

The Kinetic Mechanism of Formation of the Bacteriophage T4 DNA Polymerase Sliding Clamp

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DNA replication in bacteriophage T4 requires the assembly of a structure called the “sliding clamp” near the 3' end of the DNA strand that is to be extended. This structure is a trimer ring of the T4 gene 45 product (gp45) and serves to regulate the processivity of the DNA polymerase within the T4 DNA replication system. The placement of this ring is performed by an ATPase complex of the products of T4 genes 44 and 62 (gp44/62) that consists of four gp44 subunits and one gp62 subunit. In an effort to understand the role of ATP hydrolysis in processes occurring during the formation of the phage T4 DNA sliding clamp, we have performed direct substrate and product binding experiments and steady-state and presteady kinetic experiments on the gp44/62-gp45 system. Substrate (ATP) and product (ADP) binding studies show that the gp44/62 complex binds $4(\pm 1)$ ATP molecules with a K_d of $34(\pm 12)$ μM , and $3.7(\pm 0.3)$ ADP molecules with a K_d of $14(\pm 7)$ μM . The binding of the other reaction product (inorganic orthophosphate) could not be detected. Presteady-state kinetic analysis of ATP hydrolysis during the sliding-clamp-loading process indicates a biphasic progress curve, consisting of an initial rapid “burst” phase with an amplitude of four ATP molecules per gp44/62 complex and a rate of 15 s^{-1} , followed by a second slower phase corresponding to the steady-state rate of ATP hydrolysis by this complex. The rate of the burst phase is kinetically consistent with the previously observed rate of T4 DNA polymerase holoenzyme formation. The burst amplitude depends solely on the concentration of gp44/62 ATP binding sites present. These results suggest that the formation of a single T4 sliding clamp requires the hydrolysis of four ATP molecules by one gp44/62 complex in a process requiring 0.5 to 1 second. A model describing the clamp-loading process is discussed in the context of these results.

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Introduction

A crucial quality for the physiological function of a DNA polymerase is processivity, which deter-

mines the extent to which the polymerase resists dissociation from the DNA template it is engaged in copying (Fairfield *et al.*, 1983; Nossal & Alberts, 1983; Nossal, 1992; Young *et al.*, 1992; von Hippel *et al.*, 1994). Highly processive DNA synthesis allows the rapid and efficient replication of large genomes. In the bacteriophage T4 replication complex, this quality is imparted to DNA polymerase by the action of two protein complexes. The first appears to form a proteinaceous ring encircling the DNA (Gogol *et al.*, 1992), and it has been shown to be a trimeric complex of the product of T4 gene 45 (gp45; Sanders *et al.*, 1994). This ring forms a complex with DNA polymerase, thereby preventing dissociation of the polymerase from the DNA, yet allowing longitudinal movement of the

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Abbreviations used: gp44/62, the complex of the products of T4 genes 44 and 62 in the ratio 4:1; gp45, the trimer of the product of T4 gene 45; TNP-ATP, 2'-(or 3')-O-(trinitrophenyl)adenosine-5'-triphosphate; KP_i, potassium phosphate; KOAc, potassium acetate; P_i, inorganic orthophosphate; PEI-F, polyethyleneimine, with fluorescent indicator; TEAB, triethylammonium bicarbonate; ssDNA, single-stranded DNA; TLC, thin-layer chromatography.

ring-polymerase complex (Reddy *et al.*, 1993; Kaboord & Benkovic, 1993). Because of these characteristics, this processivity control structure has long been termed a "sliding clamp" (Huang *et al.*, 1981).

The sliding clamp is capable of assembling with T4 polymerase on DNA under macromolecular crowding conditions (Reddy *et al.*, 1993), but under normal *in vitro* conditions such spontaneous assembly does not occur. Rather this is usually accomplished by the second of the processivity protein complexes, which contains four gp44 subunits and one gp62 subunit (gp44/62; Jarvis *et al.*, 1989b). The gp44/62 complex appears to function as a sliding-clamp-loading ATPase enzyme (Kaboord & Benkovic, 1993, 1995, 1996). The hydrolysis of ATP has long been known to be required for the clamp-loading process (Piperno & Alberts, 1978), but the detailed mechanism whereby this hydrolysis is coupled to the loading of the sliding clamp onto the DNA and the polymerase is not understood.

The T4 DNA replication system is archetypal in the sense that this mode of attaining processive synthesis is conserved in both bacterial and eukaryotic replication systems (O'Donnell, 1987, 1992; Brush *et al.*, 1995). The clamp cognates of the gp45 trimer in *Escherichia coli* and *Saccharomyces cerevisiae* are the β subunit dimer of DNA polymerase III and the PCNA trimer, respectively. Analogs of the gp44/62 complex also exist as the γ complex of *E. coli*, and the RF-C complex in *S. cerevisiae*. The crystal structures of both of the other clamp proteins have been determined (Kong *et al.*, 1992; Krishna *et al.*, 1994). The overall structure of these clamps is that of a ring, which presumably encircles DNA in a manner similar to that of the T4 clamp (Gogol *et al.*, 1992).

Since the structure of gp45 is that of a ring of three subunits, this poses the intriguing question of how a topologically closed ring of gp45 subunits can be induced to encircle DNA. A mental image of the reaction almost obligatorily includes conformational distortion ("opening" or partial disassembly) of the protein ring, and suggests that the role of gp44/62 and ATP hydrolysis is to produce the required ring distortion. Indeed, previous results indicate that relatively large changes in protein conformation do occur during clamp loading (Hockensmith *et al.*, 1987, 1993; Capson *et al.*, 1991). Any ultimate picture of the mechanism of clamp formation must include a dynamic description of both the protein structural changes involved and the chemical state of ATP during the various stages of the clamp-loading process. We are pursuing both avenues of study at the present time. Experiments designed to examine structural changes in the loading process are presented in the companion paper (Latham *et al.*, 1996).

Here we discuss experiments that have been undertaken to elucidate the kinetic mechanism of ATP hydrolysis by the clamp-loading complex, gp44/62. We present binding experiments that

determine the number of ATP molecules bound by the gp44/62 complex. This result, in combination with presteady-state kinetic experiments that determine the number of these bound ATP molecules that are used during the clamp-loading process, together with ADP-ATP exchange studies and binding experiments with ADP, illuminate the core mechanistic events that occur in the clamp-loading process. Finally, steady-state kinetic studies are used to shed light on an additional aspect of ATP hydrolysis by gp44/62.

Results

Binding of ATP to gp44/62

The gp44/62 complex contains four gp44 subunits, each potentially capable of binding ATP (Jarvis *et al.*, 1989a; Rush *et al.*, 1989). Direct binding studies of the ATP substrate (and the ADP and inorganic orthophosphate (P_i) products) to gp44/62 have not previously been performed. We have undertaken such studies in order to determine both the stoichiometry and the strengths of binding of these components. Figure 1A shows the combined results of two typical [γ - ^{32}P]ATP binding experiments, using 5 μ M gp44/62 complexes. The high concentration of gp44/62 used in these experiments necessitated the use of a large volume of gp44/62 stock solution for each binding point. The gp44/62 storage buffer contained 50 mM potassium phosphate (KP_i). Since it was conceivable that P_i , a product of the gp44/62 ATPase reaction, could have an effect on the binding of ATP, the gp44/62 stock solution was dialyzed against storage buffer minus P_i for these experiments. The data may be fitted to a simple binding curve (defined by equation (2), see Experimental Procedures) with a plateau value ($n\epsilon$) of 21(\pm 1) μ M (4(\pm 0.2) ATP molecules per gp44/62 complex), and a K_d of 38(\pm 8) μ M. This indicates that there are four identical binding sites for ATP on the gp44/62 complex.

To determine whether P_i affected the binding of ATP to gp44/62, binding experiments with 23 mM P_i present were also carried out. Figure 1B presents the combined ATP binding data of five experiments performed in the presence of P_i . The data were again fitted to equation (2), with a plateau value of 17(\pm 2) μ M (3.4(\pm 0.4) ATP molecules per gp44/62 complex) and a K_d of 30(\pm 15) μ M. No differences, beyond experimental error, were observed in the measured dissociation constants and stoichiometry of ATP binding in the presence and absence of P_i . Since P_i is a product of the ATPase reaction and would be expected to interfere with ATP binding if present at the active site, these data indicate that the dissociation constant of P_i is >23 mM. In support of this, efforts to use these titration procedures to detect direct binding of $^{32}P_i$ to gp44/62 were completely unsuccessful. However, the sensitivity of this technique limits useful P_i concentrations to the submillimolar range.

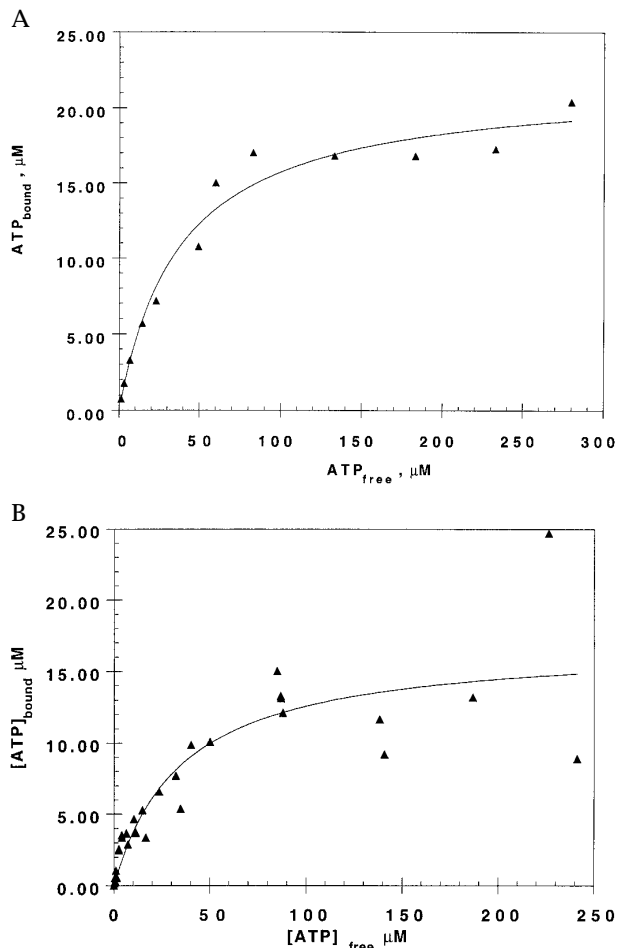


Figure 1. A, Binding of ATP to gp44/62 in the absence of P_i . Titrations were performed in 50 mM Hepes (pH 7.5), 2.5 mM $MgSO_4$, 0.2 mM DTT, 23% (v/v) glycerol, using the Centricon binding technique of Stitt (1988). The data were combined with two separate experiments and fitted to equation (2), with $n\epsilon = 21(\pm 1)$ μM and $K_d = 38(\pm 8)$ μM . B, Binding of ATP to gp44/62 in the presence of 23 mM P_i . Titrations were performed in 50 mM Hepes (pH 7.5), 2.5 mM $MgSO_4$, 0.2 mM DTT, 23% glycerol, 23 mM KP_i . The data were fitted to equation (2), with $n\epsilon = 14(\pm 2)$ μM and $K_d = 22(\pm 6)$ μM .

All of the above binding experiments were carried out in the presence of 2.3 mM Mg^{2+} . Since the hydrolysis of ATP by isolated gp44/62 is very slow ($k_{cat} < 0.5 \text{ min}^{-1}$ at room temperature, see below), and each point on the binding curve was completed within two minutes, very little of the starting ATP was converted to ADP during the experiment. Given that the binding constant of ADP is comparable to that of ATP (see the next section), we conclude that the curves in Figures 1 and 2 satisfactorily reflect the binding of ATP to gp44/62.

Finally, a study of the effect of ATP on the intrinsic fluorescence of gp44/62 (data not shown) was also performed to provide a different measure of ATP binding. A slight (ca 5% at plateau) quenching of gp44/62 fluorescence by ATP was observed, and a dissociation constant of

$20(\pm 10)$ μM was measured using this technique. This result is consistent with the ATP binding constant measured by Centricon filtration (see above).

Binding of ADP to gp44/62

Figure 2 shows the binding of 5 μM gp44/62 to [α - ^{32}P]ADP in the presence and absence of P_i . Both sets of data are adequately fitted by binding equation (2), with a plateau value of $21(\pm 0.7)$ μM ($4(\pm 1)$ ADP molecules per gp44/62 complex) and a K_d of $16(\pm 7)$ μM . It is interesting that the product of the ATPase reaction, ADP, binds gp44/62 with an affinity that is comparable to, and perhaps slightly greater than, that of ATP. The presence of P_i had no discernible effect on the binding of ADP to gp44/62, again indicating a very high K_d for this product of the ATPase reaction.

Stoichiometry of 2'-(or 3')-O-(trinitrophenyl)ATP (TNP-ATP) binding to gp44/62

A further independent measure of the stoichiometry of ATP binding to gp44/62 was sought. The fluorescent ATP analog TNP-ATP has been used in other systems to study ATP-enzyme interaction (Hiratsuka, 1976; Moczydlowski & Fortes, 1981a,b). The fluorescence of the attached trinitrophenyl group is very sensitive to the surrounding solvent environment, being severely quenched in water and less quenched in organic solvent. Thus this compound usually shows a fluorescence enhancement in going from bulk solution to an enzyme ATP-binding pocket (Hiratsuka, 1976). Fluorescence emission spectra of 1 μM TNP-ATP in the presence and absence of 1.1 μM gp44/62 are presented in Figure 3A. This result shows that the expected change in fluorescence intensity also applies to the gp44/62 system, in that TNP-ATP shows a \gg fivefold fluorescence enhancement upon binding to the gp44/62 complex (Figure 3A, curve b; curve a represents the baseline fluorescence spectrum of the probe free in solution).

A fluorescence titration in which TNP-ATP is added to a fixed (1 μM) concentration of gp44/62 is shown in Figure 3B. The stoichiometry of binding of this ATP analog to gp44/62 may be estimated by the intersection of the asymptote to the binding curve at low TNP-ATP concentrations and the asymptote to the plateau. It is apparent from Figure 3B that the intersection point is approximately 2 μM , which corresponds to two molecules of TNP-ATP bound per gp44/62 complex.

Steady-state kinetics of gp44/62

The steady-state kinetic parameters of the gp44/62-gp45 system have been presented elsewhere (Jarvis *et al.*, 1989b; Berdis & Benkovic, 1996). Nonetheless, in preparation for the presteady-state kinetic studies, some steady-state experiments were performed here as well. All experiments were

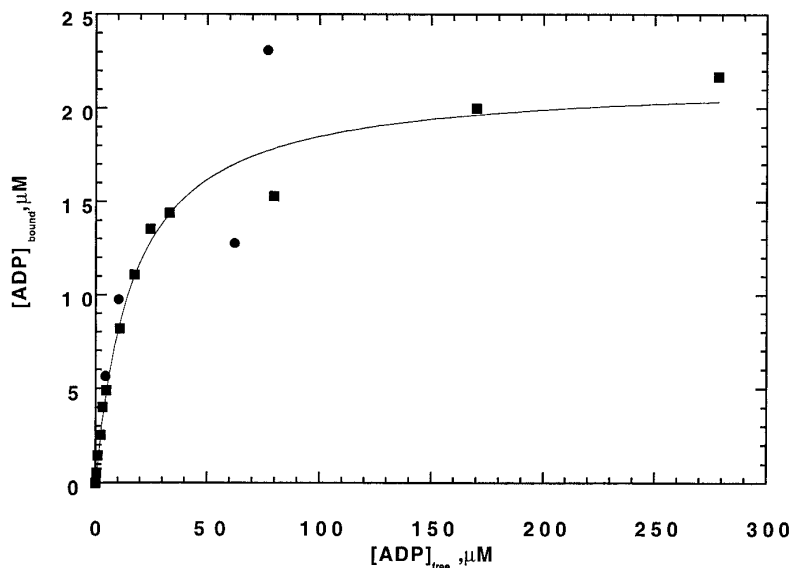


Figure 2. Binding of ADP to gp44/62. Titrations were performed in (■) 50 mM Hepes (pH 7.5), 2.5 mM MgSO₄, 0.2 mM DTT, 23% glycerol, and (●) the same buffer plus 23 mM KPi. The combined data were fitted to equation (2), with $n\epsilon = 21(\pm 1) \mu\text{M}$ and $K_d = 17(\pm 2) \mu\text{M}$.

performed assuming that the K_m for the DNA cofactor (in units of molecules of template-primer DNA) was $<100 \text{ nM}$ (Jarvis *et al.*, 1989b), and were run at a DNA concentration of $1 \mu\text{M}$. A K_m for ATP of ca $200 \mu\text{M}$ was also assumed for all reactions (Jarvis *et al.*, 1989b; Berdis & Benkovic, 1996).

Figure 4A shows representative time courses for the hydrolysis of ATP by gp44/62 alone, by gp44/62 + gp45, by gp44/62 + DNA, and by gp44/62 + gp45 + DNA (the complete system), all at initial ATP concentrations of $100 \mu\text{M}$. As expected, gp44/62 in isolation has the lowest ATPase activity of the set. The data can be fitted adequately to a single exponential function ($[P_i] = [ATP]_0(1 - e^{-kt})$), yielding a basal rate of $0.36 \mu\text{M}/\text{minute}$ ($0.006 \mu\text{M}/\text{second}$) at this ATP concentration. The rate of ATP hydrolysis by gp44/62 + gp45 is 30-fold faster than that catalyzed by gp44/62 alone, corresponding to a rate of $10 \mu\text{M}/\text{minute}$. This gp44/62 + gp45 ATPase rate is also adequately fitted by a single exponential model.

The addition of 50/30-mer DNA to gp44/62 increases the basal rate of the system tenfold (to $2.7 \mu\text{M}/\text{minute}$). The gp44/62 + DNA data do not fit a single exponential function with an end point at $100 \text{ mM } P_i$, but rather a biphasic kinetic model such as that defined by equation (4); see Experimental Procedures. After the initial phase, characterized by a rate of $2.7 \mu\text{M}/\text{minute}$, the reaction slows to approximately the same rate as seen with gp44/62 alone (ca. $1 \mu\text{M}/\text{minute}$).

The complete system (gp44/62 + gp45 + 50/30-mer DNA) produces the largest ATPase rate (Mace & Alberts, 1984a; Jarvis *et al.*, 1989b). As seen for the gp44/62 + DNA ATPase results, these data also fit a biphasic kinetic model with an initial fast rate of $156 \mu\text{M}/\text{minute}$ followed by a slow phase of $0.5 \mu\text{M}/\text{minute}$. The rate of the slow phase is again of the same order of magnitude as that seen with the gp44/62 ATPase alone. Figure 4B shows

steady-state time courses for the complete reaction at different ATP concentrations. The K_m for ATP in the complete reaction was measured as $400(\pm 238) \mu\text{M}$, and the k_{cat} was determined to be $248(\pm 50) \text{ min}^{-1}$. This K_m value, measured at 22°C , is in reasonable agreement with an earlier value of $\sim 200 \mu\text{M}$, measured at 37°C by Jarvis *et al.* (1989b).

Presteady-state ATPase kinetics

In order to probe the initial events leading to formation of the T4 sliding clamp, presteady-state kinetic studies of the gp44/62 ATPase were conducted. Three key characteristics of the reaction were under scrutiny. (1) Are the rates of ATP hydrolysis and subsequent events (equation (4)) sufficiently different to give the time course a biphasic nature? (2) If this is the case, can the size of the initial phase (the "burst") be used to estimate the number of ATP molecules bound and hydrolyzed during the burst? (3) Is the rate of the initial phase consistent with the observed rate of holoenzyme formation measured by Kaboord & Benkovic (1993)?

Figure 5 shows the results of a quench-flow experiment with $1.1 \mu\text{M}$ gp44/62, $1 \mu\text{M}$ gp45 and $1 \mu\text{M}$ 50/30-mer DNA, at $200 \mu\text{M}$ ATP. These data clearly show that the complete ATPase reaction is biphasic in nature, suggesting that events after ATP hydrolysis are rate limiting and lead to the steady state. The data of Figure 5 were fitted to equation (4) (see Experimental Procedures) with a burst amplitude of $6(\pm 1) \mu\text{M}$, a rapid initial phase of 600 min^{-1} (10 s^{-1}), and a slow phase of 190 min^{-1} (3.0 s^{-1}). The rate of the slow phase is very close to the projected initial steady-state rate for this ATP concentration (see above). Results from a number of such presteady-state experiments at various concentrations of ATP and gp45 are summarized in Table 1.

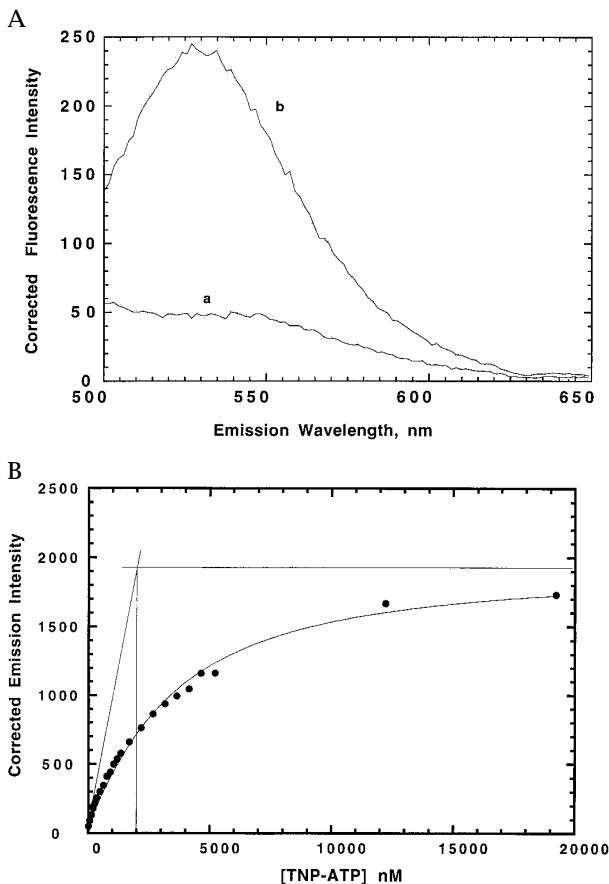


Figure 3. A, The effect of gp44/62 on the fluorescence of TNP-ATP. Curve a, 1 μ M TNP-ATP in 50 mM Hepes (pH 7.5), 50 mM KOAc, 0.5 mM MgSO₄, 5 mM KP_i, 5% glycerol. Curve b, 1 μ M TNP-ATP + 1 μ M gp44/62 in the same buffer. B, Binding of TNP-ATP to gp44/62. Measured in 50 mM Hepes (pH 7.5), 50 mM KOAc, 0.5 mM MgSO₄, 5 mM KP_i, 5% glycerol. The line was fitted to equation (3) (see Experimental Procedures), with $F_{\max} = 1905(\pm 56)$, $n = 2$, and $K_d = 2.4(\pm 0.2)$ μ M.

Several things are apparent from these data. The first is that the burst amplitude is relatively independent of ATP concentration, down to 100 μ M. Since the burst amplitude, unlike the steady-state rate, is a direct reflection of the binding of ATP, this means that the dissociation constant for ATP is less than 100 μ M, which is in good agreement with the dissociation constant of 38 μ M determined from direct binding experiments (Figures 1 to 3). The average burst amplitude for the ATPase reactions from Table 1 is $4.4(\pm 1)$ μ M, corresponding to four ATP molecules per 4:1 gp44/62 complex. The rate of the burst phase is 5 to 30 μ M/second; therefore this phase takes approximately 0.2 to 1 second to complete. We argue below that this reflects a role for the burst phase, and by implication, four ATP molecules, in the formation of the T4 sliding clamp. Another important observation is that the burst amplitude is independent of the three concentrations of gp45 used, but is directly dependent on the concentration of gp44/62 (see Discussion).

Discussion

The work of a number of researchers has suggested that ATP is used to form the clamp as part of the DNA polymerase holoenzyme, rather than to maintain it or to move it along DNA as synthesis progresses (Piperno & Alberts, 1978; Newport *et al.*, 1980; Huang *et al.*, 1981; Jarvis *et al.*, 1991). On the other hand, other work (Newport *et al.*, 1980; Selick *et al.*, 1987; Jarvis *et al.*, 1991) indicates that ATP is required periodically during DNA synthesis on primed single-stranded templates. The latter set of studies suggested the possibility that this periodic requirement for ATP might reflect a timing, or "clocking", mechanism in which ATP hydrolysis drives the formation of an activated state of the accessory proteins complex that is characterized by a finite decay constant or relaxation time. This decay constant was envisioned to determine the frequency of further activation events as DNA synthesis proceeded. Based on what is now known about the formation of the DNA polymerase holoenzyme in this and other systems, the most likely candidate for the "activated complex" is the loaded gp45 sliding clamp itself. In these terms the "clock decay constant" would then correspond to the dissociation rate of the clamp from the holoenzyme-DNA complex.

Kinetic data on the chemical and structural events leading up to the formation of the T4 sliding clamp are essential for an understanding of the role of ATP hydrolysis in the clamp-loading process. No data on the presteady-state ATPase kinetics of the gp44/62-gp45 system had been available (however see Berdis & Benkovic, 1996). We have attempted to fill this gap using a dual approach. Elucidation of the kinetics of ATP usage in forming the sliding clamp represents a first step. The identification and characterization of the structural intermediates in the process, and their kinetics of interconversion, is also necessary. These two lines of research converge in the connection between the rates of ATP hydrolysis and the rates of structural changes. Here, we focus on the kinetic mechanism of ATP hydrolysis as the first phase in clarifying the role of this substrate in the placement of the gp45 clamp onto DNA. In the companion paper (Latham *et al.*, 1996) we approach some aspects of the related structural problems.

The gp44/62 complex contains four gp44 ATPase subunits and has the ability, in principle, to bind four ATP molecules per complex. The actual binding affinities of the sites in multisubunit enzymes can have profound mechanistic consequences (Lazdunski, 1974; Stitt, 1988; Geiselmann *et al.*, 1993). Our work on the processivity clamp-loading system that is presented here shows that the gp44/62 complex binds four ATP molecules, even in the presence of up to 23 mM P_i (Figures 1A, B and 3B), with an average dissociation constant of $34(\pm 12)$ μ M. There is no evidence of binding site heterogeneity in the binding of either

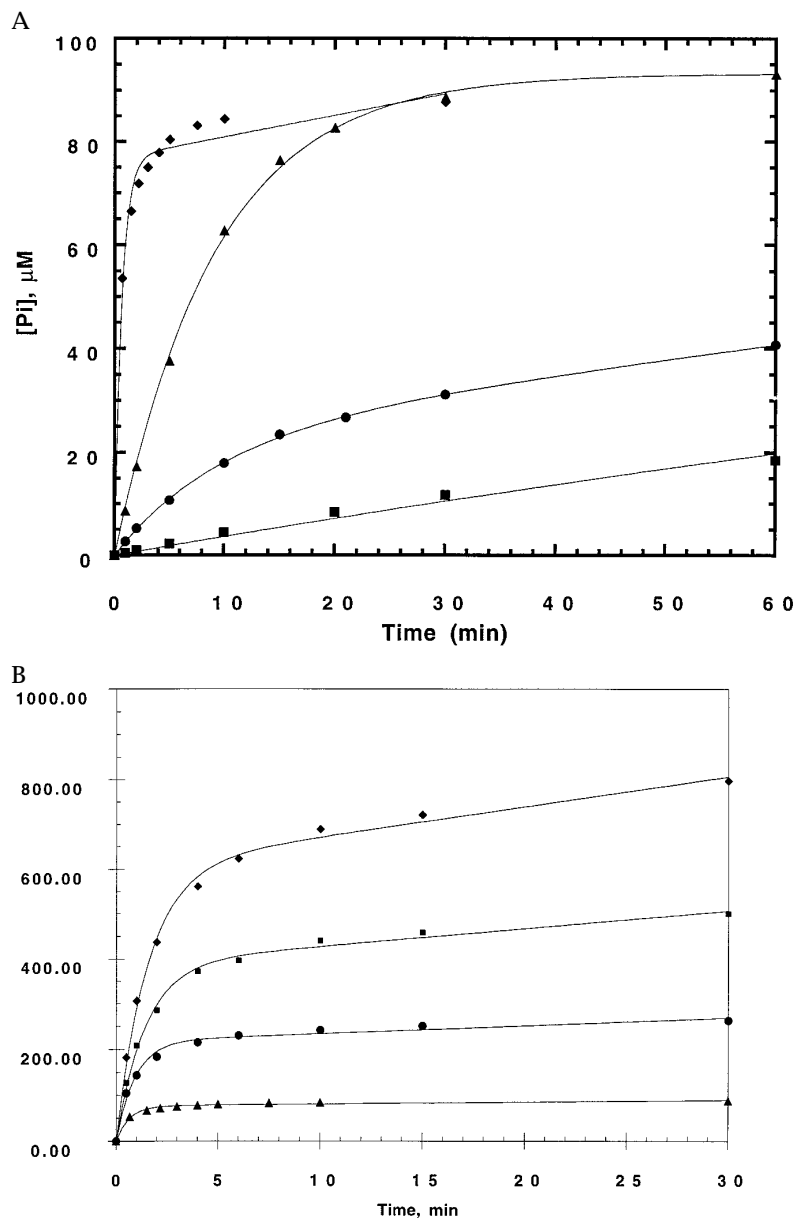


Figure 4. A, Steady-state time courses for the four combinations of components in the T4 sliding clamp system. All experiments were performed at 100 μM ATP as described in Experimental Procedures. (■) 1.1 μM gp44/62 alone; (▲) 1.1 μM gp44/62 + 1 μM gp45; (●) 1.1 μM gp44/62 + 1 μM 50/30-mer DNA; (◆) 1.1 μM gp44/62 + 1 μM gp45 + 1 μM 50/30-mer DNA. The first two curves were fitted with an exponential function while the third and fourth curves were fitted to the biphasic model described by equations (4) and (5) (see Experimental Procedures). B, Steady-state time courses for the ATPase activity of the gp44/62 (1.1 μM) + gp45 (1 μM) + 50/30-mer DNA (1 μM) accessory proteins system at various concentrations of ATP. (▲) 100 μM ATP; (●) 300 μM ATP; (■) 600 μM ATP; (◆) 1 mM ATP. All curves were fitted to the biphasic model described by equations (4) and (5).

ATP or ADP. The dissociation constant of $34(\pm 12)\ \mu\text{M}$ is considerably smaller than the measured K_m for ATP of the fully activated ATPase complex (Jarvis *et al.*, 1989b), but is consistent with burst amplitudes obtained in the presteady-state experiments (see below).

The binding of ADP to the gp44/62 complex is much like that of ATP, but shows a slightly smaller dissociation constant of $16(\pm 7)\ \mu\text{M}$ (Figure 2). The presence of P_i does not affect the stoichiometry or affinity of ADP binding. As mentioned above (see Results), the fact that neither ATP nor ADP binding is affected by the presence of up to 23 mM P_i shows that the dissociation constant of P_i is $>23\ \text{mM}$. Using the measured binding constants for ATP and ADP, the overall equilibrium constant of $\sim 2.7 \times 10^6\ \text{M}$ for the reaction $\text{ATP} \rightleftharpoons \text{ADP} + \text{P}_i$ (Alberty, 1968;

Shikama & Nakamura, 1973), and the estimate of 23 mM for the dissociation constant of P_i , we can calculate the approximate "internal" equilibrium constant for the overall gp44/62-catalyzed ATPase reaction $\text{E}\cdot\text{ATP} \rightleftharpoons \text{E}\cdot\text{ADP}\cdot\text{P}_i$ as approximately $2.8 \times 10^8\ \text{M}^{-1}$.

To further study the internal thermodynamics of the reaction we attempted to use gp44/62 to catalyze the reverse reaction of ATP synthesis from ADP and P_i at constant concentrations of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ and concentrations of P_i up to 200 mM. No conversion of $[\alpha\text{-}^{32}\text{P}]\text{ADP}$ into $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ was observed under any of the conditions tested (data not shown), further supporting the idea that P_i binding is extremely weak or that the internal forward equilibrium constant is very large.

The binding of the fluorescent ATP analog TNP-ATP shows an apparent heterogeneity in the

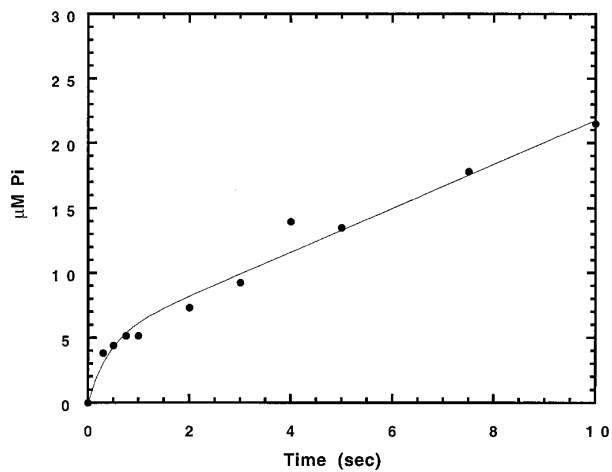


Figure 5. Presteady-state time course for the ATPase activity of the gp44/62 (1.1 μM) + gp45 (1 μM) + 50/30-mer DNA (1 μM) accessory proteins system at 200 μM ATP. The data were fitted to the biphasic model of equations (4) and (5).

ATP binding sites of gp44/62. The gp44/62 complex binds two TNP-ATP molecules (Figure 3B). This could indicate a true binding site heterogeneity that is not detectable in either the direct binding studies on ATP and ADP, or it could suggest that the large trinitrophenyl groups of the two bound TNP-ATP molecules occlude the remaining two ATP binding sites. The latter explanation would suggest that two of the binding sites are quite close in space. The fact that TNP-ATP is not a substrate for gp44/62 suggests that the presence of four filled ATP sites is necessary for activity, or may indicate that the binding mode of the analog is different from that of the substrates and products. Further investigation of this issue is required.

The steady-state rates of ATP hydrolysis by gp44/62 and gp44/62 + gp45 that we measured here are in rough agreement with previously determined values (Mace & Alberts, 1984a,b; Jarvis *et al.*, 1989b). The rates determined in this study are

probably somewhat smaller than those of the latter authors because they were collected at 22°C rather than at 37°C. However, when the template-primer DNA cofactor was included in the system, the situation became more complex. Both the gp44/62 and the gp44/62 + gp45 ATPase systems initially showed rates consistent with previous results, but these initial phases were followed by slower phases whose rates were about equal to those catalyzed by the gp44/62 complex in isolation (Figure 4A and B). The meaning of this phenomenon is unclear, but some possibilities can be ruled out. Thus we can state that this slow phase does not reflect any nuclease contamination of the proteins, since both protein preparations were assayed for such contamination when initially purified. Furthermore, since the kinetics of gp44/62 and gp44/62 + gp45 do not show the slowdown of the apparent rate (as manifested by the appearance of biphasic kinetics), the proteins are not inherently unstable over these reaction times.

One possibility is that this anomaly is due to the buildup of ADP at later times in the reaction. We have performed assays of the complete accessory protein system in the presence of increasing concentrations of ADP. We find that ADP does severely inhibit the ATPase activity of gp44/62 under these conditions, and that the onset of this inhibition occurs when the ratio of ADP to ATP is about 3:1 (data not shown). This is similar to the ADP:ATP ratios at which the slow second phase appears (Figure 4A and B). The work of Hockensmith *et al.* (1993) has shown that gp44/62 does not bind to DNA in the presence of gp45 and ADP. Presumably this effect is due either to a stable gp44/62-gp45-ADP complex, or to a sequestering of DNA by gp45, which binds to DNA in the presence of ADP (Hockensmith *et al.*, 1993). The latter mode of inhibition could explain why the rate of the slow second phase appears to be comparable to the rate of ATP hydrolysis by the gp44/62 complex alone, since the gp44/62 would be prevented from binding to DNA by competition with gp45. The above authors have also noted that gp44/62 does bind to DNA in the presence of ADP, although this binding is clearly different from that observed in

Table 1. Kinetic characteristics of presteady-state ATP hydrolysis by gp44/62 + gp45 + primer-template DNA

Reaction components			Burst amplitude (μM)	Burst rate ($\mu\text{M s}^{-1}$)	Steady-state rate ($\mu\text{M s}^{-1}$)
ATP (μM)	gp44/62 (μM)	gp45 (μM)			
100	1.1	1.1	3.8(\pm 0.5)	4.9	1.32
200	1.1	1.1	6.3(\pm 1)	13.6	1.7
500	1.1	1.1	4.4(\pm 1.6)	9.4	1.5
1000	1.1	1.1	5.2(\pm 1.4)	33	1.9
1000	1.1	2.2	5.3(\pm 0.5)	47	2.0
1000	1.1	0.5	5.1(\pm 1)	30	1.7
100	0.55	0.5	1.5(\pm 0.5)	2	0.62

Reactions were run in HEPES-Pol buffer at room temperature in the quench-flow apparatus described in Experimental Procedures. Measures of precision were obtained from the correlation matrix of the non-linear fits of individual time courses. In some cases the values shown represent averages of two to three measurements.

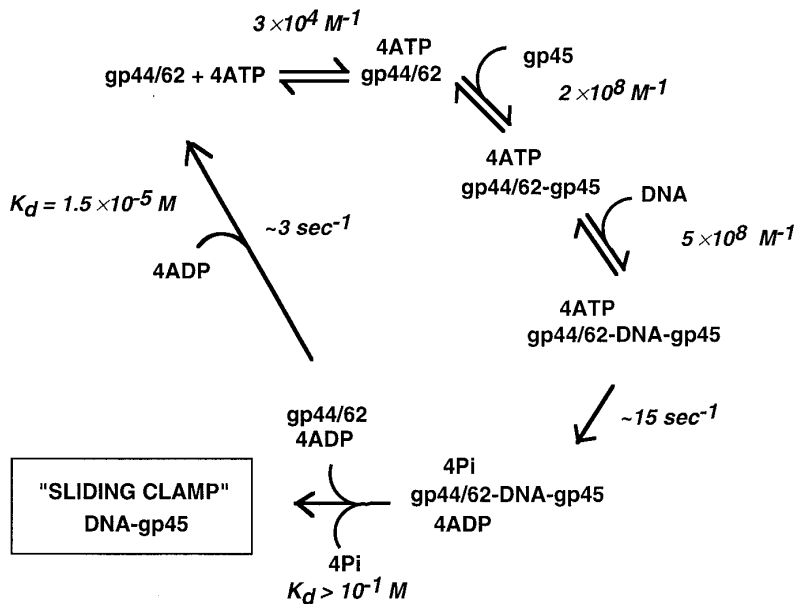


Figure 6. A proposed model of the mechanism of ATP hydrolysis by the fully activated gp44/62 ATPase, based on the data presented here. The process begins with the binding of four ATP molecules to the gp44/62 complex, with a binding constant of $3 \times 10^4 \text{ M}^{-1}$ (obtained from Figure 1). This is followed by the rapid binding of gp45 and DNA to the ATP-gp44/62 complex, as discussed in the text. The binding constant of $2 \times 10^8 \text{ M}^{-1}$ for the interaction of these complexes is from Latham *et al.* (1996), the binding constant for template-primer DNA to the activated complex is the reciprocal of the Michaelis constant for the activation of the gp44/62 + gp45 ATPase by DNA (Jarvis *et al.*, 1989b). The rate-limiting step during the observed fast phase (see

Results and Figure 5) is the hydrolysis of the four ATP molecules, the result of which is to place the gp45 ring into position to encircle the DNA. Although the exact point at which P_i dissociates from gp44/62 is not certain, its high dissociation constant suggests that it dissociates rapidly after ATP hydrolysis. The gp44/62 then dissociates from the loaded gp45 clamp, which in turn dissociates from the DNA. The gp44/62 complex is recycled in the slow phase by the rate-limiting dissociation of the ADP product. The dissociation constant from gp44/62 shown for ADP is from Figure 2.

the presence of ATP or the non-hydrolyzable ATP analog $\text{ATP}\gamma\text{S}$. The data in Figure 5A on the gp44/62 + DNA system would suggest that the binding of gp44/62 to DNA promoted by ADP produces a complex incapable of hydrolyzing ATP properly. Although these ideas are consistent with our data, further work must be done to examine this phenomenon.

Presteady-state studies on the fully activated gp44/62 ATPase reveal two of the processes involved in loading the sliding clamp. These are a fast phase involving ATP hydrolysis with a rate of 5 to 30 s^{-1} , and a slower phase following the hydrolysis step with a rate of 3 to 5 s^{-1} . This latter phase corresponds to the initial steady-state rate. The amplitude of the fast phase is $4.4 (\pm 1.0) \mu\text{M}$ (an average of the values in Table 1, omitting the last one), which corresponds to four ATP molecules hydrolyzed per gp44/62 complex. The duration of the fast phase is 0.2 to 1 second, which is slightly faster than the rate of T4 DNA polymerase holoenzyme formation as measured by Kaboord & Benkovic (1993). The fast phase is therefore implicated as the rate-limiting step in the formation of the sliding clamp. Our data also show that all four ATP molecules bound to the gp44/62 complex are hydrolyzed during the clamp-loading process.

This rate information, combined with the binding data, allows us to write a fairly detailed kinetic mechanism for the clamp loading reaction, as summarized in Figure 6. The clamp-loading process is defined as the steps beginning with the initial binding of ATP to gp44/62 at the point labelled "SLIDING CLAMP" in Figure 6. The fact

that preincubation of the gp44/62 and gp45 proteins with DNA template-primer cofactor was not necessary to obtain the fast phase (see Experimental Procedures) suggests that the order of reaction as written is at least kinetically accessible, and that DNA binding is not rate limiting.

The binding of ATP can also be shown not to be rate limiting, since the burst amplitude is independent of ATP concentrations in excess of the measured dissociation constant of $34 \mu\text{M}$. The results of Latham *et al.* (1996), and an earlier observation (M. Reddy, personal communication), suggest the existence of a stable gp44/62-gp45 complex only during ATP hydrolysis. For this reason we write the ATP binding step first in Figure 6. In addition, we have not observed a burst of four ATP molecules per gp44/62 complex by gp44/62 + gp45 in the absence of DNA (data not shown). Therefore the binding of the DNA cofactor is placed before the hydrolysis of ATP in the mechanism. The rate-limiting step for the burst phase then must represent either the binding of the two protein complexes, or the hydrolysis of the four ATP molecules. We assume that after the hydrolysis of ATP (in approximately one second) the gp45 trimer encircles the DNA and is ready to bind the DNA polymerase, since Kaboord & Benkovic (1993) observed that holoenzyme formation also occurs at approximately this rate.

The fact that the burst amplitude is independent of the concentration of gp45 means that the rate-limiting step in the steady-state phase is dependent on events occurring within the gp44/62 complex. This conclusion was also reached by

Berdis & Benkovic (1996) in their studies on the role of ATP in DNA polymerase holoenzyme formation. When the ratio of gp45 to gp44/62 is 0.5:1, a burst amplitude of one gp44/62 equivalent is observed. This means that two rounds of gp45 loading are occurring during the burst phase. Therefore during the first half of the burst phase, half of the gp44/62 is used to load all of the gp45 onto DNA. The loaded gp45 is then released from gp44/62. Finally, gp45 dissociates from the DNA, to be reused by the second half of the gp44/62 in the remainder of the burst phase. None of the above mentioned steps can be slower than the rate of the burst phase, thus they cannot be rate limiting. The simplest and most direct candidate for the rate-limiting step in the slow phase must therefore be the dissociation of either ADP or P_i . We consider ADP dissociation to be the more likely candidate in view of the high estimate of the dissociation constant of P_i . We cannot say with certainty that gp44/62 dissociates from DNA after the loading process, but this does seem likely in view of the small size of the DNA cofactor used in our experiments. Since the gp45 should cover most of the double-stranded portion of the 50/30-mer DNA (Munn & Alberts, 1991;

Polyethyleneimine, with fluorescent indicator (PEI-F), cellulose thin-layer chromatography (TLC) plates were obtained from EM Separations Technology (Gibbstown, NJ). DE52 DEAE cellulose and P11 phosphocellulose were from Whatman LabSales (Hillsboro, OR). Q-Sepharose was obtained from Pharmacia-LKB Biotechnology, Inc. (Uppsala, Sweden). Hydroxyapatite was purchased from Bio-Rad (Hercules, CA). Single-stranded DNA-cellulose was prepared by the method of Alberts & Herrick (1971). Radioactive materials were purchased from DuPont New England Nuclear (Boston, MA). DNA oligonucleotides were synthesized and purified by either DNAExpress (Ft. Collins, CO) or Oligos, Etc. (Wilsonville, OR). The Centricon filters were from the Amicon Corp. (Beverly, MA). TNP-ATP was purchased from Molecular Probes, Inc. (Eugene, OR). The flame photometry standard for the phosphate assay (Lanzetta *et al.*, 1979) was obtained from Bacharach (Pittsburgh, PA).

DNA template-primer cofactors

The DNA used in these experiments was a duplex primer-template construct made by the annealing of two synthetic oligonucleotides: a 50-mer DNA template strand and a 30-mer primer strand. The sequence and structure of this construct is:

```
5' -CCGCAGCCTCGCAGCCGTCCAACCAACTCA-3'
3' -GGCGTCGGAGCGTCGGCAGGTTGGTTGAGTAGGTCTTGTGTTTGGCCGGTCA-5'
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Capson *et al.*, 1991), any gp44/62 remaining on the DNA must be bound to the 20-base single-stranded section. It seems difficult to fit an additional gp44/62 into this complex. Even if the gp44/62 moved to the double-stranded portion after dissociation of gp45, it should block further loading reactions. These conclusions provide a basis for the structure-based arguments for ADP dissociation as the rate-limiting step put forward in the companion paper (Latham *et al.*, 1996).

The interpretations presented here (Figure 6) provide a detailed mechanism for the kinetics of the chemical events associated with the loading of the T4 sliding clamp onto template-primer DNA (and thus presumably also onto the gp43 polymerase). Approaches to the exploration of the physical changes that accompany these chemical events, and their kinetics, are presented in the companion paper (Latham *et al.*, 1996). Further extensions and refinements of these studies should provide a complete description of the operation of this central regulatory system of DNA replication.

Experimental Procedures

Reagents and materials

All chemicals and reagents used in these experiments were obtained from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), or Boehringer-Mannheim (Indianapolis, IN), and were used without further purification. QAE-Sephadex was from Sigma.

Synthesis of [α - 32 P]ADP

[α - 32 P]ADP was synthesized from [α - 32 P]ATP by hydrolysis with myosin. Reaction mixture volumes were typically 20 μ l and contained 80 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 100 μ Ci [α - 32 P]ATP, and 1.2 μ g myosin. This mixture was incubated at 25°C for 20 minutes, then applied to a 1 cm \times 14.5 cm column of QAE-Sephadex that had been equilibrated in 0.1 M triethylammonium bicarbonate (TEAB; pH 7.7). After loading, the column was eluted with a linear gradient (150 ml) increasing in TEAB (pH 7.7) concentration from 0.1 to 1.0 M. Fractions of 2 ml were collected. Fractions containing the largest peak of radioactivity were collected, pooled and evaporated in a Speed-Vac twice to remove TEAB. The product was redissolved in 50 mM Hepes (pH 7.5) and used from this solution.

Proteins

E. coli bacterial strains containing the plasmids pTL151W, pTL45W and pTL43, which overproduce both gp44/62 and gp45, gp45 and gp43, respectively, were the generous gifts of Drs T. C. Lin and W. Konigsberg at Yale University. The T4 accessory proteins were purified from *E. coli* strains containing pTL151W. Lysis of the cells and centrifugation of the lysate was performed according to Reddy *et al.* (1992). The cleared cell lysate was applied to a 5 cm \times 23 cm column of DE52 DEAE-cellulose pre-equilibrated in 40 mM Tris-HCl (pH 7.4), 1 mM $MgCl_2$, 12.5% (v/v) glycerol, and 1 mM DTT. Under these conditions, gp45 bound to DE52 (Jarvis *et al.*, 1989a), and gp44/62 passed through the column. At this point the purification of the two protein complexes proceeded independently. Continuing with the gp45 purification, the DE52 column

was washed, then eluted with a linear NaCl gradient (2 l) from 50 mM to 0.2 M, collecting 15 ml fractions. Fractions containing gp45 were pooled and dialyzed versus 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 1 mM DTT, 25% glycerol. The dialyzed was passed over a 1.2 cm × 15 cm column of single-stranded (ss)DNA-cellulose. The flow-through fraction from this column was applied to a 1.5 cm × 20 cm column of Q-Sepharose, washed, and eluted with a linear NaCl gradient (600 ml) from 0 to 0.4 M. Fractions of 4 ml were collected. Fractions containing gp45 were dialyzed into 20 mM Hepes (pH 7.5), 50 mM potassium acetate (KOAc), 0.5 mM DTT, 50% glycerol. Purification of gp44/62 was continued by passing the flow-through fraction from the DE52 column over a 2.5 cm × 30 cm column of ssDNA-cellulose. The flow-through from this column was applied to a 2.5 cm × 36 cm column of P11 phosphocellulose, washed, and eluted with a linear KP_i gradient (2 l) from 20 to 150 mM, collecting 8 ml fractions. Fractions containing gp44/62 were pooled and dialyzed into 50 mM KP_i (pH 7.0), 5 mM $MgSO_4$, 0.5 mM DTT, 50% glycerol.

Purification of gp45 was carried out by the method of Morris *et al.* (1979), from bacteria containing the plasmid pTL45W. This procedure was modified as follows: (1) no ammonium sulfate precipitation was performed and (2) the norleucine-Sepharose and second DEAE-cellulose column were replaced with a single Q-Sepharose column. Chromatography on this Q-Sepharose column was performed as described above. Concentrations of gp44/62 are reported in units of 4:1 gp44:gp62 complexes, while those for gp45 are reported as the concentration of gp45 trimers. All concentrations were determined spectrophotometrically using calculated molar extinctions: gp43, $\epsilon_{280\text{ nm}} = 1.28 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$; gp44/62, $\epsilon_{280\text{ nm}} = 1.23 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$; and gp45, $\epsilon_{280\text{ nm}} = 5.72 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Jarvis *et al.*, 1990).

ATPase substrate and product binding assays

The binding of ATP and ADP to gp44/62 was assayed by the method of Stitt (1988). All binding assays were performed at room temperature with a gp44/62 concentration of 5 μM in 50 mM Hepes (pH 7.5), 23% glycerol, 0.2 mM DTT, and varying amounts of ATP or ADP spiked with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\alpha\text{-}^{32}\text{P}]\text{ADP}$.

Some of the binding mixtures contained some inorganic phosphate (as KP_i , pH 7.0), or $MgSO_4$, due to the presence of these components in the gp44/62 storage solution. When P_i was removed by dialysis, the total removal of P_i was confirmed by the assay of Lanzetta *et al.* (1979), with the modification that Sterox, which is no longer available, was replaced by a flame photometry standard solution. The activity of dialyzed protein preparations was checked against the stock protein using the ATPase assay described below for the steady-state kinetics experiments, and was found to be indistinguishable from the stock activity.

Each binding mixture (150 μl) was made up immediately before use. A 100 μl aliquot of this solution was placed in a Centricon-100 or Centricon-30 filter device, and approximately 50 μl was forced through the filter with dry compressed air. Three aliquots from the filtrate and from the original solution were spotted onto PEI-F cellulose TLC plates or sheets of DE81 paper, or directly quantified by scintillation counting. Radioactivity on the TLC plates or DE81 paper was quantified on an AMBIS-4000 radioactivity scanner (Scanalytics, Boston, MA). Free and bound ATP or ADP was determined by

measuring the average radioactivity in the three filtrate spots (cpm_{free}), and in the three original mixture spots ($\text{cpm}_{\text{total}}$), and applying the relations:

$$\frac{\text{cpm}_{\text{free}}}{\text{cpm}_{\text{total}}} [\text{AT(D)P}]_{\text{total}} = [\text{AT(D)P}]_{\text{free}}$$

$$[\text{ATP}_{\text{bound}}] = [\text{ATP}_{\text{total}}] - [\text{ATP}_{\text{free}}] \quad (1)$$

Data were fitted to the equation:

$$[\text{AT(D)P}]_{\text{bound}} = \frac{n\epsilon [\text{AT(D)P}]_{\text{free}}}{[\text{AT(D)P}]_{\text{free}} + K_d} \quad (2)$$

where n is the number of sites per gp44/62 complex, and ϵ is the total concentration of gp44/62 complexes.

Fluorimetric binding studies with TNP-ATP

The binding of TNP-ATP to gp44/62 was monitored by fluorescence titration in an SLM-8000 fluorescence spectrophotometer. Aliquots of TNP-ATP were added, with constant stirring, to 2 ml of a 1 μM solution of gp44/62 in 25 mM Hepes (pH 7.5), 160 mM KOAc, 6 mM $MgCl_2$, 5 mM DTT, 5% glycerol, in a 3 ml cuvette. Titrations were performed at 22°C (room temperature). Steady-state fluorescence emission spectra of TNP-ATP were taken after each addition of TNP-ATP. Using an excitation wavelength of 460 nm, TNP-ATP has an emission maximum at 540 nm. Fluorescence intensity at 540 nm was plotted as a function of added TNP-ATP, and the low and high concentration asymptotes were used to determine the binding stoichiometry. The fluorescence data were also fitted to the equation:

$$F = F_{\text{max}} \left(\frac{A - \sqrt{A^2 - 4n\epsilon [\text{TNP-ATP}]_{\text{tot}}}}{2n\epsilon} \right)$$

$$A = [\text{TNP-ATP}]_{\text{total}} + n\epsilon + \frac{1}{K_a} \quad (3)$$

where K_a is the association constant, F_{max} is the plateau value, and F is the measured fluorescence at $[\text{TNP-ATP}]_{\text{total}}$. TNP-ATP was found not to be a substrate for the gp44/62 or the gp44/62 + gp45 + DNA systems.

Steady-state kinetic measurements

All steady-state kinetic assays were performed at 22°C (room temperature) with 1 μM gp44/62 in Hepes-Pol buffer (25 mM Hepes (pH 7.5), 160 mM KOAc, 6 mM $MgCl_2$, 5 mM DTT). Reactions were initiated by the addition of either gp44/62, or a mixture of gp44/42 and gp45. Reactions were quenched by the addition of an equal volume of either 0.1 M HCl or 0.5 M EDTA (pH 8.0). Both quenching methods gave identical results, and 0.1 M HCl was used most frequently. Aliquots from the quenched reaction were delivered to a PEI-F cellulose TLC plate, and developed in 0.3 M KP_i (pH 7.0). Radioactive spots containing P_i and ATP were quantified using an AMBIS radioactivity scanner.

Presteady-state kinetic measurements

Presteady-state measurements were made using an Update Instruments (Madison, WI) quench flow apparatus modified in our laboratory. Fluid flow parts from Upchurch Scientific (Oak Harbor, WA) and a custom mixer constructed from plexiglass by the University of Oregon machine shop were used to build a modified instrument based on the design of Johnson (1986). The

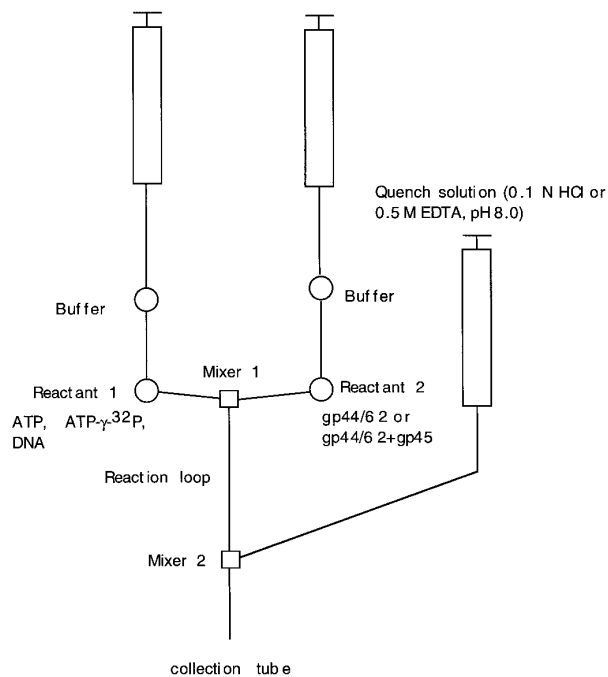
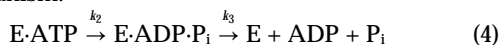


Figure 7. Scheme showing the basic design of the presteady-state measurement experiments.

basic design of the experiments is shown in Figure 7. Enzyme solutions (either gp44/62 or gp44/62 + gp45) in one syringe of the apparatus, and ATP (containing a trace amount of [γ - ^{32}P]ATP) or ATP + DNA in the other syringe were mixed, travelled through a reaction loop of varying length, and then were mixed with a quench solution consisting of either 0.1 M HCl or 0.5 M EDTA (pH 8.0). Indistinguishable results were obtained with either quench solution. Experiments were also performed by incubating DNA and protein(s) in one syringe and ATP in the other. The results with this premixing protocol were the same as those obtained with the other strategy described.

Buffers and reaction conditions were the same as described above for the steady-state measurements. The concentrations of gp44/62 and DNA were always 1 μM , other protein and ATP concentrations were as specified in Results. Products of the reaction were analyzed as for the steady-state measurements. Data were fitted, using the mechanism:



to the equation (Gutfreund, 1972):

$$\epsilon \left[k_2 \left(\frac{k_3}{k_3 + k_2} \right) t + \left(\frac{k_2}{k_2 + k_3} \right)^2 (1 - e^{-(k_2 + k_3)t}) \right] \quad (5)$$

ATP synthesis reactions

Reactions designed to detect the synthesis of ATP by gp44/62 + 45 or gp44/62 + 45 + DNA were performed. Reactions were initiated by adding a solution of gp44/62 and gp45 to a mixture containing 2 μM DNA, 50 μM ADP, and 20 to 200 mM KPi , in Hepes-Pol buffer. The final concentrations of gp44/62 and gp45 were both 2 μM in a reaction volume of 20 μl . Reactions were incubated at 37°C for varying times, then quenched by the addition of an equal volume of 0.1 M HCl. The reactions were then

applied to PEI-F cellulose TLC plates and developed in 0.3 M KPi (pH 7.0). The developed TLC plates were quantified on an AMBIS radioanalytic scanner.

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