled and diluted 5-fold with S buffer.

The diluted fractions were applied to a 5-ml packed hydroxypapitate column equilibrated in S buffer containing 200 mM NaCl. The column was developed with a 25-bed-volume linear gradient from 0 to 100% phosphate buffer (150 mM potassium phosphate, pH 7.0, 1 mM DTT, 0.1% Nonidet P-40, 10% glycerol). Gp62 eluted at 50 mM phosphate buffer. The fractions containing gp62 were pooled, and their volume was doubled by the addition of 3.0 M ammonium sulfate.

The diluted fractions were applied to a 1-ml packed hydrophobic interaction column equilibrated with a solution of 2.0 M ammonium sulfate in HIC buffer (50 mM MOPS, pH 7.5, 1 mM DTT, 0.5 mM EDTA, 10% glycerol). A 30-bed-volume linear gradient from 2.0 to 0 M ammonium sulfate in HIC buffer was applied to the column. Gp62 eluted near the end of the gradient, at approximately 200 mM ammonium sulfate. Fractions containing gp62 were pooled, concentrated in a 10,000 molecular weight cutoff ultrafiltration tube, and dialyzed into storage buffer (30 mM potassium phosphate, pH 7.5, 1 mM DTT, 1 mM EDTA, 50% glycerol).

DEAE Chromatography—Purified gp44/62 (60 μg), gp44 (55 μg), or gp44 (55 μg) and gp62 (6 μg) were suspended in a total volume of 250 μl of DE buffer (40 mM Tris, pH 7.9, 1 mM MgCl2, 1 mM DTT, 0.1% Nonidet P-40, 10% glycerol) and incubated for 1 h at 15 °C. Protein solutions were applied to a 1-ml DEAE column equilibrated in DE buffer, and the flow-through was collected. Elution was with 400 μg NaCl in DE buffer. Equal volumes of flow-through and eluent were acetonite-precipitated and analyzed. The relative amounts of gp44 per lane were scanned by comparing stained bands on a ColorOne scanner (Apple) set on 600 dots/inch resolution in the gray-scale mode. The scanned images were quantified as described in Torgov et al. (26).

Isoelectric Point Determination and Two-dimensional Electrophoresis—Purified gp44 and gp62 were mixed at a ratio of 2:gp62 subunits/gp44 tetramer in reconstitution buffer (20 mM MOPS, pH 7.0, 2 mM DTT, 0.2% Nonidet P-40, 10% glycerol) and incubated overnight at 4 °C. Aliquots of the reconstitution mixture were mixed with an excess of isoelectric focusing (IEF) sample buffer (12.5% glycerol, 2% Nonidet P-40, 0.4% pH 3/10 ampholytes, 1.0% pH 8/10 ampholytes, and 0.6% pH 9/11 ampholytes). Aliquots were loaded on first-dimension isoelectric focusing gels (0.8% agarose, 2% Nonidet P-40, 5% glycerol, 5% sorbitol, and ampholytes of the desired pH range mixture at a final concentration of 2%) and run for 1600 V-h. The catholyte and anolyte were 40 mM NaOH and 20 mM HEPES, respectively. To determine a pH profile, two or three typical first-dimension gels were cut into 3-mm sections. Each section was macerated in 20 μl of deionized water, and the pH of the resulting solution was measured. First-dimension gels were equilibrated for 10 min in SDS sample buffer (36) and placed into the well of a second-dimension gel. Second-dimension gels were silver-stained or electroblotted and subjected to detection by chemiluminescence. Blots were usually cut in half and processed separately for gp44 (top half) and gp62 (bottom half).

Immunoprecipitation—Purified gp44/62 (15 μg), gp44 (13 μg), gp62 (2 μg), or gp44 (13 μg) and gp62 (2 μg) was suspended in 100 μl of reconstitution buffer (25 mM HEPES, pH 7.0, 5 mM DTT, 0.1% Nonidet P-40, 1 mM ATp, 6 mM magnesium acetate, 10% glycerol) and incubated for 1 h at 15 °C. Then 900 μl of binding buffer (10 mM HEPES, pH 7.5, 2% Nonidet P-40) and 6 μg of anti-gp62 antibody were added to each sample. The samples were incubated for 1 h at 4 °C with inversion. Washing of Dynal sheep anti-rabbit IgG-coated magnetic beads in binding buffer was repeated twice, and then the magnetic beads were added to each sample, and incubation was continued for another h. Each sample was washed three times with 10 mM HEPES, pH 7.5, 0.1 M NaCl, 2% Nonidet P-40 and three times with 10 mM HEPES, pH 7.5, 1.0 mM NaCl, 0.2% Nonidet P-40. Precipitated proteins were eluted by inversion for 40 min with 20 μl of sodium citrate pH 2.5.

ATPase Assays—ATPase assays were performed using a pyruvate kinase and lactic acid dehydrogenase-coupled enzyme system as described in Janzen (34) using 250 mM gp44/62, 250 mM gp44 tetramers, or 25 mM each gp44 tetramers and gp62 monomers. When titrations of gp62 into gp44 were performed, 250 mM gp44 tetramers was used with either 0, 31.25, 62.5, 125, 187.5, 250, 325, or 500 mM gp62 subunits. Preincubation of gp44 and gp62 subunits before assaying for activity was found to have no effect upon the ATPase rate obtained.

RESULTS

Purification of Individual Subunits—Expression vectors for the individual subunits of the CLC were constructed utilizing arabinose-inducible vectors of the pBAD series (33). Constructs...
were used to transform a strain of E. coli (LMG194) that cannot metabolize arabinose. The T4 gene 44 was cloned into pBAD24 to yield pCLC44 (34). E. coli harboring the plasmid pCLC44 could be induced to produce large amounts of soluble gp44. The gp44 subunits were purified to $>95\%$ homogeneity (data not shown) using the method of Rush et al. (27).

Translation of gene 62 is coupled to translation of the upstream gene 44, either by sequestration of the gene 62 translation initiation region (TIR) in a hairpin structure or through a naturally weak TIR. Reported attempts at expression of gene 62 required maintenance of a portion of the upstream reading frame for gp62 synthesis (27). Recently, synthesis of gp62 has been observed in the absence of translational coupling via Western blotting techniques (26). To optimize expression of gene 62 we tested different configurations for the TIR of gene 62. The first construct has the exact sequence of gene 62 from 97 nucleotides upstream of the start codon to the stop codon amplified directly from the natural sequence and cloned into the medium copy number, ampicillin-resistant plasmid pBAD18 to yield pCLC62H$^+$ (H$^+$ = putative hairpin preserved). Expression from this construct was taken as the basal level. A second construct was designed that disrupted the proposed secondary structure around the gene 62 TIR. The sequence from 20 nucleotides upstream of the gene 62 start codon to the stop codon was amplified and cloned into pBAD18 resulting in pCLC62H$^-$ (H$^-$ = putative hairpin disrupted). Surprisingly, the construct with the deletion in the hairpin exhibited a 4-fold lower amount of expression than the basal level (Fig. 2). The Shine-Dalgarno region for gene 62 is weak by comparison to the consensus. To increase the efficiency of translation initiation, an optimized Shine-Dalgarno region was placed 5 nucleotides upstream of the gene 62 start codon. This construct was cloned into pBAD18 to yield pCLC62S (S = synthetic). Maximum amounts of gp62 expression were observed with the pCLC62S vector, levels more than 12-fold higher than basal expression. These results suggest that a weak TIR is the important determinant for coupling of gene 62 translation to that of gene 44. The results also imply that the putative hairpin may not actually form in vivo. The construct pCLC62S was used as a source for all gp62 purification attempts.

Although induction of pCLC62S enabled us to produce significant amounts of gp62, we found that solubility of the protein was limited. Solubility decreased as temperature increased, leading to sequestration of gp62 in inclusion bodies (34). Thus a short induction period at low temperature ($30^\circ$C) was chosen for expression of gp62 to minimize formation of inclusion bodies. Induced cells were lysed, and the soluble fraction was subjected to the following chromatographic steps: DEAE, sulfonic acid, hydroxylapatite, and hydrophobic interaction chromatography (Fig. 3). The protocol yielded approximately 70 $\mu$g of gp62/liter of cells induced.

**Physical Evidence for Reconstitution**—Anion exchange chromatography demonstrated a change in the binding character of the gp44 subunit. When DEAE chromatography was performed at pH 7.9, 95% of the individual gp44 subunits bound to the media, whereas 95% of the intact CLC was found in the flow-through fraction (Fig. 4). The clamp loader subunits were incubated together at a ratio of 0.75 gp62 subunits/gp44 tetramer and applied to the DEAE column. A decrease in the proportion of gp44 that bound to the column was observed, with nearly 50% of the gp44 subunits appearing in the flow-through (Fig. 4). A similar shift in gp62 elution position could be observed using cation exchange chromatography (result not shown).

IEF and two-dimensional gel electrophoresis also give evidence for in vitro reconstitution of the CLC. When intact CLC is subjected to IEF in the presence of 1.5% CHAPS (dissociating conditions), gp44 and gp62 migrated as separate subunits (Fig. 5A). The gp44 subunit exhibited an isoelectric point of 6.7, whereas gp62 migrated with a pI of 9.4. Individual subunits, when run separately in the presence or absence of CHAPS, migrated with similar isoelectric points (result not shown). The pIs determined for the gp44 and gp62 subunits agree with predicted values (27). When the CLC was subjected to IEF in the absence of CHAPS (native conditions), the subunits migrated with identical pIs of 8.4 (Fig. 5B). This value is similar to the experimentally determined value for CLC (21). Gp44 and gp62 subunits were mixed, then run under IEF native conditions. The majority of both subunits migrated as a complex (Fig. 5C). IEF gives evidence that the reconstituted complex is

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**Reconstitution of the T4 gp44/62 Complex**

Figure 2: Expression of gp62 from different translation initiation regions. A, equal amounts of E. coli cells harboring either pCLC62H$^+$ (H$^+$), pCLC62H$^-$ (H$^-$), or pCLC62S (S) were induced, lysed, and fractionated via SDS-polyacrylamide gel electrophoresis on a 15% gel. The proteins were electrophoresed onto a nitrocellulose membrane, then subjected to Western blotting techniques with detection by chemiluminescence. A gp62 marker (M) was included along with an uninduced cell lysate (U) as controls. The amount of gp62 in each lane was quantified using the NIH Image software as described in Torgov et al. (26). B, schematic representations of each gene 62 construct illustrating the differences in the TIRs are shown. The amount of expression achieved for each construct relative to expression from the natural TIR is indicated for each construct. opt SD, optimized Shine-Dalgarno region.

Figure 3: Purification of the gp62 subunit of T4 CLC. Equal amounts of protein (2.0 $\mu$g) from each stage of gp62 purification from the soluble cell fraction were fractionated via SDS-polyacrylamide gel electrophoresis using a 15% gel. Visualization was achieved by staining with Coomassie Brilliant Blue. The fractions analyzed were the cell lysate (L), the DEAE-cellulose flow-through (DE), the sulfonic acid pool (S), the hydroxylapatite pool (HTP), and the hydrophobic interaction chromatography pool (HIC). Intact CLC (M) was used as a marker with the positions of the gp44 and gp62 subunits indicated.
FIG. 4. Reconstitution of the CLC reduces the amount of gp44 bound to a DEAE column. Purified gp44, the intact CLC, or gp44 incubated with gp62 were subjected to DEAE-cellulose chromatography at pH 7.9. The flow-through (FT) and eluted (Bound) proteins were fractionated on a 12% SDS-gel and visualized with Coomassie Brilliant Blue staining. The positions of gp44 and gp62 are indicated. Individual gp44 subunits bound to the DEAE column, whereas the gp44/62 complex had little affinity for DEAE under the conditions employed. Interaction of gp44 with gp62 resulted in a decrease in the affinity of gp44 for DEAE.

FIG. 5. The in vitro reconstituted complex migrates with an isoelectric point similar to native CLC. Two-dimensional gel electrophoresis was performed as described under "Experimental Procedures." The pH profile for each gel is shown, and the second dimension positions of gp44 and gp62 are marked. A, intact CLC was treated with CHAPS before first dimension electrophoresis. B, intact CLC was analyzed without pretreatment. C, individually purified gp44 and gp62 subunits were mixed and incubated before first-dimension electrophoresis. The lower half of the blot was given a longer exposure to visualize the two populations of gp62; the portion of gp62 marked with the arrowhead on the left was reconstituted into CLC, whereas the portion marked with the arrowhead on the right were individual gp62 monomers.

stable, since once dissociated, the subunits would migrate at their individual pl's.

Immunoprecipitation was also used to investigate the in vitro reconstitution of the CLC. In the presence of 0.6 mM magnesium polyvalent antibody against the CLC efficiently precipitated individual gp44 or gp62 subunits. Under the same conditions, the efficiency of immunoprecipitation of intact CLC was significantly and reproducibly reduced (Fig. 6, three left lanes). When the gp44 and gp62 subunits were incubated together before immunoprecipitation, neither subunit precipitated efficiently, a pattern indicative of complex formation (Fig. 6, right lane). The reduction in immunoprecipitation of the CLC compared with that of individual subunits could be due to sequestration of epitopes upon complex formation or to conformational changes altering epitopes after complex assembly.

Enzymatic Activity of Reconstituted gp44/62—The CLC possesses an intrinsic ATPase activity that resides in the gp44 subunits; however, the ATPase activity of purified gp44 subunits differs from that of intact CLC. The ATP hydrolysis activity of gp44 subunits is known to exhibit a higher rate of hydrolysis than the CLC in the presence of DNA (27). The results found with our gp44 preparation matched this expectation (Table I). Similar to CLC behavior (30, 32), the gp44 subunits did not exhibit cooperative behavior with respect to either ATP or DNA binding, as evidenced by the ATP hydrolysis activity exhibiting Michaelis-Menten kinetics (result not shown). Our purified gp44 subunits possessed a $K_m$ for ATP of 190 ± 20 $\mu M$ in the presence of DNA. This value resembles the $K_m$ for ATP reported for CLC under similar condition, 260 ± 40 $\mu M$ (30) or 125 ± 16 $\mu M$ (15). The $K_m$ for DNA was similar for gp44 and CLC, approximately 500 $\mu M$ nucleic DNA with double-stranded character (30). The greatest difference between the ATPase activities of gp44 and CLC was the response to the sliding clamp (gp45). The ATPase activity of the CLC exhibited stimulation in the presence of gp45 that is similar to that observed when DNA is present. When both cofactors are present, the stimulation of the CLC was synergistic. However, gp44 exhibits little to no stimulation in the presence of gp45, and there is no synergistic stimulation observed when both gp45 and DNA cofactors are present.

A stoichiometric mixture of one gp44 tetramer per gp62 subunit exhibited a pattern of stimulation of the ATPase activity by cofactors that indicated reconstitution (Table I). The ATPase rate in the presence of DNA was lower than that observed from gp44 tetramers but higher than would be expected if all gp44 subunits had been converted to CLC. Of more significance is the fact that the ATPase activity exhibited stimulation in the presence of gp45 as well as stimulation in the presence of both gp45 and DNA that was higher than the products of both single cofactors. Since the gp44 and gp62 mixture displayed synergistic stimulation of the ATPase activity in the presence of both cofactors, some active CLC must have formed. Accounting for the ATP hydrolysis rate of individual gp44 subunits and the CLC, approximately 35% of native complex activity was achieved. The gp62 preparation alone exhibited no ATPase activity in the absence of gp44, regardless of the presence of gp45 and DNA.

It was possible that only a subset of purified gp62 subunits were involved in the CLC reconstitution or that the reconstituted complex formed with a different stoichiometry than that of native CLC. To address this question we titrated gp62 into a fixed amount of gp44 subunits. The ATP hydrolysis rate in the presence of both gp45 and DNA was then measured. The ATPase activity observed for gp44 tetramers when zero gp62...
subunits were present was taken as the minimum rate. The ATP hydrolysis rate increased as the number of gp44 equivalents per gp44 rose from zero to one (Fig. 7) and then began to plateau as superstoichiometric amounts of gp62 were added. It appears that the reconstituted complex is forming a composition similar to the native 4:1 stoichiometry.

**DISCUSSION**

Using transformed *E. coli*, we were able to demonstrate the production and purification of the bacteriophage T4 gp62 in the absence of gp44 subunits. Translation from the T4 genome of gene 62 is coupled to that of the upstream gene 44. Initial reported attempts to express gp62 using a plasmid vector required maintenance of an upstream reading frame (27). Using immunologic techniques, it was recently shown that a small amount of translation does occur from the natural gene 62 TIR in the absence of complete gp44 translation (26). Therefore we started creating constructs with the natural TIR of gene 62 including the putative hairpin structure (pCLC62H). It was expected that disruption of this putative secondary structure would increase expression, but the construct in which the upstream portion of the hairpin stem was deleted (pCLC62H2) exhibited a 4-fold decrease in expression level. Using Zucker and Genemee folding algorithms available over the Internet, we examined predicted folding patterns of the gene 44-gene 62 intercistronic region and found no convincing evidence for the putative hairpin structure that had been proposed. The gene 62 expression results, in addition to the facts that uncoupled translation of gene 62 occurs (26) and the related phage RB69 does not exhibit a possible hairpin between genes 44 and 62 (28), imply that the putative hairpin structure (37) does not have a physical existence. Thus, our results are in congruence with the recent suggestion by the Karam (and co-workers) laboratory (28) that the majority of the translational coupling of genes 44 and 62 is due to the naturally weak TIR of gene 62.

Assembly of the gp44/62 complex had been thought to occur as a direct consequence of the translational coupling of these two genes, either through directed subunit interaction or decrease of diffusion distances (29). Assembly of the CLC when the subunits were expressed from separate plasmids demonstrated that translational coupling is not a requirement for the formation of the CLC (28). A translational capture model, which required co-expression of genes 44 and 62, has also been suggested for the formation of the CLC (34). We were able to achieve reconstitution of the gp44/62 complex by mixing individually purified native subunits, arguing that the CLC forms via a self-assembly mechanism. The reconstituted complex demonstrated every ATPase activity observed in the native complex including synergistic stimulation by DNA and gp45 (see Table 1). If co-folding of subunits were required for the assembly of the CLC, we would expect to detect virtually no synergistic stimulation in the ATPase assay. Previous studies have indicated that when co-expression of subunits has proven to be an absolute necessity for heterooligomeric complex formation, as with bacterial luciferase, only minimal activity (1%) could be achieved without concerted refolding (38, 39). ATPase rates observed during the reconstituted assay were lower than those observed when the native complex was used. However, testing of the ATPase activity was performed directly on the mixture of gp44 and gp62 subunits. If the reconstituted CLC had been isolated away from stray subunits before the ATPase assay, absolute rates as high as those seen for purified native CLC may have been achieved. Alternatively, the activity we observed is the maximum that can be achieved with reconstitution. For example, the human CLC reconstituted from co-expressed subunits and then purified only exhibited 30% of the native activity (40).

**TABLE I**

**ATP hydrolysis rates**

The rate values were determined under steady-state conditions as described under “Experimental Procedures.” Measurements were repeated at least three times. Uncertainty is expressed as 1 sample S.D.

| Enzyme preparation | Cofactor | Cofactor | Cofactor |
|--------------------|----------|----------|----------|
| 250 nM gp44/62     | gp45     | DNA      | gp45 + DNA |
| 250 nM gp44        | 4.0 ± 2.3 | 35.1 ± 4.3 | 39.6 ± 5.4 | 1450 ± 200 |
| 250 nM reconstituted* | 10.8 ± 5.9 | 42.0 ± 5.6 | 53.0 ± 2.3 | 580 ± 28 |

* The ATPase assay for reconstitution of the CLC was performed directly on a mixture containing 250 nM gp44 tetramers and 250 nM gp62 monomers.

Translational coupling of genes 44 and 62 has been cited as a determinant of the four gp44 subunits to one gp62 subunit CLC stoichiometry (28). The stoichiometry of the complex assembled when subunits are expressed in trans was found to be consistent with the known stoichiometry, even though there was no strict control over the relative amounts of proteins synthesized (34). The results (see Fig. 7) of gp62 titration into a fixed concentration of gp44 subunits plateau near the known stoichiometry. This argues that stoichiometry of the gp44/62 complex is determined by self-assembly. Thus although translational coupling may limit the stoichiometry of subunits synthesis (26), it is not a determinant of the stoichiometry in the assembled complex.

The T4 bacteriophage gp44/62 protein complex is the simplest example of a multisubunit clamp loader. Although yeast, human, and *E. coli* cells all possess five subunit clamp loaders with four of the subunits containing some degree of homology to each other, the T4 CLC has four identical subunits (7). The human replication factor C CLC has been reconstituted from simultaneously expressed subunits, but it cannot be assembled in vitro without at least a core of three co-folded subunits (41). The γ-complex of *E. coli* (γ86′γ9) has been reconstituted from purified subunits, resulting in a complex that was functional in DNA replication assays. Since one of the purified subunits was stored in 4 M urea and the first incubation step occurred in 0.5 M urea, co-folding cannot be completely excluded from the γ-complex assembly pathway (42). The γ subunit, which acts as a scaffold for γ-complex assembly, also exists as a tetramer when purified but is dimeric in the γ-complex (43). The gp44 subunits are isolated as tetramers and may serve as a preformed core for clamp loader assembly. Native CLC subunits only dissociate under denaturing conditions (21, 31). It is uncertain whether the subunits of the reconstituted complex are...
as tightly associated as in the native case. However, our IEF results (Fig. 5C) argue for a strong interaction rather than an equilibrium association, as the subunits remain associated against the force of first dimension electrophoresis. The immunoprecipitation results (Fig. 6) further argue for a strong interaction, since the decrease in efficiency of subunit precipitation upon formation of the CLC suggests that there may be a substantial conformation change in the gp44 subunits upon reconstitution of the CLC.

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REFERENCES
1. Young, M. C., Reddy, M. K., and von Hippel, P. H. (1992) Biochemistry 31, 8675–8690
2. Nossal, N. G. (1992) FASEB J. 6, 871–878
3. Bertram, J. G., Bloom, L. B., Turner, J., O'Donnell, M., Beechem, J. M., and Goodman, M. F. (1998) J. Biol. Chem. 273, 24564–24574
4. Bloom, L. B., Turner, J., Kelman, Z., Beechem, J. M., O'Donnell, M., and Goodman, M. F. (1996) J. Biol. Chem. 271, 30699–30708
5. Lee, S. H., Kwong, A. D., Pan, Z. Q., and Hurwitz, J. (1991) J. Biol. Chem. 266, 584–602
6. Tsurimoto, T., and Stillman, B. (1991) J. Biol. Chem. 266, 1950–1960
7. O'Donnell, M., Onrust, R., Dean, F. B., Chen, M., and Hurwitz, J. (1993) Nucleic Acids Res. 21, 1–3
8. Cullmann, G., Fien, K., Kobayashi, R., and Stillman, B. (1995) Mol. Cell. Biol. 15, 4661–4671
9. Kabaord, B. F., and Benkovic, S. J. (1995) Curr. Biol. 5, 149–157
10. Reddy, M. K., Weitzel, S. E., Daube, S. S., Jarvis, T. C., and von Hippel, P. H. (1995) Methods Enzymol. 262, 466–476
11. Berdis, A. J., and Benkovic, S. J. (1997) Biochemistry 36, 2733–2743
12. Latham, G. J., Bacheller, D. J., Pietroni, P., and von Hippel, P. H. (1997) J. Biol. Chem. 272, 31677–31684
13. Pietroni, P., Young, M. C., Latham, G. J., and von Hippel, P. H. (1997) J. Biol. Chem. 272, 31664–31676
14. Kabaord, B. F., and Benkovic, S. J. (1996) Biochemistry 35, 1084–1092
15. Berdis, A. J., and Benkovic, S. J. (1996) Biochemistry 35, 9253–9265
16. Gogol, E. P., Young, M. C., Kubasek, W. L., Jarvis, T. C., and von Hippel, P. H. (1992) J. Mol. Biol. 224, 395–412
17. Reddy, M. K., Weitzel, S. E., and von Hippel, P. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3211–3215
18. Sexton, D. J., Carver, T. E., Berdis, A. J., and Benkovic, S. J. (1996) J. Biol. Chem. 271, 28045–28051
19. Kroutil, L. C., Frey, M. W., Kabaord, B. F., Kunkel, T. A., and Benkovic, S. J. (1998) J. Biol. Chem. 273, 135–146
20. Jarvis, T. C., Paul, L. S., and von Hippel, P. H. (1989) J. Biol. Chem. 264, 12709–12716
21. Barry, J., and Alberts, B. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 2717–2721
22. Morris, C. F., Hama-Inaba, H., Mace, D., Sinha, N. K., and Alberts, B. (1979) J. Biol. Chem. 254, 6787–6796
23. Pipperno, J. R., and Alberts, B. M. (1978) J. Biol. Chem. 253, 5174–5179
24. Hsu, T., and Karam, J. D. (1990) J. Biol. Chem. 265, 5303–5316
25. Karam, J., Bowles, M., and Leach, M. (1979) Virology 94, 192–203
26. Torgov, M. Y., Janzen, D. M., and Reddy, M. K. (1998) J. Bacteriol. 180, 4339–4343
27. Rush, J., Lin, T. C., Quinones, M., Spicer, E. K., Douglas, I., Williams, K. R., and Konigsberg, W. H. (1989) J. Biol. Chem. 264, 10943–10953
28. Yeh, L. S., Hsu, T., and Karam, J. D. (1986) J. Bacteriol. 160, 2905–2913
29. Gerald, W. L., and Karam, J. D. (1984) Genetics 107, 537–549
30. Jarvis, T. C., Paul, L. S., Heekensmith, J. W., and von Hippel, P. H. (1989) J. Biol. Chem. 264, 12717–12729
31. Spicer, E. K., Nossal, N. G., and Williams, K. R. (1984) J. Biol. Chem. 259, 15425–15432
32. Mace, D. C., and Alberts, B. M. (1984) J. Mol. Biol. 177, 279–293
33. Guzman, L. M., Belin, D., Carson, M. J., and Beckwith, J. (1995) J. Bacteriol. 177, 4121–4130
34. Janzen, D. M., Torgov, M. Y., Abbott, S. N., and Reddy, M. K. (1999) Virology 260, 64–73
35. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
36. O'Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
37. Trojanowska, M., Miller, E. S., Karam, J., Stormo, G., and Gold, L. (1984) Nucleic Acids Res. 12, 5979–5993
38. Sinclair, J. F., Waddle, J. J., Waddill, E. F., and Baldwin, T. O. (1993) Biochemistry 32, 5036–5044
39. Waddle, J. J., Johnston, T. C., and Baldwin, T. O. (1987) Biochemistry 26, 4917–4921
40. Uhmann, F., Cai, J., Flores-Rozas, H., Dean, F. B., Finkelstein, J., O'Donnell, M., and Hurwitz, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6521–6526
41. Cai, J., Uhmann, F., Gibbs, E., Flores-Rozas, H., Lee, C. G., Phillips, B., Finkelstein, J., Yao, N., O'Donnell, M., and Hurwitz, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12986–12991
42. Onrust, R., Finkelstein, J., Naktinis, V., Turner, J., Fang, L., and O'Donnell, M. (1995) J. Biol. Chem. 270, 13348–13357
43. Onrust, R., Finkelstein, J., Turner, J., Naktinis, V., and O'Donnell, M. (1995) J. Biol. Chem. 270, 13366–13377