Role of Adenosine 5′-Triphosphate Hydrolysis in the Assembly of the Bacteriophage T4 DNA Replication Holoenzyme Complex†

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ABSTRACT: Steady-state and pre-steady-state rates of ATP hydrolysis by the 44/62 accessory protein were determined to elucidate the role of ATP hydrolysis in bacteriophage T4 holoenzyme complex formation. Steady-state ATPase measurements of the 44/62 protein under various combinations of 45 protein, DNA substrate, and T4 exo$^-$ polymerase indicate that although the 44/62 protein synergistically hydrolyzes ATP in the presence of 45 protein and DNA substrate, the ATPase activity of 44/62 is diminished substantially upon the formation of the holoenzyme complex. The decrease in activity is primarily in k_{cat} while the K_m for ATP is changed unsubstantially by the various combinations. Data suggest that the decrease in the rate of ATP hydrolysis upon the addition of T4 exo$^-$ polymerase in the presence of 45 protein and DNA substrate is due to formation of a stable holoenzyme complex consisting of only the 45 protein and T4 exo$^-$ polymerase in a 1:1 ratio. The 44/62 protein acts catalytically to load 45 protein onto the DNA substrate and does not remain a component of the holoenzyme complex. Pre-steady-state kinetic analysis of the ATP hydrolysis reaction catalyzed by the 44/62 protein loading the 45 protein onto the DNA substrate in the absence or presence of polymerase is biphasic, in which a burst in ATP hydrolysis precedes the steady-state rate of ATP hydrolysis. An identical burst in ATP consumption is obtained under either condition, indicating that ATP hydrolysis is not required to load polymerase into the holoenzyme complex. The data suggest one turnover of ATP at each of the four ATPase active sites of the 44/62 protein per 45 protein loaded. ATP hydrolysis by the 44/62 protein under conditions of holoenzyme complex formation is the rate-limiting step in holoenzyme complex formation. The process of holoenzyme formation appears to be identical for leading and lagging strand synthesis.

The holoenzyme of the bacteriophage T4 DNA replication system is assembled from the phage DNA polymerase (the product of gene 43) and the accessory proteins (the products of genes 44, 45, and 62) [reviewed by Nossal (1992) and Young et al. (1992)]. The 44/62 protein is a tightly associated complex, with a subunit stoichiometry of 4:1 (Jarvis et al., 1989a) and possesses DNA-dependent ATPase activity stimulated by the 45 protein (Mace & Alberts, 1984b). A key feature of the holoenzyme is its high processivity for both DNA synthesis and 3′ → 5′ nucleotide excision relative to the polymerase (Alberts & Frey, 1970; Mace & Alberts, 1984a,b). Since the holoenzyme for at least leading strand synthesis consists of only the 45 protein and the 43 polymerase, the former acts as a "sliding clamp" (Huang et al., 1981) to bind the polymerase to the DNA at the primer/template junction and the 44/62 protein acts as a "clamp loader" for the 45 protein. Although the 44/62 protein has dual enzymatic activities that can be studied simultaneously, i.e., the hydrolysis of ATP and the formation of the holoenzyme complex, detailed kinetic analysis of this accessory protein has been difficult primarily due to the complexities of the numerous possible protein−protein and protein−DNA interactions within this system.

Despite the complexities of the replication machinery, the T4 polymerase system is an attractive target for studying the dynamics of DNA replication and the role of various accessory proteins in achieving highly efficient DNA replication since a bacteriophage T4 replication fork can be constructed from purified components that catalyze leading and lagging strand synthesis at *in vivo* rates and accuracies (Piperno & Alberts, 1978). The 44/62 protein and 45 accessory proteins have their counterparts in *Escherichia coli* and eukaryotes where the clamp loader is the γ complex (Maki & O'Donnell, 1987) or RF-C complex (Lee et al., 1991) and the sliding clamp is the β subunit (Stukenburg et al., 1991) or PCNA[1] (Tsurimoto & Stillman, 1991). The three-dimensional structures of the β subunit and PCNA showed the proteins to be in the shape of rings with a central cavity large enough to enclose duplex DNA (Krishna et al., 1994; Kong et al., 1992). For the T4 system, cryoelectron microscopy revealed presumably the 45 protein encircling the DNA (Gogol et al., 1992) so that one anticipates on the basis of subunit organizational similarities among the replication systems that this sliding clamp will have a structure analogous to the β subunit and PCNA. Thus, one of the more difficult questions to answer is how the closed, ring-shaped processivity factor can be loaded onto circular DNA. The most likely possibility is that the 44/62 protein opens the 45 protein so that it can be loaded onto DNA, and

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[1] Abbreviations: PCNA, proliferating cell nuclear antigen; Bio, biotin; TBE, Tris-HCl/borate/EDTA; EDTA, ethylenediaminetetracetate sodium salt; dNTP, deoxynucleotide triphosphate.

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this process is mediated by ATP hydrolysis by the 44/62 protein. Thus, accurate measurements of the amount and the rate of ATP hydrolyzed by the 44/62 protein in achieving this process are of paramount importance.

Cross-linking (Capson et al., 1991) and footprinting (Munn & Alberts, 1991) experiments have shown that the 44/62 protein and 45 protein are found at the primer/template junction in the presence of ATP. The dynamics of ATP hydrolysis by the 44/62 protein required for the formation of this complex are unknown as is the exact mechanism of ATP-coupled stimulation of polymerase processivity or the exact number of ATP molecules that must be hydrolyzed to form the productive holoenzyme complex. One possible model invokes the formation of an ADP-bound state of the 44/62 protein in conjunction with the 45 protein to form a sliding clamp. This complex then stabilizes the binding of polymerase (the sliding clamp's affinity for DNA may be stabilized by polymerase in a reciprocal manner), decreasing the dissociation rate of the polymerase and potentiating the high processivity of the holoenzyme conformation (Jarvis et al., 1989b). The holoenzyme complex consisting of the 44/62 and 45 accessory proteins in conjunction with the polymerase would then be locked into a conformation that prevents further ATP hydrolysis during the lifetime of the holoenzyme complex. Consistent with this model, nonhydrolyzable analogs of ATP cause the accessory proteins to bind tightly to the primer/template junction although they do not act to increase the processivity of polymerase since the sliding clamp conformation that requires bound ADP is inaccessible (Jarvis et al., 1989b).

In contrast to this model, Huang et al. (1981) determined that a "periodic" ATP hydrolysis event was required to "sustain" the processivity of the holoenzyme during DNA elongation, consistent with an "activated" holoenzyme complex that supports highly processive DNA synthesis (Newport et al., 1980). Termination of replication and dissociation of the holoenzyme complex would occur only when the accessory proteins return to the "unactivated" or prehydrolysis state. The net result is a timing mechanism in which highly processive DNA synthesis has some limited duration. A favorable feature of this mechanism is that the polymerase and/or holoenzyme complex would be prone to dissociation upon encountering the 5′ end of the previously synthesized Okazaki fragment during discontinuous DNA synthesis of the lagging strand.

Both of these models assume that the 44/62 and 45 proteins remain stably associated even upon the addition of polymerase to form the holoenzyme complex (44/62:45:Pol complex). In this report, a detailed steady-state analysis of the ATPase activity of the 44/62 protein is presented to address these models. Data obtained indicate that the 44/62 protein acts catalytically to assemble the holoenzyme complex, invalidating any models invoking a 44/62-containing holoenzyme complex. Pre-steady-state kinetic analysis of ATP hydrolysis by the 44/62 protein has been performed in an attempt to determine the stoichiometry of ATP hydrolyzed to form the holoenzyme complex as well as to compare the rate of ATP hydrolysis to the rate of holoenzyme complex formation. Processive DNA synthesis reactions have been performed in parallel using the defined forked primer/template to define further the relationship between ATP hydrolysis and the assembly of the functional holoenzyme complex.

METHODS AND MATERIALS

Materials. [γ-^{32}P]ATP, [α-^{32}P]ATP, and [α-^{32}P]dCTP were purchased from New England Nuclear. Unlabeled dNTPs were obtained from Pharmacia (ultrapure). All oligonucleotides, including those containing biotin derivatives, were synthesized by Operon Technologies (Alameda, CA) and purified as previously described by Capson et al. (1992). RNA oligonucleotides were a kind gift from Henri Buc (Pateur Institute). Hybrid DNA−RNA oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX). ATP, phosphoenolpyruvate, $MgCl_2$, $Mg(OAc)_2$, and all buffers were from Sigma. NADH was from Boehringer Mannheim. All other materials were obtained from commercial sources and were of the highest available quality. T4 polynucleotide kinase was from United States Biochemicals. Lactate dehydrogenase and pyruvate kinase were from Sigma. These enzymes were dialyzed against 25 mM Tris, pH 7.5, and stored in 20% glycerol at −20 °C. The T4 exonuclease-deficient polymerase D129A (Asp-219 to Ala mutation) was purified as previously described (Frey et al., 1993). The T4 accessory proteins 44/62 and 45 were a gift from Barbara F. Kaboord (The Pennsylvania State University). Both the 44/62 protein and 45 protein were purified from overproducing strains obtained from William Konigsberg (Yale University). The concentration of the 44/62 and 45 proteins are reported as units of 4:1 complex and trimer, respectively, in agreement with the stoichiometry reported by Jarvis et al. (1989a).

Primer/Template Construction. The template strand of the biotin-labeled primer/template was constructed from two shorter oligonucleotides (Bio-23-mer plus 39-mer). Purified 3′-biotin-labeled 23-mer was 5′-phosphorylated by T4 polynucleotide kinase according to the manufacturer's protocol. The two template oligonucleotides were then ligated by T4 DNA ligase with the annealed primer strand (34-mer) serving to bridge the two substrate oligonucleotides. Completion of the ligation reaction was determined by 5′-labeling an aliquot of the ligation reaction with [γ-^{32}P]ATP and analyzing the sample oligonucleotide on a 16% acrylamide/8 M urea/90 mM Tris/65 mM boric acid/2.5 mM EDTA, pH 8.3, sequencing gel to examine formation of 62-mer. The duplex was then purified as described by Capson et al. (1992) and quantitated as described by Kuchta et al. (1987).

Lagging strand forked primer/templates (Bio-33/62/36-mers) were constructed, purified, and quantitated in an identical manner described above.

Steady-State ATP Hydrolysis Measurements. All assays were carried out using either a Gilford 250 spectrophotometer equipped with a strip-chart recorder or a Cary 114 spectrophotometer. A coupled assay system was used to monitor the formation of ADP, consisting of 3 mM phosphoenolpyruvate, 200 μM NADH, 6 units of lactate dehydrogenase, and 6 units of pyruvate kinase. The disappearance of NADH was measured at 340 nm and accurately reflects the rate of ATP hydrolysis by the 44/62 protein. The temperature was maintained at 25 °C using a circulating water bath to heat the thermospacers of the cell compartment. Reaction cuvettes were 1 cm in path length and either 1 or 0.3 mL in volume. All cuvettes were incubated for at least 5 min in a water bath prior to initiation of reaction. A typical assay for measuring the ATPase activity of the 44/62 protein contained 250 nM 44/62 protein, 250 nM 45 protein, 250 nM primer/template, 1 μM streptavidin, 1 mM ATP, 10 mM

$Mg(OAc)_2$, and the coupled assay reagents in a buffer consisting of 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol. All assays reflect initial velocity conditions with less than 10% of the limiting reactant utilized over the time course of the reaction.

Pre-Steady-State ATP Hydrolysis Measurements. ATPase measurements of the 44/62 protein under various protein and primer/template conditions were performed in a rapid quench instrument described by Johnson (1986). A typical assay for measuring the ATPase activity of the 44/62 protein contained 250 nM 44/62 protein, 250 nM 45 protein, 250 nM primer/template, 1 μM streptavidin, 1 mM ATP, 50 nM [γ-^{32}P]ATP or [α-^{32}P]ATP, and 10 mM $Mg(OAc)_2$ in a buffer consisting of 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol at 25 °C. The reactions were quenched with 1 M HCl, extracted with phenol/$CHCl_3$ (1:1), and neutralized with 3 M NaOH in 1 M Trizma base. Samples were analyzed for ATP hydrolysis by one of two methods. The first method consists of washing the radioactive samples with heat-activated, acid-washed charcoal and collecting the supernatant by centrifugation using 0.45 μM Millipore filtration units. This process was repeated three times to remove any remaining radiolabeled ATP, and the samples were then counted by standard liquid scintillation counting techniques. Samples were also analyzed using thin-layer chromatography with a 0.3 M potassium phosphate solvent. Samples were scanned for product ($^{32}P_i$) formation using a Molecular Dynamics PhosphorImager and were essentially [γ-^{32}P]ATP free. The second method involved separating unhydrolyzed [γ-^{32}P]ATP from $^{32}P_i$ or [α-^{32}P]ADP from unhydrolyzed (α-^{32}P]ATP by thin-layer chromatography directly with no prior treatment. Samples were analyzed for product formation using a Molecular Dynamics PhosphorImager in which [γ-^{32}P]ATP from $^{32}P_i$ and [α-^{32}P]ATP from [α-^{32}P]ADP are easily distinguished. Product formation was quantitated by measuring the ratio of hydrolyzed product and unhydrolyzed substrate. The ratios of product formation are corrected for substrate in the absence of enzyme (zero point). Corrected ratios are then multiplied by the concentration of total ATP used in each assay to yield the amount of ATP hydrolyzed. Identical results were obtained using either method.

Complex Formation and DNA Synthesis Assays. All reactions were performed at 25 °C in a buffer consisting of 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol. All concentrations are final reaction concentrations. Reactions were initiated by mixing 500 nM primer/template, 1 μM streptavidin, 10 μM dCTP, and varying concentrations of ATP in assay buffer with 100 nM T4 exo$^-$ polymerase, 550 nM 44/62 protein, and 550 nM 45 protein. This mixture was allowed to incubate for 15 s, and then the remaining dNTPs (10 μM each) and the single-stranded salmon sperm DNA trap (1 mg/mL) were added. At variable times, aliquots were removed and quenched in either 0.5 M EDTA or 1 M HCl. Acid-quenched samples were extracted with phenol/$CHCl_3$ (1:1) and neutralized with 3 M NaOH in 1 M Trizma base. The polymerization products were analyzed on 16% sequencing gels as described by Mizrahi et al. (1986). Gel images were obtained with a Molecular Dynamics PhosphorImager. Product formation was quantitated by measuring the ratio of radiolabeled extended and nonextended primer. The ratios of product formation are corrected for substrate in the absence of polymerase (zero point). Corrected ratios are then multiplied by the concentration of primer/template used in each assay to yield total product. The amount of complex formation is equivalent to the amount of fully elongated product (62-mer) as previously described (Benkovic & Kaboord, 1995).

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. Lineweaver−Burk data were fitted using eq 1 and

$$v = VA/(K_a + A) \quad (1)$$

computer programs developed by Cleland (1979), where v is the initial velocity, V is the maximum velocity, A is the reactant concentration, and K_a is the Michaelis constants for A.

Data obtained for pre-steady-state ATP hydrolysis by 44/62 were fit to eq 2, where A is the burst amplitude, k is the first-order rate constant, B is the steady-state rate, and C is a defined constant.

$$y = Ae^{-kt} + Bt + C \quad (2)$$

RESULTS AND DISCUSSION

Assays for the Dual Enzymatic Activity of the 44/62 Protein. The ATPase activity of the 44/62 protein is measured by two distinct methods to obtain accurate and unambiguous results. Steady-state ATPase measurements used a spectrophotometric ATPase assay in which the production of ADP is coupled to NAD formation. Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, and NADH are used as coupling reagents, and the decrease in absorbance at 340 nm resulting from the formation of NAD from NADH provides an accurate method of measuring ATP hydrolysis. Another important feature of the coupled assay system is that ATP is constantly regenerated so that the concentration of ATP remains essentially unchanged during the course of the reaction.

Pre-steady-state ATPase measurements of the ATPase activity of the 44/62 protein were performed using either [γ-^{32}P]ATP or [α-^{32}P]ATP as the reporter molecule. This method of analyzing ATP hydrolysis is extremely sensitive, able to detect product formation in the nanomolar to picomolar range which is necessary for pre-steady-state kinetic measurements. Nearly identical steady-state rates for ATP hydrolysis by the 44/62 protein are obtained using either method for measuring ATP hydrolysis (±5%).

Processive DNA synthesis measurements were performed by examining the ability of the 44/62 protein to productively load the 45 protein onto the DNA primer/template. This can be accomplished by measuring the strand displacement ability of the holoenzyme complex using the defined Bio-34/62/36-mer primer/template (Figure 1) as holoenzyme will yield a 62-mer product while the enzymatic action of polymerase alone will yield smaller products up to the forked strand (<44-mer). Since ATP hydrolysis by the 44/62 protein is required to load the 45 protein onto the primer/template junction, holoenzyme complex formation can thus be measured as a function of ATP hydrolysis.

Kinetic Parameters for the 44/62 Protein. It is assumed that only the 44/62 protein contains ATPase activity such that increases in the rate of ATP hydrolysis in the presence of the 45 protein still reflect that of the intrinsic ATPase activity of the 44/62 protein. In the absence of gene 62 protein, the gene 44 protein is an oligomer that possesses DNA-stimulated ATPase activity but cannot be stimulated

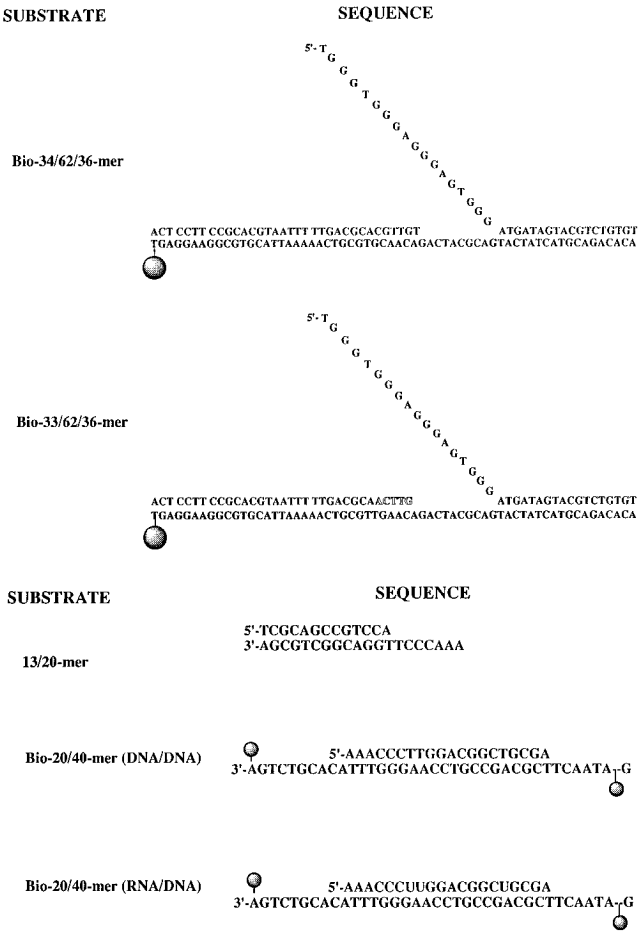


FIGURE 1: Substrates used for both ATPase and processive DNA synthesis measurements. The last five bases on the Bio-33/62/36-mer substrate are either RNA or DNA.

by the 45 protein (Rush et al., 1989). This suggests that the 62 protein is required for coupling the interaction of the 44 protein with the 45 protein. Furthermore, plasmids encoding gene 62 without gene 44 do not produce the 62 protein, presumably due to highly coordinated synthesis of the 44/62 gene product (Rush et al., 1989).

Kinetic parameters for the Bio-34/62/36-mer primer/template were measured as a function of ATP hydrolysis by the 44/62 protein in the presence of stoichiometric 45 protein by varying the concentration of Bio-34/62/36-mer primer/template, yielding an apparent K_m for primer/template of 20 $\pm$ 1 nM for the 44/62 and 45 protein complex and a k_{cat} of 1.4 $\pm$ 0.2 s^{-1}. Thus, a concentration of 250 nM Bio-34/62/36-mer was used for all subsequent assays such that the concentration of primer/template was maintained at $10K_m$.

The effect of 45 protein concentration on the rate of ATP hydrolysis by the 44/62 protein in the presence of 250 nM 34/62/36-mer primer/template was measured, varying the concentration of the 45 protein. The increase in the rate of ATP hydrolysis by 250 nM 44/62 protein is linear up to a concentration of 500 nM 45 protein.

Kinetic parameters for ATP hydrolysis by the 44/62 protein alone cannot accurately be measured since the rate of ATP hydrolysis is barely detectable using the coupled assay system (3 nM/s). In the presence of a stoichiometric 1:1 ratio (250 nM) of 44/62 and 45 proteins, $k_{cat} = 0.17 \pm 0.03$ s^{-1} and $K_{ATP} = 250 \pm 130$ μM. In the presence of a stoichiometric 1:1 ratio (250 nM) of 44/62 and Bio-34/62/36-mer, similar data analyses yield a k_{cat} value of 0.024 $\pm$ 0.001 s^{-1} and a K_{ATP} of 125 $\pm$ 20 μM. The combination of 44/62, T4 exo$^-$ polymerase, and Bio-34/62/36-mer likewise yields a k_{cat} value of 0.036 $\pm$ 0.003 s^{-1} and a K_{ATP} of 50 $\pm$ 40 μM. The similarity in k_{cat} value for 44/62 and Bio-34/62/36-mer with and without T4 exo$^-$ suggests that the 44/62 protein does not interact with the polymerase in the absence of 45 protein. Although there appears to be a 2−3-fold decrease in the value of K_{ATP}, the high standard errors indicate that the Michaelis constant for ATP is still in the range of 100 μM. Thus, the Michaelis constant for ATP is not altered significantly in the presence of any effector alone (DNA, 45 protein, T4 exo$^-$) while k_{cat} increases nearly 60-fold in the presence of 45 protein and DNA as compared to DNA substrate alone. The 7-fold higher k_{cat} value obtained in the presence of 45 protein alone as opposed to in the presence of DNA substrate alone could reflect a small DNA contamination carried over from purification of the 45 protein since a combination of DNA and 45 protein synergistically activates the 44/62 protein (described below). Control experiments were performed in which DNase I and S1 nuclease were added to the reaction mixture to degrade any endogenous double- or single-stranded DNA. Identical steady-state rates were obtained in the absence and presence of DNase I and S1 nuclease, indicating that the higher ATPase activity of the 44/62 protein in the presence of the 45 protein is not caused by DNA contamination. A reasonable explanation for this phenomenon is that the 44/62 protein hydrolyzes ATP in a process that opens and/or closes the ring-shaped 45 protein even in the absence of DNA substrate.

In the presence of a 1:1:1 ratio (250 nM) of 44/62, 45 protein, and Bio-34/62/36-mer, a reciprocal plot varying ATP levels yields a k_{cat} value of 1.4 $\pm$ 0.2 s^{-1} (in satisfactory agreement with the k_{cat} value obtained by varying DNA) and a K_{ATP} of 130 $\pm$ 10 μM. The large ca. 60-fold increase in k_{cat} when the 45 protein and duplex DNA are both present is indicative of synergistic activation of the 44/62 protein (Jarvis et al., 1989b). Again, the Michaelis constant for ATP obtained under these conditions does not vary significantly from the two aforementioned conditions.

In the presence of a 1:1:1 ratio (250 nM) of 44/62, 45 protein, Bio-34/62/36-mer, but with 1 μM streptavidin, the steady-state data fit a k_{cat} value of 0.66 $\pm$ 0.04 s^{-1} and a K_{ATP} of 140 $\pm$ 30 μM. In this case, k_{cat} is decreased by a factor of 2 due to the presence of streptavidin binding the 3′-biotin moiety while the K_m for ATP is unchanged. Several possibilities are consistent with the decrease in k_{cat} for the 44/62 protein. The decrease in k_{cat} could reflect the inability of the 45 protein to translocate off the end of the duplex DNA in the presence of streptavidin. Since the loaded 45 protein must have a limited lifetime on the primer/template, it is possible that a significant population of the 45 protein is sequestered for a short period of time on this primer/template, resulting in a diminished rate of ATP hydrolysis by the 44/62 protein since there is a decreased amount of 45 protein to reload back onto the primer/template. Alternatively, the datum may be interpreted as the formation of the 44/62:45 protein complex on the primer/template that does not require ATP hydrolysis. Since the 44/62 protein is sequestered into this complex, k_{cat} would also decrease. Although it is not possible to distinguish between these two mechanisms from the data presented here, data described in the following sections indicate that the 44/62 protein does

Table 1: Kinetic Parameters for the 44/62 Protein[a]

condition	K_{ATP} (μM)	k_{cat} (s^{-1})
44/62 protein	nd[b]	nd
44/62 and 45 proteins	250 ± 130	0.17 ± 0.03
44/62 protein and Bio-34/62/36-mer	125 ± 16	0.024 ± 0.003
44/62 protein, T4 exo$^-$, and Bio-34/62/36-mer	50 ± 40	0.036 ± 0.003
44/62 and 45 proteins and Bio-34/62/36-mer	130 ± 70	1.43 ± 0.19
+1 μM streptavidin	140 ± 30	0.66 ± 0.04
44/62 and 45 proteins, Bio-34/62/36-mer, and T4 exo$^-$	220 ± 30	1.76 ± 0.09

[a] Assays for measuring the ATPase activity of the 44/62 protein contained stoichiometric concentrations of 44/62 protein, 45 protein, primer/template, and T4 exo$^-$ polymerase, using 1 mM ATP, 10 mM $Mg(OAc)_2$, and the coupled assay reagents in a buffer consisting of 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol. [b] nd = not determined.

not remain stably associated with the 45 protein once the holoenzyme complex is formed.

In the presence of a 1:1:1:1 ratio (250 nM) of 44/62, 45 protein, Bio-34/62/36-mer, and T4 exo$^-$ polymerase, but no streptavidin, a k_{cat} value of 1.7 ± 0.1 s^{-1} and a K_{ATP} of 220 ± 30 μM are obtained. Attempts to measure kinetic parameters for the 44/62 protein in the presence of a 1:1:1:1 ratio (250 nM) of 44/62, 45 protein, Bio-34/62/36-mer, and T4 exo$^-$ polymerase but in the presence of streptavidin could not be performed due to a severe decrease in the ATPase activity of the 44/62 protein upon holoenzyme complex formation (described in more detail below). The kinetic parameters for ATP processing under these variable conditions are collected in Table 1.

T4 exo$^-$ Polymerase and the 45 Protein Form a Stoichiometric Complex That Does Not Require Additional ATP Hydrolysis by 44/62. Experiments were performed in an attempt to determine the rate of ATP hydrolysis by the 44/62 protein under conditions of holoenzyme complex formation. The rate of ATP hydrolysis by the 44/62 protein in the presence of stoichiometric 45 protein and Bio-34/62/36-mer in the presence of streptavidin is 200 nM/s while in the absence of streptavidin, a rate of 430 nM/s is obtained. This 2-fold rate decrease in the presence of streptavidin is again consistent with either the 44/62:45 complex or the 45 protein alone being "hindered" from rolling off the end of the duplex DNA by the presence of a physical block. ATPase measurements were then obtained using stoichiometric quantities of accessory proteins, DNA substrate in the presence of steptavidin, and T4 exo$^-$ polymerase (1:1:1:1) in which the polymerase was added last. The rate of ATP hydrolysis prior to the addition of polymerase was 200 nM/s, and this rate eventually decreased to a limiting value of 20 nM/s upon the addition of polymerase. The decrease in ATP hydrolysis is suggestive of formation of a stable holoenzyme complex that once produced does not require ATP hydrolysis. The decrease in ATP hydrolysis under conditions of holoenzyme complex formation is not due to any physical, inhibitory interactions of streptavidin with the 44/62 protein since large ATP hydrolysis rates are obtained with the 45 protein in the presence of streptavidin using primer/templates devoid of biotin moieties (data not shown).

A control experiment was performed under analogous conditions except that streptavidin was omitted from the reaction mixture. Under these conditions, the rate of ATP hydrolysis is 430 nM/s, and this rate does not decrease upon the addition of a stoichiometric quantity of T4 exo$^-$ polymerase. This suggests that, in the absence of the physical blocks, holoenzyme complex formation is unstable since the high rate of ATP hydrolysis is indicative of the 45 protein being constantly reloaded onto the DNA substrate. The lack of stable holoenzyme complex formation in the absence of the streptavidin block is consistent with previous reports in which the presence of streptavidin is absolutely required for holoenzyme complex formation (Kaboord & Benkovic, 1993). Consequently, steady-state kinetic parameters for the 44/62 protein using stoichiometric quantities of accessory proteins, T4 exo$^-$ polymerase, and Bio-34/62/36-mer in the presence of streptavidin were not obtained due to the "shut down" in ATP hydrolysis under these conditions.

Table 2: ATP Hydrolysis Rates by the 44/62 Protein during Holoenzyme Complex Formation[a]

T4 exo$^-$ polymerase added (nM)	rate (nM/s)	T4 exo$^-$ polymerase added (nM)	rate (nM/s)
0	200 ± 10	150	80 ± 5
50	175 ± 10	200	67 ± 3
75	150 ± 7	250	20 ± 3
100	105 ± 8	500	20 ± 3
125	99 ± 5		

[a] Assays for measuring the ATPase activity of the 44/62 protein contained stoichiometric concentrations of 44/62 protein, 45 protein, and primer/template in the presence of 1 μM streptavidin varying T4 exo$^-$ polymerase, using 1 mM ATP, 10 mM $Mg(OAc)_2$, and the coupled assay reagents in a buffer consisting of 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol.

To further evaluate the formation of a stable accessory protein:T4 exo$^-$ polymerase:DNA complex that is suggested by the decrease in k_{cat} noted with the combination experiments, steady-state ATP hydrolysis measurements were performed in which T4 exo$^-$ polymerase was added incrementally to obtain a final concentration stoichiometric with the concentrations of the 44/62 and 45 proteins and primer/template. All assays were performed using the coupled assay system with 1 mM ATP and 10 mM $Mg(OAc)_2$ with the concentrations of the 44/62 protein, 45 protein, and Bio-34/62/36-mer maintained constant at 250 nM and varying the concentration of T4 exo$^-$ polymerase. The rate of ATP hydrolysis by 44/62 in the absence of T4 exo$^-$ polymerase is 200 nM/s and decreases as the amount of T4 exo$^-$ polymerase is added, reaching a level rate of 20 nM/s when the concentration of polymerase is equal to or greater than 1 equiv of accessory proteins and primer/template.[2] ATP hydrolysis rates upon the addition of polymerase are summarized in Table 2. Plotting the data as the rate of ATP hydrolysis versus equivalents of T4 exo$^-$ polymerase results in a linear titration curve with a break at 1 equiv of polymerase added (Figure 2). The rate of ATP hydrolysis does not decrease further even upon the addition of 2 equiv of T4 exo$^-$ polymerase, suggesting that a 1:1 complex of accessory proteins and polymerase is formed.

The rate of 20 nM/s is significantly higher than the rate of ATP hydrolysis by 44/62 protein alone or in the presence

[2] The shutdown in ATPase activity by the 44/62 protein does not occur immediately after the addition of T4 exo$^-$ polymerase. Representative data are depicted in Figure 3 in which the initial rate of ATP hydrolysis is diminished substantially upon the addition of polymerase, but the rate of ATP hydrolysis does not reach the limiting value of 20 nM/s until approximately 60 s.

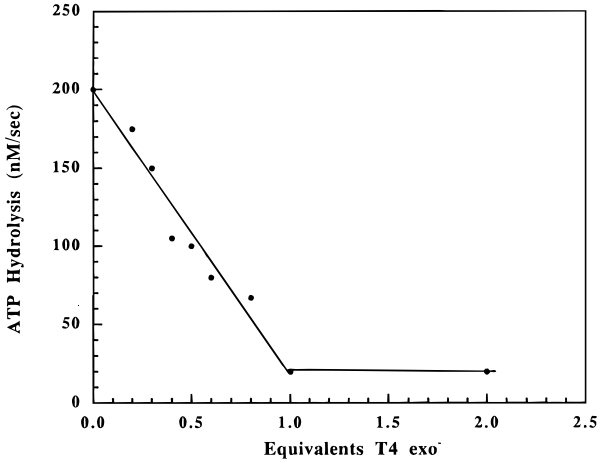


FIGURE 2: Titration curve for the formation of the bacteriophage T4 holoenzyme complex. See text for discussion.

of DNA substrate. However, dissociation of the holoenzyme complex has been determined to be biphasic, in which the off-rate for 70% of the holoenzyme complex is 0.002 s^{-1} while the remaining 30% dissociates at a rate of 0.03 s^{-1} (Kaboord & Benkovic, 1995). From this datum, one can calculate an ATPase rate of approximately 0.4 nM/s from the slow dissociating species (250 nM 44/62 × 0.002 s^{-1} × 70%) and a rate of 2.3 nM/s from the fast dissociating species (250 nM 44/62 × 0.03 s^{-1} × 30%). Thus, the rate of 20 nM/s most likely reflects the sum of rates caused by 44/62 reloading any 45 protein that has dissociated from the holoenzyme complex (∼3 nM/s) and the ATPase rate of 44/62 protein in the presence of duplex DNA (∼3−6 nM/s).

The 44/62 Accessory Protein Loads the 45 Protein onto DNA in a Catalytic Manner. The above data suggest that a stable accessory protein:T4 exo⁻ polymerase:DNA complex is formed since the rates of ATP hydrolysis decrease linearly until a stoichiometric amount of T4 exo⁻ polymerase is added. However, it is not clear if the decrease in ATP hydrolysis is due to depletion of 44/62 protein, i.e., the 44/62 protein remains part of the complex, or if the ATPase activity of 44/62 is decreased since there are decreasing levels of 45 protein free in solution to be loaded onto the DNA substrate; i.e., the 44/62 protein does not remain part of the holoenzyme complex. To determine if the 44/62 protein remains part of the holoenzyme complex, steady-state ATPase assays were performed in which the concentration of the 44/62 protein and Bio-34/62/36-mer were held constant at 250 nM with a streptavidin concentration of 1 μM while the concentration of 45 protein was fixed at 100 nM. In these assays, T4 exo⁻ polymerase was varied at 0, 125, and 250 nM to obtain a stoichiometric ratio of polymerase to first 45 protein and then 44/62 protein. The rate of ATP hydrolysis in the absence of polymerase is 220 nM/s and decreases to a limiting rate of 20 nM/s when either 125 or 250 nM T4 exo⁻ polymerase was added to the reaction (Figure 3). Since the concentration of polymerase (125 and 250 nM) used was greater than that of the 45 protein present (100 nM) but not the 44/62 protein complex, the decreased rate of ATP hydrolysis to the level of 20 nM/s indicates that the 45 protein remains part of the holoenzyme complex. In this case, the 44/62 protein loads the 45 protein onto the primer/template, and when enough polymerase is present to capture all the 45 protein in the stable, holoenzyme complex, the ATPase activity of the 44/62 protein is diminished since no free 45 protein remains to be loaded onto the DNA. These

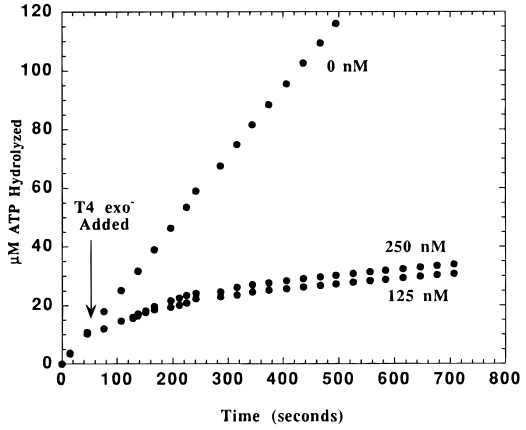


FIGURE 3: Titration curve for the formation of the bacteriophage T4 holoenzyme complex in which the concentration of 45 protein was maintained at 125 nM while the concentrations of Bio-34/62/36-mer and 44/62 protein were fixed at 250 nM. Streptavidin was maintained at 1 μM while the ATP concentration was fixed at 1 mM. Prior to the addition of polymerase, the steady-state rate of ATP hydrolysis was 200 nM/s. T4 exo⁻ polymerase was added (arrow), and the ATPase activity decreased to eventually reach a limiting rate of 20 nM/s.

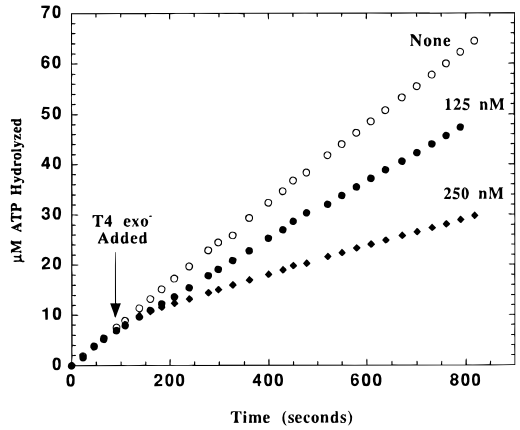


FIGURE 4: Titration curve for the formation of the bacteriophage T4 holoenzyme complex in which the concentration of 44/62 protein was maintained at 50 nM while the concentrations of Bio-34/62/36-mer and 45 protein were fixed at 250 nM. Streptavidin was maintained at 1 μM while the ATP concentration was fixed at 1 mM. Prior to the addition of polymerase, the steady-state rate of ATP hydrolysis was 80 nM/s. T4 exo⁻ polymerase was added (arrow), and the ATPase activity decreased to eventually reach a limiting rate of 20 nM/s.

experiments by themselves do not rule against the 44/62 protein remaining part of the holoenzyme complex as well since the loss of ATPase activity correlates with increasing complex formation, i.e., formation of the stable 44/62:45:Pol complex.

To demonstrate that 44/62 protein does not remain part of the holoenzyme complex, steady-state ATPase assays were performed in which the concentration of 45 protein and Bio-34/62/36-mer were now held fixed at 250 nM (at a streptavidin concentration of 1 μM) while the concentration of 44/62 protein was fixed at 50 nM. In these assays, the T4 exo⁻ polymerase was varied at 0, 125, and 250 nM to obtain finally a 1:1:1 stoichiometry for DNA:45 protein:polymerase (Figure 4). The rate of ATP hydrolysis in the absence of polymerase is 80 nM/s and decreases linearly until 1 equiv of T4 exo⁻ polymerase was present (250 nM) with respect to the 45 protein but not with respect to the 44/62 protein. The lower ATPase rates obtained under these conditions reflect the lower concentration of the 44/62 protein used.

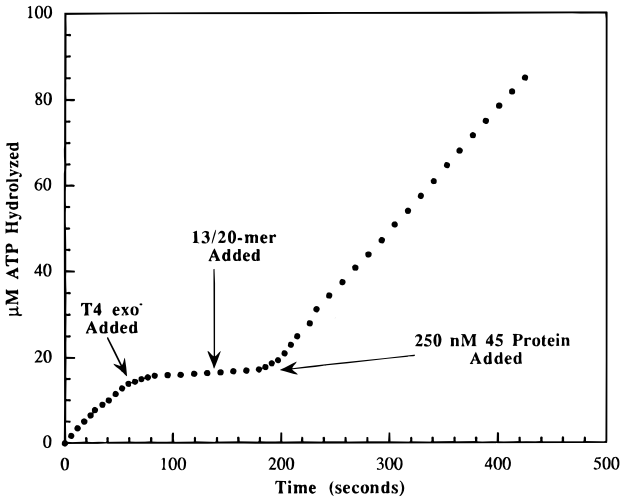


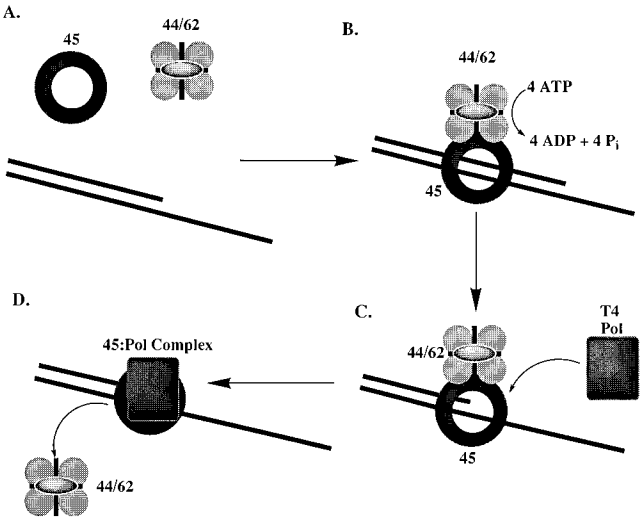
FIGURE 5: The catalytic nature of the 44/62 protein is demonstrated in which the holoenzyme complex is formed using stoichiometric concentrations of 44/62 protein, 45 protein, and Bio-34/62/36-mer (250 nM). Streptavidin was maintained at 1 μM while the ATP concentration was fixed at 1 mM. Prior to the addition of T4 exo$^-$ polymerase, the steady-state rate of ATP hydrolysis was 210 nM/s. At the time indicated (arrow), T4 exo$^-$ polymerase (250 nM) was added upon which the ATPase activity of the 44/62 protein eventually decreased to a rate of 20 nM/s. Excess DNA was added, resulting in a lack of ATPase stimulation of the 44/62 protein. An additional 250 nM 45 protein was added, resulting in a large increase in ATP activity of the 44/62 protein.

The concentrations of polymerase used in these experiments are greater than the concentration of 44/62 protein so that if the 44/62 protein had remained part of the complex, the rate of ATP hydrolysis would have reached the minimum value ATPase rate of 20 nM/s at 125 nM T4 exo$^-$ polymerase.

To further demonstrate the catalytic nature of the 44/62 protein, steady-state ATPase assays were performed using stoichiometric quantities of 44/62 protein, 45 protein, and Bio-34/62/36-mer (250 nM) using a streptavidin concentration of 1 μM while maintaining the concentration of ATP at 1 mM. The steady-state rate of ATP hydrolysis by the 44/62 protein under these conditions is 210 nM/s. To form the holoenzyme complex, 250 nM T4 exo$^-$ polymerase was added, upon which the rate of ATP hydrolysis by the 44/62 protein decreases to a limiting rate of 20 nM/s (Figure 5). Once this limiting ATPase rate was obtained, 1 μM duplex DNA devoid of biotin moieties (13/20-mer) was added, resulting in no substantial increase in the ATPase activity of the 44/62 protein. An excess of 45 protein (250 nM) was then added, resulting in a large increase in ATP hydrolysis, corresponding to a steady-state rate of 290 nM/s. The increase in ATP hydrolysis upon the addition of 45 protein indicates that the 44/62 protein does not remain part of the holoenzyme complex but instead acts catalytically to load the newly added 45 protein onto the primer/template devoid of biotin moieties. Since the 13/20-mer DNA substrate does not contain biotin moieties, the large rate enhancement in ATPase activity is consistent with the 44/62 protein continually loading the 45 protein onto the primer/template. If the 44/62 protein remained part of the stable holoenzyme complex formed on the biotin-labeled DNA substrate, there would not have been an increase in ATP hydrolysis upon the addition of 45 protein since the 44/62 would have been sequestered and unable to interact with the newly added 45 protein.

These results are consistent with the 44/62 protein acting catalytically to load the 45 protein onto the DNA primer/template in an ATP-dependent manner. Once the polymerase binds the 45 protein to form the stable holoenzyme complex, the 44/62 protein does not remain associated with the holoenzyme complex. These data confirm the work of Kaboord and Benkovic (1995), which demonstrates the catalytic nature of the 44/62 protein.

Scheme 1: Proposed Model for Holoenzyme Complex Formation[a]



[a] The 44/62 protein loads the 45 protein onto duplex DNA in an ATP-dependent process (step B). In this initial process, each of the four active sites of the 44/62 protein hydrolyzes ATP to open and/or close the ring-shaped 45 protein. Once loaded onto the DNA, the 45 protein or the 44/62:45 protein complex binds polymerase in an ATP-independent manner to form the stable holoenzyme complex (step C). Once the holoenzyme complex is formed, the 44/62 protein does not remain stably associated with the complex but can act catalytically to commence another loading cycle (step D).

Newport et al. (1980) proposed that stimulated ATP hydrolysis by the 44/62 protein in the presence of 45 protein could produce an activated holoenzyme complex required for processive DNA synthesis. Decay of this activated holoenzyme would terminate processive DNA synthesis by virtue of dissociation of the holoenzyme complex. Likewise, Selick et al. (1987) proposed a similar mechanism in which the ADP$-$P$_i$-bound high-energy state of the holoenzyme complex potentiates processive DNA synthesis. Again, the decay of this high-energy intermediate would result in dissociation of the holoenzyme and thus terminate DNA synthesis. The detailed kinetic analysis provided here demonstrates that the 44/62 protein acts catalytically to assemble the holoenzyme complex. By virtue of its catalytic nature, the 44/62 protein does not remain associated with the holoenzyme complex upon formation. Thus, the kinetic evidence provided here indicates that there is not an activated or high-energy species dependent upon ATP hydrolysis to potentiate the processive nature of the holoenzyme complex.

The steady-state data presented above lead to the proposed model for holoenzyme complex formation depicted in Scheme 1. In this postulated mechanism, the 44/62 protein loads the 45 protein onto the DNA substrate in an ATP-dependent process that most likely involves opening (and closing) the ring-shaped 45 protein (step B). Once loaded onto the primer/template, the 45 protein is essentially "locked" onto the primer/template by the binding of polymerase to form the stable holoenzyme complex (step C). The data presented in this report cannot conclusively rule out the existence of a 44/62:45 protein "preinitiation" complex that

precedes the binding of polymerase. However, the data clearly indicate that the 44/62 protein does not remain stably associated with the 45 protein once polymerase has bound to form the holoenzyme complex (step D).

Since the 44/62 protein does not remain associated with the holoenzyme complex, termination of processive DNA synthesis is therefore an ATP-independent process, and some other mechanism for the decay and dissociation of the holoenzyme complex must be invoked. Since the 45 protein is highly unstable on duplex DNA in the absence of polymerase as implied by the high steady-state ATP hydrolysis rates exhibited by the 44/62 protein, we postulate that dissociation of the holoenzyme either depends on the intrinsic nature of the polymerase to dissociate from the primer/template and/or the 45 protein or is due to the 45 protein's ability to dissociate from the primer/template and/or the polymerase. This process would require opening of the closed ring in order for the 45 protein to be released from the DNA for recycling. Re-formation of the holoenzyme complex would require the ATPase activity of the 44/62 protein to reload the 45 protein back onto the primer/template.

Pre-Steady-State ATP Hydrolysis by the 44/62 Protein. The steady-state data presented above yield important mechanistic information regarding the role of ATP hydrolysis in the formation of the stable holoenzyme complex by describing ATPase rates for the 44/62 protein under conditions of holoenzyme complex formation. However, this kinetic technique does not allow for the accurate determination of the absolute amount of ATP hydrolyzed by the 44/62 protein during holoenzyme complex formation nor does it allow for the determination of the rate for ATP hydrolysis during holoenzyme complex formation, which is approximately 2 s (Kaboord & Benkovic, 1993).

Pre-steady-state measurements of ATP hydrolysis by the 44/62 protein under conditions of holoenzyme complex formation, however, will yield this information. In general, rapid kinetic techniques allow the measurement of events at the active site and examination of individual steps of a complex reaction sequence. In a chemical quench-flow experiment, the time dependence of ATP hydrolysis can be measured by mixing 44/62 protein with ATP and stopping the reaction milliseconds later with the addition of acid. The addition of acid frees phosphate from the enzyme, and the time dependence of phosphate production might appear as a burst of P_i release preceding the steady-state accumulation of P_i. The amplitude of the burst generally provides a direct measurement of the concentration of E:ADP:P_i formed in the initial turnover while the rate of the approach to the steady state equals the first-order rate constant for ATP hydrolysis. Accordingly, pre-steady-state ATP hydrolysis rates for the 44/62 protein were determined under a variety of protein and DNA conditions using a rapid-quench instrument (Johnson, 1986) in order to determine the exact number of ATP molecules hydrolyzed per 44/62 molecule in the loading of the 45 protein for the formation of the holoenzyme complex as well as to measure the rate of ATP hydrolysis under these conditions.

For pre-steady-state ATPase measurements of the 44/62 protein under conditions of complex formation, stoichiometric quantities of accessory proteins, primer/template, and polymerase were used. Thus, the concentration of 44/62 was 250 nM as were the concentrations of 45 protein, Bio-34/62/36-mer, and T4 exo$^-$ polymerase (when present) unless otherwise noted. A low concentration of either [γ-^{32}P]ATP or [α-^{32}P]ATP (50 nM) was present to yield an accurate signal for ATP hydrolysis yet still remain sufficiently low to be considered insignificant to the total ATP pool of 1 mM. Rapid-quench assays were performed according to the protocol of Johnson (1986) in which MgATP and Mg[γ-^{32}P]ATP or Mg[α-^{32}P]ATP were placed in one syringe while the 44/62 protein in the absence and presence of the other replicative proteins and/or DNA substrate was placed in the other (Scheme 2). At various reaction times, the samples were quenched as previously described and analyzed for ATP hydrolysis. It is assumed that there is a negligible isotope effect from [γ-^{32}P]ATP used as the reporter molecule.

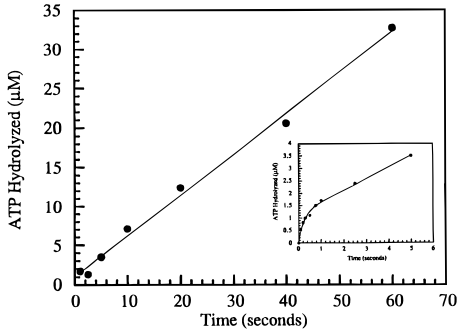
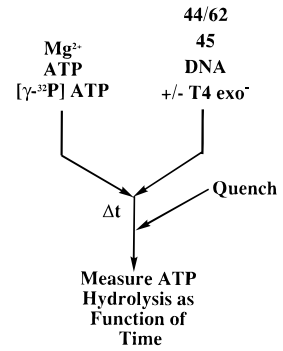


FIGURE 6: Pre-steady-state time course for ATP hydrolysis by 44/62 protein in the presence of stoichiometric 45 protein and Bio-34/62/36-mer with 1 μM streptavidin. The insert depicts the time course from 0 to 5 s to more accurately depict the burst amplitude and first-order rate constant.

Scheme 2: Representative Protocol for Pre-Steady-State ATPase Measurements of the 44/62 Protein under Conditions of Loading the 45 Protein onto DNA Substrates and for the Formation of the Holoenzyme Complex



Control reactions were performed in which ATP and [γ-^{32}P]ATP were reacted against a solution devoid of proteins. The amount of ATP hydrolysis due to rapid mixing in the rapid-quench instrument was negligible, and no burst in ATP hydrolysis was detected although a small steady-state rate of ATP hydrolysis was noted (data not shown). Representative time courses for ATP hydrolysis by the 44/62 protein in the presence of stoichiometric quantities of 45 and Bio-34/62/36-mer as well as in the presence of stoichiometric quantities of 45, Bio-34/62/36-mer, and T4 exo$^-$ polymerase are depicted in Figures 6 and 7. Data obtained using [γ-^{32}P]ATP or [α-^{32}P]ATP are identical.

The time course for ATP hydrolysis by 250 nM 44/62 protein in the absence of 45 protein, T4 exo$^-$ polymerase, and DNA substrate is linear and does not reveal a burst in ATP hydrolysis (data not shown). The rate of ATP hydrolysis is approximately 6 nM/s, consistent with the low steady-state rate of ATP hydrolysis previously determined (3 nM/s).

Table 3: Summary of Pre-Steady-State Data Obtained for the Hydrolysis of ATP by the 44/62 Protein[a]

condition	burst amplitude (μM ATP)	k_{ATPase} (s^{-1})	SS_{ATPase}[b] (nM/s)	SS_{ATPase}[c] (nM/s)
(1) 44/62 (250 nM)	nd[d]	nd	<6	3
(2) 44/62, 45 (250 nM), Bio-34/62/36-mer (250 nM)	1.1 ± 0.5	1.3 ± 0.2	300 ± 20	200 ± 10
(3) 44/62 (125 nM), 45 (250 nM), Bio-34/62/36-mer (250 nM)	0.5 ± 0.1	nd	80 ± 15	85 ± 8
(4) 44/62 (500 nM), 45 (250 nM), Bio-34/62/36-mer (250 nM)	1.1 ± 0.3	1.2 ± 0.2	200 ± 15	220 ± 20
(5) 44/62 and 45 (250 nM), T4 exo$^-$ (250 nM), 34/62/36-mer (250 nM)	1.6 ± 0.4	0.8 ± 0.2	200 ± 20	20 ± 3

[a] Assays for measuring the ATPase activity of the 44/62 protein contained varying concentrations of 44/62 protein at stoichiometric concentrations of 45 protein, primer/template, and T4 exo$^-$ polymerase (with 1 μM streptavidin), using the rapid quench procedure described in Materials and Methods. [b] Steady-state ATPase rates determined from the rapid-quench experiment. [c] Steady-state ATPase rates determined using the coupled assay system. [d] nd = not detected.

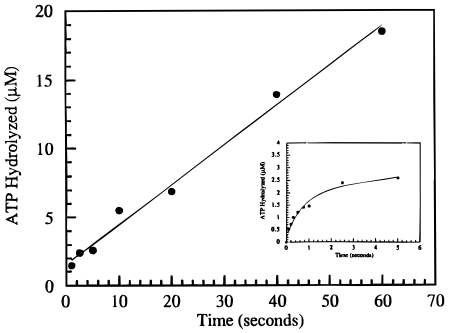


FIGURE 7: Pre-steady-state time course for ATP hydrolysis by the 44/62 protein in the presence of stoichiometric 45 protein, Bio-34/62/36-mer, and T4 exo$^-$ polymerase with 1 μM streptavidin. The insert depicts the time course from 0 to 5 s to more accurately depict the burst amplitude and first-order rate constant.

In the presence of 45 protein, Bio-34/62/36-mer, and streptavidin, the time course for ATP hydrolysis by the 44/62 protein shown in Figure 6 (and insert) is biphasic and represents an average of five determinations with proteins purified on different occasions. The burst amplitude of ATP hydrolysis in this case is equal to 1.1 ± 0.5 μM, approximately 4 times the concentration of 44/62 protein used in the assay. However, since the 44/62 protein contains four subunits of the ATPase domain (Jarvis et al., 1989a), the burst amplitude of ~1 μM is consistent with one turnover of each active site. The first-order rate constant of ATP hydrolysis under these conditions is 1.5 s^{-1}. The second phase in the time course (equivalent to the steady-state rate of ATP hydrolysis) is 300 nM/s, higher than but still consistent with the steady-state rate of 200 nM/s obtained using the coupled assay system.

To further evaluate the biphasic nature of ATP hydrolysis displayed by the 44/62 protein in loading the 45 protein onto duplex DNA, experiments were performed in which excess 44/62 protein (500 nM) in the presence of 250 nM 45 protein, 250 nM Bio-34/62/36-mer, and streptavidin was rapidly mixed versus ATP. The time course for ATP hydrolysis by the 44/62 protein is biphasic (data not shown), in which the burst amplitude of ATP hydrolysis in this case is equal to 1.1 ± 0.3 μM. The burst amplitude of ATP hydrolysis is less than the total active site concentration of the 44/62 protein but is instead limited by the amount of 44/62 protein that interacts with the 45 protein (250 nM). The first-order rate constant for the burst phase is equal to 1.2 s^{-1}. The steady-state rate of ATP hydrolysis is 200 nM/s, approximately equal to the rate obtained using stoichiometric quantities of 44/62 protein with respect to DNA and 45 protein.

Further experiments were performed in which substoichiometric 44/62 protein (125 nM) in the presence of 250 nM 45 protein, 250 nM Bio-34/62/36-mer, and streptavidin was rapidly mixed versus 1 mM ATP. The time course for ATP hydrolysis by the 44/62 protein is biphasic and represents an average of two determinations with proteins purified on different ocassions (data not shown). Extrapolation of the steady-state rate yields a burst amplitude of ATP hydrolysis in this case equal to 0.5 ± 0.1 μM, again approximately 4 times the active site concentration of 44/62 protein used. The steady-state rate of ATP hydrolysis is 80 nM/s, approximately one-half of the rate obtained using stoichiometric quantities of 44/62 protein with respect to DNA and 45 protein.

Together, these data implicate several mechanistic features of the interaction of the 44/62 protein with the 45 protein. Using stoichiometric quantities of 44/62 and 45 proteins, a burst in ATP hydrolysis equal to the active site concentration of the 44/62 protein is obtained. Likewise, when the concentration of 44/62 protein is half that of the 45 protein, a burst in ATP hydrolysis equal to the active site concentration of 44/62 protein is obtained. However, when the concentration of 44/62 protein is twice that of the 45 protein, a burst in ATP hydrolysis equal to that of 44/62 protein that can interact in a 1:1 complex with the 45 protein per loading event is obtained. Thus, the active site concentration of the 44/62 protein can accurately be measured by pre-steady-state kinetic analysis since, in all cases examined, stoichiometric amounts of ATP were hydrolyzed that were contingent upon the amount of 44/62:45:DNA complex formed.

In the presence of 45 protein, Bio-34/62/36-mer, streptavidin, and T4 exo$^-$ polymerase, the time course for ATP hydrolysis by the 44/62 protein shown in Figure 7 (and insert) is biphasic and represents an average of four determinations. The burst amplitude in this case is equal to 1.6 ± 0.4 μM, again consistent with one turnover of each active site of the 44/62 protein. The first-order rate constant of ATP hydrolysis under these conditions is 0.8 s^{-1}. The second phase in the time course (equivalent to the steady-state rate of ATP hydrolysis) is 200 nM/s, substantially higher than the steady-state rate previously obtained of 20 nM/s using the coupled assay system. The collective pre-steady-state data are summarized in Table 3.

Before the discrepancy in steady-state rates is addressed, a few pertinent conclusions can be drawn from direct comparison of the ATP hydrolysis time courses in the absence and presence of T4 exo$^-$ polymerase using stoichiometric quantities of 44/62 and 45 proteins and DNA substrate. First, the absolute amount of ATP hydrolyzed determined from the burst amplitude is within experimental error identical in both cases (~1 μM). Since the 44/62 protein contains four subunits of the ATPase domain, the burst amplitude of ~1 μM is consistent with one turnover

of each active site. The amount of ATP hydrolyzed also indicates that there is no ATP hydrolysis requirement for loading of the polymerase onto the 45 protein to form the holoenzyme complex and that all ATP requirements are for loading of the 45 protein onto the primer/template. The reason for hydrolysis of four ATP molecules by the 44/62 protein to load the 45 protein onto the primer/template is unclear at the present time. Most likely, the free energy provided by ATP hydrolysis is required for the 45 protein to productively encircle the DNA, although the mechanism of this energy transduction from the 44/62 protein complex to the 45 protein is presently unknown. It appears that the free energy from the hydrolysis of all four ATP molecules is required for the 45 protein to be clamped onto the DNA substrate, since opening and/or closing the 45 protein to encircle the DNA substrate may be a thermodynamically unfavorable process. Alternatively, the loading of 45 protein by the 44/62 protein may be highly inefficient with regard to ATP hydrolysis such that the energy of only one or two ATP hydrolysis events is coupled to productive loading of the 45 protein, and the other ATP hydrolysis events are wasted energy. Differentiation of these different mechanisms is currently under investigation.

The second piece of information gained from the pre-steady-state measurements is that the first-order rate constant for ATP hydrolysis for loading the 45 protein and holoenzyme complex formation is approximately 1 s^{-1} (1.5 s^{-1} in the absence of polymerase and 0.8 s^{-1} in the presence of polymerase). Kaboord and Benkovic (1995) have reported that the first-order rate constant for complex formation is 0.97 s^{-1}. Although the rate constants for complex formation and ATPase activity of 44/62 protein under conditions of complex formation were determined under slightly different conditions (DNA and accessory proteins were in 5-fold excess of polymerase for complex formation while DNA, accessory proteins, and polymerase were stoichiometric in the ATPase assay), the first-order rate constants are nearly identical, indicating that ATP hydrolysis is the rate-limiting step in holoenzyme complex formation. Had the rate of ATP hydrolysis been significantly faster than 1 s^{-1}, some other step after hydrolysis of ATP by the 44/62 protein would then be rate limiting in holoenzyme complex formation. The converse situation in which ATP hydrolysis would be slower than complex formation would have been nearly impossible to explain since ATP hydrolysis is absolutely required for productive holoenzyme formation (Mace & Alberts, 1984b).

The discrepancy in the steady-state rate obtained during complex formation using the coupled assay (20 nM/s) as opposed to the radioactive assay (200 nM/s) can be explained by the fact that the rate obtained using the radioactive assay over this limited time domain has not yet achieved the actual steady-state rate of ATP hydrolysis. Close inspection of the time course presented in Figure 3 also reveals the transitory nature of the decrease in ATPase activity. This transition arises from impeding the binding of residual polymerase to DNA-bound 45 protein due to the ratio of polymerase to accessory proteins and DNA substrate (1:1) used in these experiments. As a result, a small portion of 45 protein that is not in complex with the polymerase will be free to dissociate and be reloaded onto DNA, resulting in continued ATP hydrolysis. This process will continue until all 45 protein is in the holoenzyme complex, upon which the ATPase rate will be 20 nM/s (Berdis and Benkovic, unpublished observation).

It is important to note that, under these experimental conditions, at least 50% of the holoenzyme complex must be formed within 2 s since the steady-state rate of ATP hydrolysis is decreased by a factor of 2 (430 versus 200 nM/s). The comparable burst amplitudes for ATP hydrolysis by the 44/62 protein measured in the presence and absence of polymerase still indicates that additional ATP hydrolyis is not required for loading of the polymerase to form the holoenzyme complex.

Holoenzyme Complex Formation as a Function of ATP Concentration. The T4 holoenzyme complex can be assembled stoichiometrically on the forked primer/template, Bio-34/62/36-mer, in the presence of streptavidin (Kaboord & Benkovic, 1995). Processive DNA synthesis by the holoenzyme complex results in strand displacement of the 36-mer forked strand to produce fully elongated products (a mixture of 61- and 62-mers). Since the polymerase alone cannot perform strand displacement synthesis under the reaction times employed in this study, smaller products (<44-mer) are generated instead of fully elongated products.

The assembly of the holoenzyme complex was performed as a function of ATP concentration in which a solution of Bio-34/62/36 (500 nM) and streptavidin (1 μM) was incubated with 44/62 and 45 proteins (550 nM each) in the presence of varying concentrations of ATP (1, 10, 100, and 1000 μM) for 10 s. This time frame allows for each DNA molecule to be loaded with a 45 protein. T4 exo$^-$ polymerase (100 nM) was then added as well as 10 μM dCTP (the first nucleotide to be incorporated). After approximately 5 s, the remaining dNTPs (10 μM each) and single-stranded DNA (to trap free polymerase) were added to initiate DNA synthesis by any assembled holoenzyme complexes. The reaction was manually quenched at variable times by the addition of 1 N HCl.

Quantitation of the amount of 62-mer produced indicates that holoenzyme complex is formed under all concentrations of ATP except when the concentration of ATP is 1 μM (Figure 8). Furthermore, the amount of holoenzyme complex formed is the same whether 10 μM, 100 μM, or 1 mM ATP is used. Thus, a minimal amount of ATP is required for productive holoenzyme complex formation, far below the apparent Michaelis constant for ATP determined using the steady-state ATPase assays, suggesting that the K_d of ATP is at least 10-fold lower than the apparent steady-state Michaelis constant. The fact that no holoenzyme complex is formed using a concentration of 1 μM ATP appears to conflict with pre-steady-state ATPase measurements that indicate a concentration of 1 μM might be sufficient for holoenzyme complex formation. There are several possibilities to rationalize this apparent discrepancy. It is possible that the K_d for ATP of the 44/62 protein is higher than 1 μM so that the amount of ATP is not sufficient to form the holoenzyme complex. It is most likely, however, that the recycling of the 45 protein to and from the primer/template during the 10 s time frame used for the replicative assay is sufficient to deplete the ATP concentration of 1 μM before the addition of polymerase so there is no holoenzyme complex formed. Furthermore, 1 turnover of the 44/62 protein is required to load a 45 protein onto each DNA substrate. This will require a concentration of at least 2.2 μM ATP to load all the 45 protein onto the primer/template (4 ATPs $\times$ 550 nM 45 protein = 2.2 μM per loading event). Since ATP is limiting at 1 μM, only 45% of the 45 protein can theoretically be loaded onto the DNA to form the

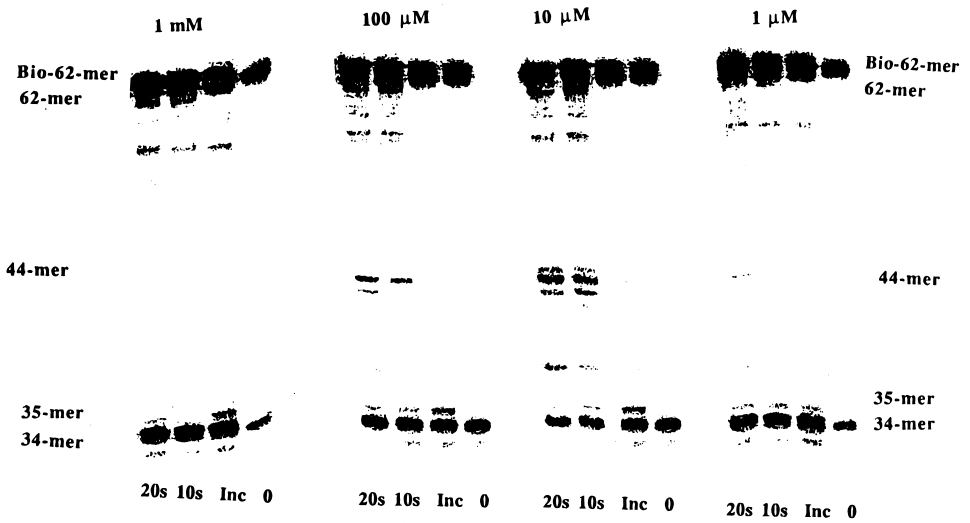


FIGURE 8: Holoenzyme formation and processive DNA synthesis measurements as a function of ATP concentration. Assays were performed as described in Materials and Methods.

holoenzyme. Thus, a combination of these two scenarios likely results in the inability to form productive holoenzyme complex at 1 μM ATP. In other cases in which there is at least 10 μM ATP present, any 45 protein that has dissociated can be recycled since there is an excess of ATP relative to the active site concentration of 44/62 protein (10 μM ATP versus 2.2 μM active site protein). It is unlikely that ATP hydrolysis is required for processive strand displacement DNA synthesis.

Lagging Strand Synthesis. Lagging strand DNA synthesis *in vivo* occurs in a discontinuous manner, in which a short RNA primer is elongated by the DNA polymerase. The average bacteriophage T4 Okazaki fragments are estimated to be about 2 kb (Nossal & Alberts, 1983), implying that a highly processive holoenzyme is required to fully extend the small RNA primer. The ATPase activity of the 44/62 protein in lagging strand synthesis was examined in detail, specifically to examine whether the 45 protein could be loaded onto an RNA primer as well as to determine if a stable holoenzyme complex could be formed at an RNA primer.

Experiments were performed to examine the rate of ATP hydrolysis by the 44/62 protein in loading the 45 protein onto a 20/40-mer RNA/DNA primer/template which contained a biotin label at the 3′ end as well as at the 5′-penultimate position of the template (Figure 1). In the presence of 250 nM 44/62 and 45 protein, 250 nM 20/40-mer Bio-RNA/DNA in the absence of streptavidin, and 1 mM ATP, the steady-state rate of ATP hydrolysis is 890 nM/s. The steady-state ATPase rate under identical conditions but in the presence of 1 μM streptavidin is 350 nM/s using the Bio-RNA/DNA substrate. The approximately 2-fold decrease in the steady-state ATP hydrolysis rate is again consistent with data previously presented, suggesting that the physical blocks hinder the 45 protein or the 44/62: 45 protein complex from rolling off the end of the duplex.

For direct comparison, the rate of ATP hydrolysis was measured under identical conditions except a Bio-20/40-mer DNA/DNA substrate was used. The steady-state ATPase rate in the absence of streptavidin is 850 nM/s, while in the presence of 1 μM streptavidin, the steady-state rate decreases by a factor of 2 to a value of 470 nM/s. Since the rates of ATP hydrolysis by the 44/62 protein in loading the 45 protein onto either RNA/DNA or DNA/DNA substrates are nearly identical, it is reasonable that the 45 protein is loaded effectively using either type of substrate. Thus, ATPase measurements are consistent with a role for the accessory proteins in lagging strand DNA synthesis since it appears that the 45 protein can be loaded onto an RNA primer as well as possessing the ability to translocate across the RNA/DNA substrate. ATPase rates are summarized in Table 4.

Table 4: Summary of ATPase Rates for Leading and Lagging Strand Holoenzyme Complex Formation[a]

condition	RNA (nM/s)	DNA (nM/s)
Bio-20/40-mer		
without streptavidin	890 ± 50	850 ± 40
with 1 μM streptavidin	350 ± 40	470 ± 30
Bio-33/62/36-mer		
without streptavidin	300 ± 50	400 ± 30
with 1 μM streptavidin	170 ± 20	200 ± 20
with 1 μM streptavidin and T4 exo$^-$ polymerase[b]	50 ± 5	20 ± 3

[a] Assays for measuring the ATPase activity of the 44/62 protein contained stoichiometric concentrations of 44/62 protein, 45 protein, primer/template, and T4 exo$^-$ polymerase, using 1 mM ATP, 10 mM $Mg(OAc)_2$, and the coupled assay reagents in a buffer consisting of 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol. [b] 250 nM T4 exo$^-$ polymerase added last.

Steady-state ATPase measurements were then performed using a primer/template designed to mimic the priming of an Okazaki fragment (Figure 1, Bio-33/62/36-mer). This oligonucleotide contained a hybrid oligo primer strand consisting of 28 deoxyribonucleotides attached to 5 ribonucleotides at the 3′ end. The template strand was a biotinylated 62-mer, and the forked DNA strand was a 36-mer. An analogous primer/template consisting of all DNA substrates was constructed and used as a control.

The rate of ATP hydrolysis by the 44/62 protein in the presence of stoichiometric 45 protein and Bio-33/62/36-mer RNA/DNA in the absence of streptavidin is 300 nM/s, while in the presence of 1 μM streptavidin, a rate of 170 nM/s is obtained. Control experiments using the DNA/DNA Bio-33/62/36-mer under identical initial velocity conditions and protein concentrations yield steady-state ATP hydrolysis rates of 400 nM/s in the absence of streptavidin and 200 nM/s in the presence of 1 μM streptavidin.

To determine if the holoenzyme could form on the RNA primer, ATPase measurements were obtained using stoichiometric quantities of accessory proteins, RNA/DNA substrate in the presence of streptavidin, and T4 exo$^-$ polymerase (1:

1:1:1) in which the polymerase was added last. The rate of ATP hydrolysis prior to the addition of polymerase was 170 nM/s, and this rate eventually decreased to a limiting value of 50 nM/s upon the addition of polymerase. The decrease in ATP hydrolysis is suggestive of formation of a stable holoenzyme complex that once produced does not require ATP hydrolysis. Control experiments using the DNA/DNA primer/template were performed under identical reaction conditions. The rate of ATP hydrolysis prior to the addition of polymerase was 180 nM/s, and this rate eventually decreased to a limiting value of 20 nM/s upon the addition of polymerase. Data are summarized in Table 4. In general, the steady-state data obtained using RNA/DNA substrates are identical to data obtained using DNA/DNA substrates and thus argue for a role of the accessory proteins in the formation of a stable holoenzyme complex that is most likely required for lagging strand DNA synthesis in a manner identical to that for leading strand DNA synthesis.

Attempts were made to examine the ATP requirements for lagging strand DNA synthesis. However, extension of the RNA primer by the T4 polymerase alone is very slow, in which at least several minutes are required before a single nucleotide is incorporated (data not shown). The accessory proteins do not appear to increase the rate of RNA primer extension. Thus, although the above ATPase measurements are consistent with formation of the stable holoenzyme complex, the lack of primer extension appears to be a contradiction. It is important to note that rapid nucleotide incorporation from an RNA primer in the bacteriopage T4 system has not been reported. It is highly possible that other proteins involved in replication including single-stranded binding protein (32 protein) and/or the helicase/primase complex (61 and 41 proteins) may also be essential for productive and efficient lagging strand synthesis. In any case, it does appear that the 44/62 and 45 proteins play an intricate role in the formation of a holoenzyme complex that must be required for processive lagging strand synthesis.

Comparison to Other Replication Systems. The replicative polymerase of *E. coli*, DNA polymerase III holoenzyme, is composed of 10 nonidentical subunits (Maki & Kornberg, 1988). The β subunit is the sliding clamp that holds the core polymerase to the primer/template for processive DNA synthesis and is analogous to eukaryotic PCNA and the 45 protein in the T4 replication system [reviewed by O'Donnell (1992)]. The β subunit does not clamp onto the DNA by itself but requires the γ complex to load it onto DNA. The γ complex, composed of five different subunits ($\gamma\delta\delta'\chi\psi$), is the molecular matchmaker that loads the β subunit onto DNA in an ATP-dependent manner analogous to the 44/62 protein complex. The γ subunit binds ATP and assists the δ subunit in loading the β clamp onto DNA while the δ' subunit stimulates the rate of ATP hydrolysis by the γ subunit. The χ and ψ subunits are essential for loading of the β subunit under physiological conditions although their exact role in the loading process is unknown. Once activated in the presence of ATP, Pol III holoenzyme forms a stable initiation complex with primed circular DNA that is isolatable by gel filtration and is capable of highly processive DNA synthesis (Burgers & Kornberg, 1982a). The half-life of the holoenzyme complex on circular DNA is greater than 15 min (O'Donnell & Kornberg, 1985), longer than the lifetime of the T4 holoenzyme complex using the biotinylated DNA substrate in the presence of streptavidin. However, upon completion of DNA synthesis, the Pol III holoenzyme complex is able to dissociate in less than 1 s, in which only polymerase dissociates, leaving the β subunit behind (O'Donnell & Kornberg, 1985). The β subunit by itself, similar to the 45 protein, is able to move bidirectionally on DNA and will also "roll" off linear DNA (Stukenberg et al., 1991).

The ATPase activity of the γ complex in the presence of primed DNA substrate is stimulated approximately 3-fold by the addition of the β subunit while the Michaelis constant for ATP increases approximately 2-fold under identical conditions (Onrust et al., 1991). The number of ATP molecules bound per active holoenzyme complex has been reported to be between two and three, suggesting that two ATP are hydrolyzed per complex formation (Burgers & Kornberg, 1982b). This turnover by the γ complex was within 3 s, a value that corresponds well with the rate of holoenzyme complex formation of less than 1 s (Burgers & Kornberg, 1982b).

While ATP hydrolysis is intimately involved in formation of the stable Pol III holoenzyme complex, the binding of ATP may also play a key role in the subsequent dissociation of the holoenzyme complex. The dissociation rate of isolated holoenzyme is increased in the presence of nonhydrolyzable analogs of ATP such as ATPγS and AMP-PNP, suggesting that the binding of nucleotide and not hydrolysis is a signal for dissociation of the holoenzyme complex (Burgers & Kornberg, 1983).

RF-C hydrolyzes ATP in an analogous manner to the 44/62 protein. The K_m value for ATP of RF-C is approximately 15 μM (Yoder & Burgers, 1991), substantially lower than that obtained for the 44/62 protein. While the ATPase activity of RF-C is activated 2-fold in the presence of PCNA and primed DNA substrate, the K_m for ATP is unchanged in the presence of DNA substrate and/or PCNA, similar to results obtained for the 44/62 protein in the T4 replication system. PCNA can only be efficiently loaded onto circular DNA by the ATP-dependent action of RF-C (Burgers & Yoder, 1993), and once loaded onto circular DNA, PCNA forms a stable and long-lived complex with the DNA with a half-life of 22 min at 37 °C (Podust et al., 1995). Similar to the Pol III and T4 systems, PCNA is able to move bidirectionally and roll off linear DNA (Podust et al., 1994). During DNA synthesis, the dissociation of Pol δ from the holoenzyme complex occurs at replicative pause sites while the PCNA clamp remains tightly bound (Podust et al., 1995), suggesting that the holoenzyme translocates across the DNA substrate by a distributive process in which Pol δ is recycled on the stable PCNA clamp. Although the off-rate for the Pol δ holoenzyme complex has not been directly measured, it can be implied from the above studies that the rate of dissociation of the holoenzyme complex is dictated by the release of the polymerase and not by PCNA. Thus, PCNA can remain bound to the DNA substrate similar to the β subunit but in direct contrast to the 45 protein. It is highly possible that ATP binding is required for the dissociation of PCNA in a manner similar to that indicated by the β subunit.

Common mechanistic themes are obvious when comparing all three replication systems. First, loading of the sliding clamp is an ATP-dependent process. Kinetic parameters for each clamp loader are similar in that the Michaelis constant for ATP for each protein is in the micromolar range. The ATPase activity of each clamp loader is also substantially increased upon the addition of DNA substrate and sliding clamp, although the T4 clamp loader appears to undergo a

higher degree of activation. Once loaded onto the DNA substrate, the sliding clamp can move in a birectional manner to locate a free 3′-OH to which a polymerase can bind to form a highly stable holoenzyme complex. The half-lives of the holoenzyme complexes from all three systems are on the order of several minutes. However, both PCNA and the β subunit are highly stable on duplex DNA while it appears that the T4 sliding clamp is not. Decomposition of the holoenzyme complex involves dissociation of polymerase from either PCNA or the β subunit while the T4 system likely involves dissociation of both polymerase and sliding clamp simultaneously (Kaboord and Benkovic, unpublished results). This suggests a possible inherent difference in recycling the sliding clamp that will impact coordination of leading and lagging strand DNA synthesis. It appears that the β subunit and PCNA remain bound to the DNA substrate in the absence of polymerase and require ATP binding to signal dissociation for movement to another primer/template. In contrast, the sliding clamp of the T4 system appears to be highly unstable on DNA in the absence of polymerase such that a signal is not required for dissociation and subsequent reinitiation of processive DNA synthesis. It is unclear at the present time how the energy of ATP hydrolysis is transduced from the clamp loader to load the sliding clamp onto DNA.

Summary. The ATPase activity of the 44/62 protein in the presence of 45 protein and DNA is synergistically activated. The rate of ATP hydrolysis by 44/62 protein in the presence of 45 protein, primer/template, and T4 exo$^-$ polymerase decreases until a stoichiometric complex of accessory proteins, primer/template, and polymerase is obtained. The 44/62 protein does not remain part of the holoenzyme complex but can instead act catalytically to assemble the holoenzyme complex. The 44/62 protein turns over once to either load the 45 protein onto the primer/template or to assemble the holoenzyme complex, indicating that ATP hydrolysis is not required for loading of the polymerase to the 45 protein on a DNA substrate. Furthermore, ATP hydrolysis by the 44/62 protein is the rate-limiting step in holoenzyme complex formation. Processive DNA synthesis assays reveal that a minimal amount of ATP is required for holoenzyme complex formation (4 $\times$ [45 protein]). Both the steady-state and pre-steady-state ATP hydrolysis data presented are consistent with a mechanism of holoenzyme complex formation in which the 44/62 protein loads the 45 protein onto the DNA substrate in an ATP-dependent manner (Scheme 1). This process appears to be identical for both leading and lagging strand synthesis. Polymerase can then bind to the 45 protein in an ATP-independent manner to form the stable holoenzyme complex. Comparison to other replication systems suggests that the role of ATP hydrolysis by the clamp loader accessory protein is ubiquitous for loading of the sliding clamp onto the DNA primer/template and is not required for maintaining the integrity of the stable holoenzyme complex.

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