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Mechanism of Bacteriophage T4 DNA Holoenzyme Assembly: The 44/62 Protein Acts as a Molecular Motor†

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ABSTRACT: The role of ATP hydrolysis by the 44/62 protein in formation of the stable holoenzyme DNA replication complex has been further elucidated by specifically examining the role that the 44/62 protein plays in loading the 45 protein onto the DNA substrate. A stable phospho-45 protein or phosphorylated holoenzyme complex was not detected or isolated, suggesting that the 44/62 protein may not act as a protein kinase. Product and dead-end inhibition data are consistent with an ordered kinetic mechanism with respect to product release in which phosphate is released from the 44/62 protein prior to ADP. Positional isotope effect studies support this mechanism and failed to demonstrate that ATP hydrolysis by the 44/62 protein is reversible. Steady-state ATPase assays using aluminum tetrafluoride as an inhibitor are also consistent with release of ADP being partially rate-limiting. Aluminum tetrafluoride acts to trap ADP on the enzyme after turnover, forming a stable transition state analog that dissociates slowly from the enzyme. Processive DNA synthesis does not occur using the accessory proteins in the presence of pre- or post-hydrolysis analogs of ATP nor in the presence of ADP−AlF_4, indicating that turnover of the 44/62 protein is absolutely required for formation of the holoenzyme complex. Collectively, data obtained regarding ATP hydrolysis by the 44/62 protein are described in terms of the clamp loading protein functioning as a molecular motor, similar to other systems including myosin and kinesin.

The bacteriophage T4 DNA polymerase holoenzyme is derived from the phage DNA polymerase (the product of gene 43) and its 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 is a DNA-dependent ATPase stimulated by the 45 protein (Mace & Alberts, 1984b). Together, these proteins require ATP hydrolysis to form a sliding clamp that tethers the polymerase to the primer/template, increasing the rate and processivity of both DNA synthesis and 3′ → 5′ nucleotide excision catalyzed by the polymerase (Alberts & Frey, 1970; Mace & Alberts, 1984a,b). The 44/62 protein and the 45 protein are functionally similar to the γ complex (Maki & Kornberg, 1988) and the β subunit (Stukenburg et al., 1991) in *Escherichia coli* as well as the RF-C complex (Lee et al., 1991) and proliferating cell nuclear antigen (PCNA)[1] (Tsurimoto & Stillman, 1987) in eukaryotic systems. Recent crystallographic data indicate that the 45 protein is similar to both the β subunit and PCNA, all three

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[1] Abbreviations: PCNA, proliferating cell nuclear antigen; Bio, biotin; TBE, Tris-HCl/borate/EDTA; EDTA, ethylenediaminetetraacetate, sodium salt; dNTP, deoxynucleotide triphosphate; TEAB, triethylammonium bicarbonate; AlF_4, aluminum tetrafluoride.

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of which are in the shape of a ring with a central cavity large enough to encircle duplex DNA (J. Kuriyan, personnal communication; Kong et al., 1992; Krishna et al., 1994). Although the 44/62 protein participates in loading the 45 protein onto duplex DNA, the mechanism of this loading process is still largely speculative, i.e., how is a closed circle transiently opened to concatenate the DNA.

Several possible models for ATP consumption by the 44/62 protein with regards to its interaction with the 45 protein and DNA to form the sliding clamp have been proposed. Huang et al. (1981) originally suggested that the 44/62 protein may act as a protein kinase in conjunction with the 45 protein to specifically phosphorylate the T4 polymerase, but were unable to detect any transient phosphorylation of the polymerase. They, however, did not examine whether or not the 45 protein is phosphorylated by the 44/62 protein.

It had been postulated that an ADP-bound state of the accessory proteins forms a sliding clamp, which is stabilized by polymerase (and the sliding clamp is stabilized by polymerase in a reciprocal manner), decreasing the dissociation rate of the enzyme from the DNA and potentiating the high processivity of the holoenzyme conformation (Jarvis et al., 1989b). The ADP-bound state of the accessory proteins would prevent further ATP hydrolysis during the lifetime of the holoenzyme complex. This mechanism during processive DNA synthesis can be eliminated since the 44/62 protein acts catalytically to assemble the holoenzyme complex (Kaboord & Benkovic, 1995; Berdis & Benkovic, 1996) and does not remain part of the holoenzyme complex during processive DNA synthesis. However, it is still possible that an activated ADP-bound 44/62•45 complex is formed during the process of loading the 45 protein onto duplex DNA. This activated form of the sliding clamp could potentiate the binding of polymerase to form the stable holoenzyme complex. Upon binding of polymerase, the 44/62 protein then dissociates from the complex, retaining its catalytic activity.

Elaborating on this theme, ATP binding/hydrolysis by the 44/62 protein may act as signal for the clamp loading function of the protein. In one mechanism, the 44/62 protein is activated by ATP binding/hydrolysis to bind DNA, and the activated form of the protein then facilitates loading of the 45 protein onto the DNA primer/template. Once ADP and/or P_i dissociates from the activated enzyme form, the 44/62 protein is deactivated and released to commence another loading cycle. This type of ATP/ADP switch mechanism would then be similar to the mechanistic action of the RecA protein (Kowalczykowski & Eggleston, 1994) in which the binding and hydrolysis of ATP by the RecA protein is the "on-signal" to promote RecA binding to DNA while the ADP-bound RecA state is the "off-signal" (Kowalczykowski & Krupp, 1995). Alternatively, it is possible that the energy of ATP hydrolysis drives a physical interaction between the 44/62 and 45 proteins transducing a conformation in the latter to increase its affinity for DNA. Force generation by the hydrolysis of ATP may be required for the 44/62 protein to physically open and/or close the 45 protein onto duplex DNA. This process would resemble the mechanism of ATP hydrolysis by the actin/myosin system in which motility is mediated by the energy derived from ATP hydrolysis [reviewed by Hibberd and Trentham (1986) and Johnson (1995)].

We have attempted to distinguish among these various scenarios by a more extensive analysis of ATP hydrolysis catalyzed by the 44/62 protein, focusing on the possibility of a phosphorylated 45 protein and/or holoenzyme complex, the order and kinetics of ADP and P_i release, and the detection and trapping of differing 44/62 protein conformational or liganded states with concomitant effects on holoenzyme assembly. Collectively, the data demonstrate an absolute requirement for ATP hydrolysis by the 44/62 protein for loading of the 45 protein onto duplex DNA. The 44/62 protein is described in terms of a molecular motor during loading of the sliding clamp onto DNA.

MATERIALS AND METHODS

Materials. [γ-^{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). ATP, phosphoenolpyruvate, $MgCl_2$, $Mg(OAc)_2$, ADP−morpholidate, and all buffers were from Sigma. NADH was from Boehringer Mannheim. [^{18}O]H_2O (98% purity) was obtained from Cambridge Isotopes while PCl_5 was from Aldrich Chemicals. All other materials were obtained from commercial sources and were of the highest available quality. T4 polynucleotide kinase was from United States Biochemical. 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). Both the 44/62 protein and 45 protein were purified from overproducing strains obtained from William Konigsberg (Yale University). The concentrations 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 Bio-34/62-mer and Bio-34/62/36-mer DNA substrates used in this study were constructed as previously described (Berdis & Benkovic, 1996). Duplex DNA was purified as described by Capson et al. (1992) and quantitated as described by Kuchta et al. (1987).

Assays for the Dual Functionality of the 44/62 Protein. All ATP hydrolysis assays were performed as previously described (Berdis & Benkovic, 1996). The ability of the 44/62 to catalyze holoenzyme complex formation was measured using the strand displacement assay previously described (Kaboord & Benkovic, 1995; Berdis & Benkovic, 1996).

Attempts To Measure Protein Phosphorylation. Various complexes were formed in 100-μL reaction volumes containing 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol. Reactions were initiated by mixing 250 nM Bio-34/62/36-mer, 1 mM streptavidin, and 100 mM ATP with 50 nM [γ-^{32}P]ATP in assay buffer with 250 nM 44/62 protein and 250 nM 45 protein; 250 nM T4 exo$^-$ polymerase was included where denoted. Reactions were allowed to incubate for 15 s and then passed through a 1-mL G-25 column for 2 min. Radioactivity was measured prior to and after elution and was expressed as cpm $\times$ 10^6/mL.

Formation of the holoenzyme complex was measured by the strand displacement assays described above. Formation of the holoenzyme complex was measured prior to and after elution through the G-25 resin. Control experiments using T4 exo$^-$ polymerase in the absence of the accessory proteins were also performed. Reaction products were analyzed and quantitated as described above.

Positional Isotope Effect Measurements. [^{18}O]H_3PO_4 was synthesized from [^{18}O]H_2O and PCl_5 by the method described by Risley and Van Etten (1978). The resulting [^{18}O]H_3PO_4 was purified using a Dowex AGX8 cation exchange column. The concentration of phosphate was determined spectrophotometrically by reacting [^{18}O]H_3PO_4 with ammonium molybdate in sulfuric acid and measuring the absorbance at 630 nm using known concentrations of KP_i as a standard. [^{18}O]ATP enriched in the four terminal oxygens was generated from [^{18}O]H_3PO_4 via reaction with ADP−morpholidate by the procedure of Wehrli et al. (1965) and Middlefort and Rose (1976). Briefly, the method involves condensation of ADP−morpholidate and [^{18}O]H_3PO_4 under anhydrous and anaerobic conditions for 24 h. The [^{18}O]ATP was then purified by elution from DEAE-Sephacel (2 × 40 cm) using a 1 L gradient of 0 −1 M TEAB. [^{18}O]ATP was distinguished from ADP and ADP−morpholidate by using two different coupled assay systems. The first system consisted of converting ATP to ADP using 10 mM glucose and 3 units of hexokinase in the presence of 1 mM $NADP^+$ and 4 units of glucose-6-phosphate dehydrogenase. The production of NADPH from $NADP^+$ is measured spectrophotometrically at 340 nm using an extinction coefficient of 6220 M^{-1} cm^{-1}. The second method consists of measuring ADP using 3 mM phosphoenolpyruvate and 200 μM NADH in the presence of 6 units of pyruvate kinase and 6 units of lactate dehydrogenase. The production of NAD^+ from NADH is measured spectrophotometrically at 340 nm using an extinction coefficient of 6220 M^{-1} cm^{-1}. Fractions containing [^{18}O]ATP were free of ADP, and the pooled fractions were concentrated by lyophilization. The concentration of [^{18}O]-ATP was determined using the coupled assay system described above.

Positional isotope exchange reactions were performed in 300 μL reaction volumes containing 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol. Reactions contained 250 nM 44/62 protein, 250 nM 45 protein, 5 mM [^{18}O]ATP, and 10 mM $Mg(OAc_2)_2$; 250 nM Bio-34/62/36-mer primer/template was added where denoted. The reactions were quenched by the addition of 0.5 M EDTA to obtain a final concentration of 80 mM EDTA. Proteins were removed by phenol/chloroform extraction followed by centrifugation through a Centricon 30 filtration apparatus at 5000 rpm for 20 min at 4 °C. The filtrate was then diluted 1:1 with D_2O. All NMR experiments were performed on a Bruker 500 Fourier transform spectrophotometer operating at 202.45 MHz. Data were collected between 15 000 and 25 000 scans with a pulse of 7 ms using a sweep width of 7352.9 Hz.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear unless otherwise stated. Data were fitted using the appropriate rate equations and computer programs developed by Cleland (1979). Data for competitive inhibition were fitted using eq 1:

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (1)$$

where v is the initial velocity, V is the maximum velocity, A is the reactant concentration, K_a is the Michaelis constant for A, I is the inhibitor concentration, and K_{is} is the slope inhibition constant for I. Data for Dixon plot analysis were fit to equation 2:

$$y = Ax + B \quad (2)$$

in which y is $1/v$, x is the inhibitor concentration, A is the slope, and B is the ordinate intercept. Values of K_i are obtained by determining the abscissa intercept ratio (B/A) divided by 2. Standard errors were determined by the following:

$$\mathrm{SE} = [\mathrm{int/slope}[(\mathrm{SE} \times \mathrm{int/int})^2 + \mathrm{SE} \times (\mathrm{slope/slope})^2]^{1/2}]/2 \quad (3)$$

where all values are determined by computer fit.

Data for nonlinear inhibition were fitted to eq 4:

$$y = Ae^{-kt} + B \quad (4)$$

where y is the velocity, A is the amplitude of the curve, k is the apparent first-order rate constant, and B is a defined constant.

RESULTS AND DISCUSSION

The Bacteriophage T4 Holoenzyme Complex Is Not Phosphorylated by the 44/62 Protein. Allosteric changes in proteins can be driven in some instances by protein phosphorylation. Certain transcription and replication factors are common examples of protein−DNA interactions that are either strengthened or dependent upon phosphorylation of the DNA-binding protein. For example, SV40 DNA replication is enhanced when the T antigen is specifically phosphorylated on threonine 124 by the p34cdc2 kinase (McVey et al., 1989). By analogy, it is possible that the bacteriophage T4 DNA replication system adopts a similar strategy to achieve highly processive DNA synthesis in which the 45 protein and/or DNA polymerase is specifically phosphorylated by the catalytic action of the 44/62 protein. Once phosphorylated, the 45·polymerase complex would become highly stable on duplex DNA, an absolute requirement for processive DNA synthesis.

To determine if the 44/62 protein acts as a protein kinase to phosphorylate the 45 protein, a series of experiments was performed to isolate the possible phosphorylated 45 protein as part of the holoenzyme complex. The 44/62 protein displays a high rate of ATP consumption in the presence of duplex DNA and 45 protein (Berdis & Benkovic, 1996). In the absence of T4 polymerase, this high rate of ATP consumption might be caused by the 44/62 protein acting as a kinase to phosphorylate the 45 protein, enabling the 45 protein to bind to DNA. Since the 45 protein does not remain stably associated with the DNA in the absence of polymerase, the phosphorylated 45 protein dissociates from the primer/template in a process hypothetically involving (auto)dephosphorylation. However, using the forked, biotinylated DNA substrate, it is possible to form the stable 45· polymerase complex for which ATP consumption is not required to maintain its stability (Berdis & Benkovic, 1996). Accordingly, the holoenzyme complex consisting of the

Table 1: Summary of Detection of Phosphorylated 45 Protein and/or Polymerase by Gel Filtration Techniques[a]

condition	prior to elution	after elution	relative percent
[γ-^{32}P]ATP alone	1.6[b]	0.07[c]	4.1[d]
250 nM 44/62 & 45, 250 nM Bio-34/62/ 36-mer, 1 μM Streptavidin	1.35	0.042	3.1
250 nM 44/62, 45, & T4 exo$^-$, 250 nM Bio-34/62/ 36-mer 1 μM Streptavidin	1.86	0.039	2.1

[a] All assays were performed in a buffer consisting of 25 mM Tris−OAc (pH 7.5), 150 mM KOAc, and 10 mM 2-mercaptoethanol. [b] Values represent the amount of radioactivity present before elution through the gel filtration column and are expressed as cpm $\times$ 10^6/mL. [c] Values represent the amount of radioactivity present after elution through the gel filtration column and are expressed as cpm $\times$ 10^6/mL. [d] Values represent the ratio of the amount of radioactivity present after elution versus before elution through the gel filtration column. Results are discussed in the text.

phosphorylated 45 protein and T4 exo$^-$ polymerase should thus be isolatable, since the lifetime of the holoenzyme is several minutes ($t_{1/2}$ = 7 min) (Kaboord & Benkovic, 1995).

A series of experiments in which the holoenzyme was assembled and then separated from the remaining radiolabeled [γ-^{32}P]ATP by column exclusion chromatography are summarized in Table 1. A control reaction measuring the amount of radiolabeled [γ-^{32}P]ATP retained by the column in the absence of accessory proteins showed that 4.1% of the label appeared in the void volume. This percentage was calculated from the radioactivity appearing in the void volume divided by the total radioactivity of the sample. In a second control experiment, a reaction mixture consisting of [γ-^{32}P]ATP, accessory proteins, and Bio-34/62-mer resulted in 3.1% of the radioactivity being eluted in the void volume. The lack of appreciable labeled 45 protein bound to DNA might simply reflect the instability of this binary complex and corresponding loss of phosphorylation in the absence of the polymerase. When the holoenzyme was assembled using stoichiometric concentrations (250 nM) of Bio-34/62/36-mer (in the presence of streptavidin), 44/62 protein, 45 protein, T4 exo$^-$ polymerase, and [γ-^{32}P]ATP, the percentage of label appearing in the void volume was still only 2.1%. If the 45 protein was phosphorylated and present as holoenzyme in the void volume, then at least 20% of the label should have been found in the void volume. A small aliquot of the void volume, which was run on a 12% SDS−polyacrylamide gel and Coomasie stained (data not shown), revealed all proteins were present.

Strand displacement assays were also performed to check that the holoenzyme complex was assembled under these conditions as well as to demonstrate that the holoenzyme complex was stable during elution through the gel filtration column. Briefly, holoenzyme was formed from 250 nM Bio-34/62/36-mer (1 μM streptavidin) with 250 nM 44/62 and 45 protein in the presence of 100 μM ATP. The T4 exo$^-$ polymerase (250 nM) was added, and the reaction was allowed to incubate 15 s before passage through the gel filtration column. An aliquot of the reaction solution prior to and after elution was mixed with dCTP followed by the remaining dNTPs and single-stranded DNA trap to extend any assembled holoenzyme complexes. The 62-mer product is produced in both aliquots (Figure 1A,B), indicating that holoenzyme complex was formed and stable through the elution process. A control reaction also was performed in which the polymerase and DNA in the absence of accessory proteins were passed through the gel filtration column. Aliquots of the reaction solution obtained prior to and after elution were mixed with dCTP followed by the remaining dNTPs and single-stranded DNA trap. No strand displacement products were found for either aliquot (Figure 1C,D), consistent with the anticipated behavior of the polymerase in the absence of the accessory proteins (Kaboord & Benkovic, 1995). Since phosphorylation of either the 45 protein or the polymerase was not detected, we conclude there is no direct evidence for the formation of a phosphorylated holoenzyme complex. We cannot, however, eliminate the possibility of a transiently phosphorylated 45 protein that would be responsible for holoenzyme complex assembly. If this species is formed, it must be highly unstable since the lifetime of the phosphorylated intermediate must be less than 1 s, the time required for holoenzyme assembly (Kaboord & Benkovic, 1995; Berdis & Benkovic, 1996).

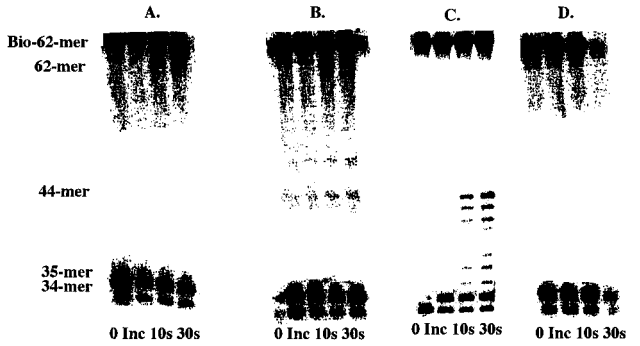
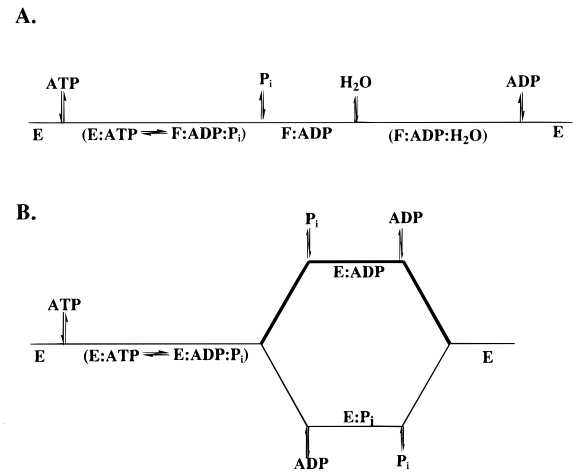


FIGURE 1: Strand displacement capabilities of the holoenzyme complex prior to elution (A) and after elution (B) through a gel filtration column. The holoenzyme was assembled, eluted, and monitored for strand displacement capabilities as described under Materials and Methods. Strand displacement products formed by the holoenzyme complex were analyzed on a 16% denaturing sequence gel and are denoted as 62-mer while shorter products (<44-mer) are produced by nucleotide incorporation by the T4 exo$^-$ polymerase alone. For comparison, strand displacement capabilities of T4 exo$^-$ polymerase in the absence of accessory proteins prior to elution (C) and after elution (D) through a gel filtration column are also shown. The zero point (0) indicates DNA·holoenzyme in the absence of dNTPs. Inc refers to incubation of the DNA·holoenzyme in the presence of dCTP while 10 and 30 refer to the time points quenched after the addition of the remaining dNTPs and single-stranded trap.

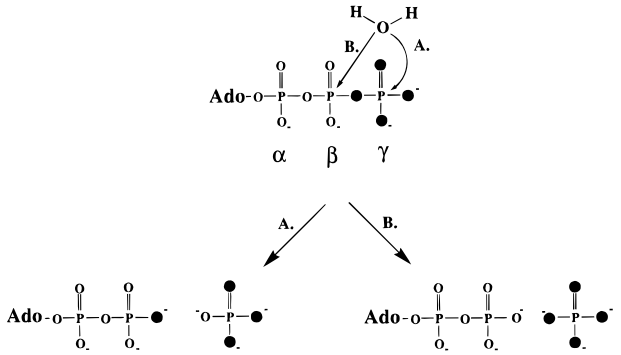
Order and Kinetics of Product Release. An alternative model for ATP hydrolysis by the 44/62 protein in the assembly of the holoenzyme complex is that either a 44/62·ADP or a 44/62·P_i complex formed after ATP hydrolysis could potentiate the formation of the sliding clamp to which the polymerase binds with high affinity. By determining the order and kinetics of product release, we attempted to distinguish different liganded states of the 44/62 protein acting during the clamp loading process. Several minimal kinetic mechanisms for ATP hydrolysis by the 44/62 protein can be described *a priori* in Scheme 1. Mechanism 1A depicts a ping-pong mechanism in which there is formation of a covalently bound ADP enzyme form and presumes attack at the β-phosphorus of the nucleotide. Mechanism 1B depicts possible sequential mechanisms featuring attack at the γ-phosphorus in which release of the products can be either random or ordered if ADP is released prior to P_i or vice versa. Several kinetic techniques were thus employed

Scheme 1: Possible Kinetic Mechanisms for Hydrolysis of ATP Catalyzed by the 44/62 Protein in the Presence of 45 Protein and Duplex DNA[a]



[a] Mechanism A depicts a ping-pong mechanism in which there is formation of a covalently bound ADP enzyme form that undergoes hydrolysis to regenerate free enzyme. Mechanism B depicts a sequential mechanism in which release of products ADP and P_i can either be random or be ordered in which P_i is released prior to ADP (boldface pathway) or vice versa.

Scheme 2: Schematic Outline of the Elucidation of the Kinetic and Chemical Mechanism for the Hydrolysis of ATP Catalyzed by the 44/62 Protein by Application of Positional Isotope Exchange Measurements[a]



[a] In both kinetic mechanisms, [^{18}O]ATP will be hydrolyzed to yield ADP and P_i. In a ping-pong kinetic mechanism, H_2O will attack the covalently bound ADP-enzyme form which will result in a lack of retention of ^{18}O in ADP (A). In a sequential mechanism, H_2O will attack the γ-phosphate, resulting in retention of ^{18}O in the β-phosphate of ADP (B). ^{18}O is denoted by blackened circles while ^{16}O is denoted by opened circles.

to distinguish among these differing kinetic mechanisms.

(1) Isotope Exchange Measurements. The ping-pong mechanism 1A can be differeniated from the sequential mechanism 1B by determining the position of bond cleavage (Scheme 2). For the ping-pong mechanism using ^{18}O γ-labeled ATP, nucleophilic attack on the β-phosphate leads to the expulsion of the ^{18}O at the β,γ position, and thus only ^{16}O should be found in the ADP while ^{18}O should be retained in the P_i. Conversely, in the sequential mechanism 1B, one would expect attack by either H_2O or an enzymic residue on the γ-phosphate, leading to ^{18}O in the product ADP and ^{16}O in P_i from the ultimate incorporation of ^{16}O from water. Figure 2A depicts the β-phosphate peak of the synthesized [^{18}O]ATP, indicating the purity of the isotopic enrichment. Analysis of the β-phosphate as well as the γ-phosphate (data not shown) indicated that greater than 95% of the ATP

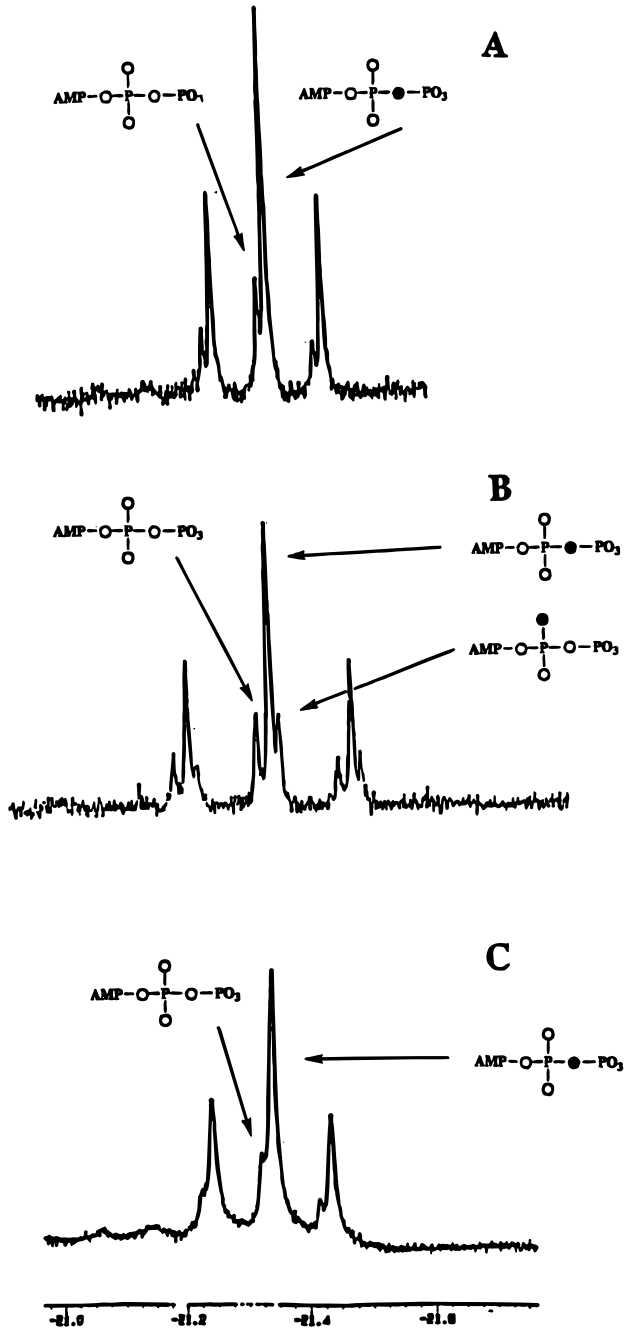


FIGURE 2: (A) ^{31}P NMR spectrum of the β-phosphorus group of 1 mM [^{18}O]ATP. (B) ^{31}P NMR spectrum of the β-phosphorus group of [^{18}O]ATP after reaction with GAR transformylase, an enzyme known to display positional isotope exchange. (C) ^{31}P NMR spectrum of the β-phosphorus group of [^{18}O]ATP after reaction with the 44/62 protein in the presence of 45 protein and duplex DNA.

generated possessed ^{18}O in all four positions of the γ-phosphate. This high isotope enrichment allowed for measurement of both the position of bond cleavage and also the positional isotope exchange by examining either the β- or the γ-phosphate peak. Positional isotope exchange measurements were performed specifically examining the ATP hydrolysis reaction catalyzed by the 44/62 protein in the presence of the 45 protein and in the presence of both the 45 protein and Bio-34/62-mer DNA substrate. The reaction was allowed to proceed to 100% completion, and the β peak of the resulting ADP was analyzed for positional isotope exchange. Comparison of the spectra of the β peak of the reacted to unreacted ATP sample revealed no accumulation

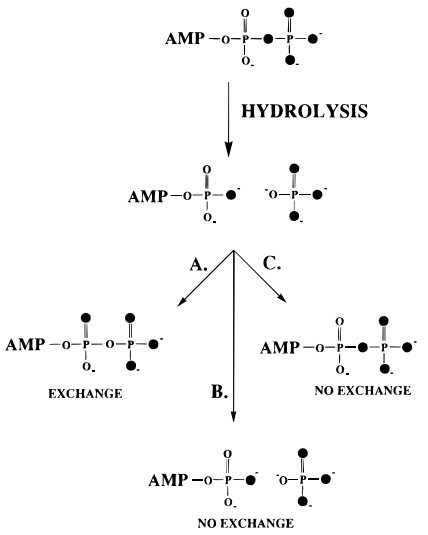
of ^{18}O that would arise from positional isotope exchange (Figure 2C).[2] Furthermore, inspection of the γ-phosphate group produced by hydrolysis for ^{18}O content showed incorporation of one ^{16}O. Thus, mechanism 1A is clearly excluded by the finding that ^{16}O from the medium appears in the P_i generated during the course of the positional isotope effect measurements. The significance in finding no detectable positional isotope exchange is discussed in more detail below.

(2) Product Inhibition Studies. Product inhibition studies were performed in an attempt to determine the order of product release as well as the location of the rate-limiting step along the reaction pathway. All ATPase activity studies of the 44/62 protein were performed in the presence of stoichiometric concentrations of 45 protein and Bio-34/62-mer DNA substrate (in the absence of streptavidin) to ensure maximal ATPase activity (Berdis & Benkovic, 1996). Inhibition studies using ADP as the product were performed by direct analysis of the hydrolysis of [γ-^{32}P]ATP as previously described (Berdis & Benkovic, 1996). The concentration of ADP was varied from 0 to 100 μM, maintaining the concentration of unlabeled ATP at 100 μM and [γ-^{32}P]ATP at 50 nM. The rate of ATP hydrolysis by the 44/62 protein decreased linearly as a function of increasing ADP concentration, and Dixon plot analysis reveals a $K_{i\ ADP} = 20 \pm 5\ \mu$M. The inhibition exhibited by ADP is consistent with this product binding to free enzyme, the same enzyme form that binds ATP.

Product inhibition studies using phosphate were performed using both the coupled assay and the radioactive assay as previously described (Berdis & Benkovic, 1996). The concentration of the 44/62 protein was 250 nM as were the concentrations of 45 protein and Bio-34/62-mer DNA substrate. ATP was maintained at 100 μM, and a concentration of 50 nM [γ-^{32}P]ATP was used in the radioactive assay. Using either method, inhibition of the ATPase activity of the 44/62 protein was not detected up to a concentration of 10 mM P_i. Since P_i does not inhibit, no conclusions can be drawn about the kinetic sequence. Since the 44/62 protein displays biphasic kinetic behavior under similar conditions (Berdis & Benkovic, 1995), catalysis is most likely not rate-limiting. Thus, the release of P_i may be likely irreversible due to a possible conformational change in the protein.

The positional isotope exchange measurements using highly enriched ATP discussed above also aided in determining further aspects of the sequential mechanism. Positional isotope exchange measurements can be used to probe any enzyme-catalyzed reaction in which functional nonequivalent groups become torsionally equivalent via a reversible reaction intermediate. In this case, the intermediate could be E·ADP·P_i or E$-P_i$·ADP (a phosphoryl enzyme). Positional isotope exchange of the ATPase reaction catalyzed by the 44/62 protein was followed by measuring the positional exchange of ^{18}O in ATP from a bridging (β, γ) to a nonbridging (β) position (Scheme 3) by NMR techniques. Because of rotational equivalency of the three β nonbridge oxygens of ADP, the re-formation of ATP results in a 67% probability that the labeled oxygen will be found at one of the β nonbridging positions of ATP, provided the reaction is reversible and the β-phosphorus is free to rotate in the active site of the enzyme. If ATP hydrolysis is irreversible, no positional isotope exchange of the ^{18}O label will occur, and the ^{18}O label will be in its original position. Myosin and other ATPases that have a slow rate of P_i release following hydrolysis demonstrate positional isotope exchange, indicating that ATP re-forms at a rate comparable to that of P_i release (Sleep et al., 1980; Sleep & Hutton, 1980; Hackney, 1988). Furthermore, the reversal of hydrolysis may also result in the incorporation of multiple water-derived oxygens (^{16}O) into P_i (provided that P_i is free to rotate) so that the extent of incorporation of water-derived oxygens can provide an estimate for the relative rate of product release versus re-formation of ATP.

Scheme 3: Schematic Outline of Positional Isotope Exchange Measurements for the Hydrolysis of ATP Catalyzed by the 44/62 Protein[a]



[a] [^{18}O]ATP is hydrolyzed, yielding ADP and P_i. Positional isotope exchange will be measured if ATP hydrolysis is reversible (A). No positional isotope exchange will occur if release of one or both products is faster than the rate of ATP-reformation (B) or if there is no rotation of the β-phosphate in the enzyme active site (C). ^{18}O is denoted by blackened circles while ^{16}O is denoted by opened circles.

Figure 2B depicts the spectra of the β-phosphorus peak of ^{18}O-labeled ATP after incubation with GAR transformylase, an enzyme known to undergo reversible ATP hydrolysis (K. Mattia, M. Warren, and S. J. Benkovic, unpublished results). Comparison of Figure 2A and Figure 2B demonstrates splitting of the β-phosphorus peak due to movement of ^{18}O from the bridging position to a nonbridging position and is diagnostic of positional isotope exchange. By contrast, Figure 2C depicts the spectra of the β-phosphorus peak of ^{18}O-labeled ATP after incubation with the 44/62 protein in the presence of stoichiometric 45 protein and DNA substrate. Analysis of the β peak of ^{18}O-labeled ATP after incubation with the 44/62 protein in the presence of stoichiometric 45 protein and DNA substrate that was allowed to proceed to 50% completion also showed a lack of positional isotope exchange (data not shown).

The lack of positional isotope exchange detected in either case indicates that either there is little rotational freedom in the enzyme active site or, most likely, the release of products is fast relative to the rate of ATP reformation. This could be caused either by an extremely slow reverse reaction (ADP·Pi $\rightarrow$ ATP) or by release of P_i followed by a conformational change so that the hydrolysis reaction

[2] We estimate that <20% isotope exchange would not have been detected.

becomes irreversible even if ADP dissociates slowly. The latter scenario would be consistent with the aforementioned product inhibition studies. In the absence of data to the contrary, we will presume the absence of a transient $E-P_i\cdot$ ADP species.

Positional isotope exchange measurements could not be performed to examine the possible formation of the 44/62·ADP·P_i complex alone or in the presence of DNA since the rate of ATP hydrolysis by the 44/62 protein under these conditions is too slow to achieve significant turnover of 5 mM ATP within a reasonable time ($\Delta t_{1/2} > 48$ h).

(3) Aluminum Tetrafluoride Inhibition. To further investigate the kinetics of ATP hydrolysis and product release, molecules that might possibly mimic the transition state for ATP hydrolysis were tested as inhibitors. Inhibition by aluminum tetrafluoride (AlF_4) was performed using the coupled assay system for detection of ATP hydrolysis. There is no inhibition of the coupling enzymes in the presence of either 1 mM aluminum nitrate, 20 mM sodium fluoride, or a mixture of 1 mM aluminum nitrate and 20 mM sodium fluoride.[3] Likewise, neither sodium fluoride nor aluminum nitrate alone at the concentrations listed above inhibits the ATPase activity of the 44/62 protein. Thus, the inhibition by AlF_4 accurately reflects the inhibition of ATP hydrolysis catalyzed by the 44/62 protein. All assays were performed using 250 nM 44/62 protein, 45 protein, and Bio-34/62-mer in the appropriate reaction buffer containing 10 mM $Mg(OAc_2)_2$ and 1 mM ATP. The concentration of aluminum nitrate was varied while a fixed concentration of 10 mM sodium fluoride was used. The time course for AlF_4 inhibition is depicted in Figure 3A. AlF_4 can best be described as a slow irreversible inhibitor since the decrease in the rate of ATP hydrolysis appears to follow a progress curve that reflects complete inactivation of the enzyme (Williams & Morrison, 1979). The rate of inhibition by AlF_4 was fit to a single exponential with a floating end point to yield apparent rate constants for inhibition. Reciprocal rate constants were then plotted versus reciprocal AlF_4 concentrations, and the resulting plot was linear, yielding an apparent K_i of 110 ± 15 μM (Figure 3B). Correcting for the concentration of ATP substrate yields a true K_i for AlF_4 of 18 ± 3 μM.

AlF_4 is a potent inhibitor of many ATPase/GTPase reactions since AlF_4 in conjunction with ADP is proposed to mimic the transition state for ATP hydrolysis (Coleman et al., 1994; Mittal et al., 1996). AlF_4 does not appear to bind to free enzyme since the decrease in the rates of ATP hydrolysis is not linear as one would expect for a typical competitive inhibitor. At face value, this inhibition is consistent with the lack of P_i inhibition. Furthermore, the coupled assay system used in these assays converts any free ADP immediately to ATP. Thus, the inhibition manifested by AlF_4 is dependent upon the formation of the 44/62·ADP enzyme form caused by the enzymatic turnover of ATP and not by the accumulation of any ADP·AlF_4 free in solution. This indicates that the AlF_4 binds to the 44/62·ADP enzyme form, inferring that the release of ADP from the 44/62 protein must be at least partially rate-limiting. Once formed, the 44/62·ADP·AlF_4 complex must be highly stable since

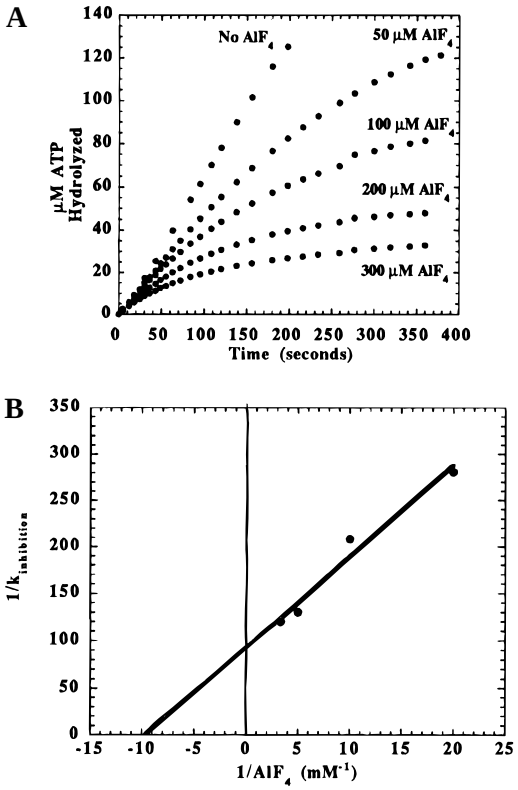


FIGURE 3: (A) Time course measuring ATP hydrolysis by the 44/62 protein in the presence of increasing concentrations of AlF_4. The time course reflects a turnover-dependent "inactivation" of the 44/62 protein by AlF_4. Assays were performed using 250 nM 44/62 protein, 250 nM 45 protein, 250 nM Bio-34/62/36-mer, 1 μM streptavidin, 1 mM ATP, 10 mM $Mg(OAc_2)_2$, 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. Aluminum nitrate was fixed at 10 mM while the concentration of sodium fluoride was varied to obtain the concentration of AlF_4 indicated. (B) Determination of the appK_i for AlF_4 by plotting reciprocal rate constants of inactivation versus reciprocal inhibitor concentrations.

complete enzyme "inactivation" is obtained at even moderate levels of AlF_4. The inhibition by AlF_4 is good evidence for an ordered product release kinetic mechanism in which P_i is released prior to ADP.[4] However, if release of ADP from the 44/62·ADP complex is at least partially rate-limiting, one would also expect that phosphate at sufficiently high concentrations should inhibit as well. The lack of P_i inhibition can most easily be explained by a large difference in affinity between P_i and AlF_4 for the 44/62·ADP species.

(4) Dead End Inhibition Studies. Dead end inhibition studies were performed to further elucidate the kinetics and dynamics of nucleotide binding. All dead end inhibition studies were performed using the coupled assay system for detecting ATP hydrolysis. These ATP analogs (Figure 4) did not inhibit the coupling enzymes, and thus the kinetics accurately reflect inhibition of ATP hydrolysis by the 44/62 protein. All assays were performed using 250 nM 44/62 protein, 45 protein, and Bio-34/62-mer DNA substrate in the appropriate reaction buffer containing 10 mM $Mg(OAc_2)_2$. For Dixon plot analysis, the concentration of ATP was maintained constant at 100 μM while the concentration of

[3] The combination of aluminum nitrate and sodium fluoride produces aluminum tetrafluoride as well as other various aluminum hydroxyfluoride complexes.

[4] It is also possible that AlF_4 binds to a covalently bound ADP form of the 44/62 protein which would arise from a ping-pong mechanism. However, the positional isotope exchange measurements described clearly ruled against a ping-pong mechanism.

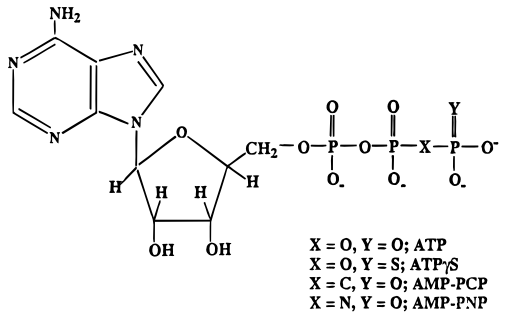


FIGURE 4: Structure of ATP and ATP analogs used for inhibition studies.

Table 2: Summary of Product and Dead End Inhibition Patterns and Inhibition Constants for the 44/62 Protein[a]

inhibitor	pattern	K_i (μM)
ADP	Dixon plot	20 ± 5
PO_4^{3-}		no inhibition up to 10 mM
AlF_4	slow-irreversible inhibition[b]	18 ± 3
ATPγS	competitive[c]	45 ± 5
AMP-PCP	competitive	2200 ± 300
AMP-PNP	competitive	1200 ± 200
AMP	Dixon plot	2400 ± 100
ADP-ribose	Dixon plot	2500 ± 300
1 mM ADP and 10 mM P_i		no attenuation of inhibition[d]
1 mM AMP and 10 mM P_i		no attenuation of inhibition
1 mM AMP and 10 mM PP_i		no attenuation of inhibition
GTP		no inhibition or substrate activity up to 2 mM

[a] All assays were performed as described under Materials and Methods. The concentration of ATP was maintained at 100 μM for Dixon plot analysis or varied from 100 μM to 1 mM for competitive inhibition patterns. [b] The inhibition displayed by AlF_4 is nonlinear and can best be described as a slow-inactivator or irreversible inhibitor. See text for discussion. [c] Competitive inhibition patterns were performed varying the concentration of ATP from 100 μM to 1 mM at several fixed levels of inhibitor, including the absence of inhibitor. [d] Slight inhibition was obtained under these conditions as the concentration of the inhibitor AMP was maintained at $0.5K_i$. However, no further inhibition was detected upon the addition of either PP_i, P_i, or AlF_4 since identical rates of ATP hydrolysis were obtained in their absence as were obtained in their presence.

inhibitor was varied. In cases where inhibition patterns were generated, the concentration of ATP was varied at several fixed concentrations of inhibitor, including the absence of inhibitor. In all cases, the initial velocities measured were linear. ATPγS (Figure 4) is a competitive inhibitor of ATP, yielding a $K_i = 45 \pm 5$ μM. Both AMP-PCP and AMP-PNP (Figure 4) also display competitive inhibition versus ATP. However, both analogs yield substantially higher K_i values than ATPγS, since the K_i for AMP-PCP is 2.2 ± 0.3 mM and that for AMP-PNP is 1.2 ± 0.2 mM. Inhibition constants for AMP, ADP-ribose, and GTP were measured using Dixon plot analysis. All inhibition constants are summarized in Table 2.

The competitive inhibition exhibited by the ATP analogs, ATPγS, AMP-PNP, and AMP-PCP, indicates that each inhibitor competes with ATP for free enzyme. While the qualitative nature of the dead end inhibition does not aid in further elucidating the mechanism of ATP hydrolysis by the accessory protein, the quantitative nature of the data does yield important information regarding nucleotide binding. As shown in Figure 4, ATPγS differs from ATP only by substitution of S for O in the γ-phosphate position. As a result, ATPγS is nonhydrolyzable and is a competitive inhibitor of the 44/62 protein, binding tightly with a K_i value of 45 μM. This value is the same order of magnitude as that of the substrate ATP (100 μM) and that of the product ADP (20 μM). The ATP analogs AMP-PCP and AMP-PNP (Figure 4) differ from ATP only at the β,γ-phosphate position in which the β,γ-oxygen of ATP is replaced either by a methylene group ($-CH_2-$) in the case of AMP-PCP or by a nitrogen in the case of AMP-PNP. Although these analogs are nonhydrolyzable, one would expect that their binding affinities should not be severely affected by these substitutions. In contrast to ATP, ADP, and ATPγS, these analogs bind with less affinity to the 44/62 protein. AMP-PNP and AMP-PCP bind approximately 12−22 times less tightly than ATP, indicating that the β,γ-phosphate portion of ATP plays an important and intricate role in nucleotide binding. This large discrimination in nucleotide binding may be caused by active site residues forming hydrogen bonds with the bridging β- and γ-oxygens of ATP. Replacement of the hydrogen bond acceptor oxygen with the methylene group or an N−H moiety would destroy these possible interactions and thus could explain the strong discrimination against these nucleotides binding. Furthermore, AMP which lacks both the β- and γ-phosphate groups binds with poor affinity and possesses a K_i value more comparable to AMP-PNP and AMP-PCP than to ATP or ADP.

Inhibition studies were also performed using various combinations of analogs in an attempt to mimic ATP or ADP. For example, AMP plus pyrophosphate and AMP plus phosphate were used to mimic ATP and ADP. These experiments examined whether one analog would increase the affinity of the second molecule for the enzyme. In all cases examined, there is no further decrease in the rate of ATP hydrolysis compared to that detected in the presence of the nucleotide inhibitor alone. Results of the combination inhibitor experiments are summarized in Table 2. While these experiments again represent a negative result, it is most likely that the 44/62 protein possesses a mechanism for discriminating hydrolyzable nucleotides from those that are not. Nucleotide discrimination would ensure that the 44/62 protein is always catalytically competent to load the 45 protein onto duplex DNA for holoenzyme assembly, an absolute requirement for efficient DNA replication *in vivo*.

ATP Turnover Is Required for Holoenzyme Complex Formation. The ability of the 44/62 protein to catalyze the formation of the holoenzyme complex was examined as a function of the kinetic steps involved with ATP hydrolysis. Ideally, we wish to identify the step in the ATP hydrolysis pathway catalyzed by the 44/62 protein associated with holoenzyme formation. In other words, is the holoenzyme formed during a prehydrolysis event (through binding of ATP or a nonhydrolyzable analog), during the hydrolysis event (driven by the free energy of hydrolysis), or during a posthydrolysis event (through a 44/62·ADP complex)? To test for these possibilities, strand displacement assays were performed under conditions that would closely mimic these possible steps along the ATP hydrolysis pathway.

Assembly of the holoenzyme was generally performed in which a solution of Bio-34/62/36-mer (500 nM) and streptavidin (1 μM) was incubated with 550 nM 44/62 protein and

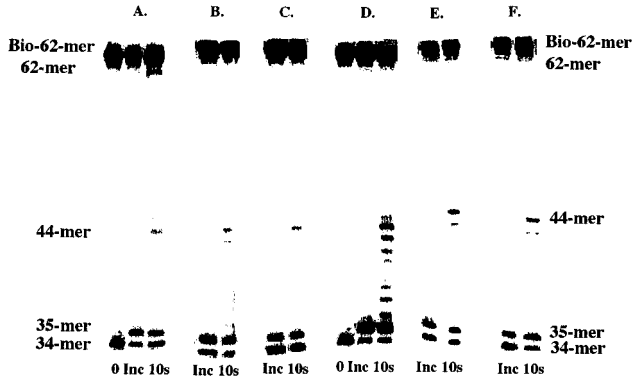


FIGURE 5: Formation of the bacteriophage T4 DNA holoenzyme complex requires ATP hydrolysis. The holoenzyme complex was assembled and assayed for strand displacement capabilities as described under Materials and Methods using ATP (A), ATPγS (B), AMP-PCP (C), ADP (D), ADP and P_i (E), and ADP and AlF_4 (F). Strand displacement products formed by the holoenzyme complex were analyzed on a 16% denaturing sequencing gel and are denoted as 62-mer while shorter products (<44-mer) are produced by nucleotide incorporation by the T4 exo$^-$ polymerase alone. The zero point (0) indicates DNA·holoenzyme in the absence of dNTPs. Inc refers to incubation of the DNA·holoenzyme in the presence of dCTP while 10 refers to the time point quenched after the addition of the remaining dNTPs and single-stranded trap.

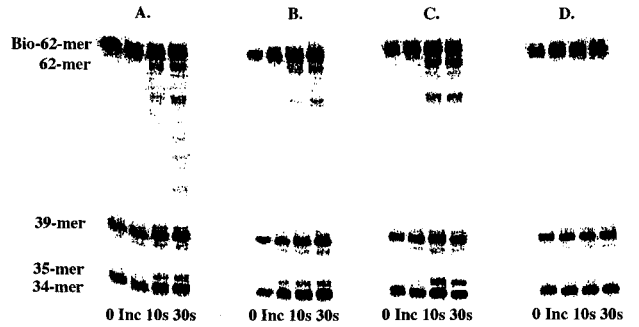


FIGURE 6: Inhibition of the 44/62 protein by AlF_4 is dependent upon ATP turnover. The holoenzyme was assembled and assayed for strand displacement capabilities as described under Materials and Methods using (A) ATP (Δt = 10 s), (B) ATP and AlF_4 (Δt = 10 s), (C) ATP (Δt = 10 min), and (D) ATP and AlF_4 (Δt = 10 min). Strand displacement products formed by the holoenzyme complex were analyzed on a 16% denaturing sequencing gel and are denoted as 62-mer while shorter products (<44-mer) are produced by nucleotide incorporation by the T4 exo$^-$ polymerase alone. The zero point (0) indicates DNA·holoenzyme in the absence of dNTPs. Inc refers to incubation of the DNA·holonenzyme in the presence of dCTP while 10 and 30 refer to the time points quenched after the addition of the remaining dNTPs and single-stranded trap. The 39-mer band present in these experiments is a contamination that arose from purification of Bio-34/62-mer. However, this small contamination does not adversely affect holoenzyme formation or the activity of the holoenzyme or polymerase itself.

45 protein in the presence of 1 mM ATP or analogs of ATP for 10 s. 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) along with single-stranded DNA were added to initiate processive DNA synthesis by any assembled holoenzyme complexes. Control reactions using 1 mM ATP as the hydrolyzable nucleotide indicated that holoenzyme complex was formed owing to production of the strand displacement product (62-mer) as shown in Figure 5A. The formation of the holoenzyme complex under these conditions is consistent with previous reports that ATP hydrolysis is absolutely required for holoenzyme formation (Jarvis et al., 1991), although the effect of nucleotide binding alone causing holoenzyme complex formation was not conclusively ruled out from these experiments. To test whether binding of ATP or an analog is sufficient for holoenzyme complex formation, strand displacement assays were performed substituting 1 mM ATPγS (Figure 5B) or 10 mM AMP-PCP (Figure 5C) for ATP. Since saturating levels of nucleotide analog were employed, all 44/62 protein should be in the 44/62·ATPγS or 44/62·AMP-PCP enzyme form. The lack of strand displacement products obtained under these conditions indicates that holoenzyme was not formed, demonstrating that a pre-ATP hydrolysis event such as nucleotide binding is not sufficient for holoenzyme complex formation.

To test whether a post-ATP hydrolysis event is sufficient for holoenzyme complex formation, strand displacement assays were performed using 1 mM ADP in place of ATP (Figure 5D). Since ADP binds with relatively high affinity (K_i = 20 μM), all 44/62 protein should be in the 44/62·ADP enzyme form. No strand displacement products were detected under these conditions, indicating that a post-ATP hydrolysis event is not sufficient for holoenzyme formation.

Structural mimics of the ATP hydrolysis transition state were also tested for their ability to support holoenzyme formation. Strand displacement products were not detected using a combination of 1 mM ADP and 10 mM phosphate (Figure 5E). Holoenzyme formation was likewise undetected in the presence of 1 mM ADP and 500 μM AlF_4 (Figure 5F). Under these conditions, ADP should bind to 44/62 protein and be trapped on the enzyme by AlF_4 to form a mimic of the transition state for ATP hydrolysis.

We note, however, that the appropriate 44/62·ADP·AlF_4 complex may not be formed by the addition of each individual component. Thus, the 44/62·ADP·AlF_4 complex was formed by enzymatic turnover of ATP by the 44/62 protein in the presence of AlF_4 to promote holoenzyme formation (or lack thereof) through this complex. Strand displacement assays were performed as described previously except that the 44/62 protein was incubated with the 45 protein and Bio-34/62/36-mer substrate (plus 1 μM streptavidin) in the presence of 1 mM ATP and 500 μM AlF_4 for 10 s prior to the addition of T4 exo$^-$ polymerase and dCTP. In this case, holoenzyme was produced, and quantitation of the 62-mer product formed indicated that the same amount was produced whether AlF_4 was added or omitted (Figure 6A,B). Thus, AlF_4 does not immediately inhibit the loading of the 45 protein by the 44/62 protein, consistent with the steady-state inhibition data indicating that AlF_4 inhibition of the 44/62 protein is dependent upon ATP turnover. The lack of immediate inhibition also indicates that AlF_4 does not inhibit the T4 exo$^-$ polymerase. The formation of holoenzyme under these conditions is consistent with previous work (Berdis & Benkovic, 1996) demonstrating that only one turnover of the 44/62 protein is required for holoenzyme formation and that ATP hydrolysis is not required for loading of polymerase.

Reactions were then performed incubating the 44/62 protein with the 45 protein and Bio-34/62/36-mer substrate (plus 1 μM streptavidin) in the presence of 1 mM ATP and 500 μM AlF_4 for 10 min prior to the initiation of DNA synthesis. Under these conditions, holoenzyme was not formed as indicated by the lack of strand displacement products (Figure 6D). A control reaction under identical

conditions but in the absence of AlF_4 yielded substantial 62-mer product (Figure 6C), indicative of holoenzyme complex formation. The lack of holoenzyme formation at long incubation times in the presence of ATP and AlF_4 is not simply due to a depletion of ATP but instead reflects the turnover-dependent nature of AlF_4 inhibition.

Collectively, these results indicate that AlF_4 does not immediately inhibit the 44/62 protein, but instead requires enzymatic turnover of ATP to form the 44/62·ADP enzyme form to which AlF_4 productively binds. Since holoenzyme complex was not formed through the 44/62·ADP·AlF_4 complex, a species resembling the transition state for ATP hydrolysis bound to the 44/62 protein is not sufficient for the assembly of the holoenzyme complex.

The 44/62 Protein Acts as a Molecular Motor. A common mechanism in achieving highly processive DNA replication is the tethering of the DNA polymerase to the primer/template by an auxillary protein generically referred to as the sliding clamp. The sliding clamp essentially decreases the rate at which the polymerase dissociates from the primer/template, thus substantially increasing the polymerase's low intrinsic degree of processivity. Crystal structures of sliding clamps from *Escherichia coli* (Krishna et al., 1994), yeast (Kong et al., 1992), and bacteriophage T4 (J. Kuriyan, personnal communication) reveal that all three processivity factors are ring-shaped and capable of encircling duplex DNA. The closed circular nature of the sliding clamps confirms the need for a clamp loading protein to achieve holoenzyme complex formation. Unfortunately, these crystal structures do not immediately provide mechanistic insight into how the clamps are loaded onto duplex DNA by the clamp loader. We have thus attempted to further elucidate the mechanism by which the clamp is loaded onto duplex DNA by examining what role ATP hydrolysis by the clamp loader plays during this process.

Several models for the role of ATP hydrolysis by the 44/62 protein in the assembly of the holoenzyme complex have been addressed in this study. First, a stable phosphorylated holoenzyme complex was not isolated or detected, suggesting that the 44/62 protein does not act as a protein kinase to phosphorylate stably the 45 protein and/or the polymerase. Second, formation of a high-energy enzyme form with bound P_i responsible for loading of the 45 protein is also unlikely. The order and kinetics of product release from the 44/62 protein during the process of clamp loading argue that phosphate is released prior to ADP, thus arguing against formation of the 44/62·P_i enzyme form. One interpretation of the lack of positional isotope exchange is that ATP hydrolysis by the 44/62 protein may be irreversible, perhaps as a consequence of the off-rate being faster than the rate of ATP re-formation. Although release of ADP appears to be partially rate-limiting as inferred by trapping of ADP on the enzyme by AlF_4, strand displacement assays revealed no holoenzyme formation under conditions in which a 44/62·ADP species should predominate. Thus, although the 44/62·ADP enzyme form may exist, it is not responsible for holoenzyme assembly. Consequently, we conclude that the energy derived from ATP hydrolysis is absolutely required for loading of the 45 protein onto duplex DNA.

The molecular motors myosin, kinesin, and dynein all hydrolyze ATP concomitant with force generation to mediate motility. By analogy, we propose that the 44/62 protein hydrolyzes ATP concomitant with force generation required for opening and/or closing the ring-shaped 45 protein upon which the clamp is loaded onto duplex DNA. The ATP hydrolysis pathway for the 44/62 protein is very similar in nature to that of the classical molecular motors. Myosin, kinesin, and dynein all possess a low basal rate of ATP hydrolysis that is increased more than 1000-fold in the presence of motile cofactor [reviewed by Johnson (1995)]. The ATPase activity of the 44/62 protein is similarly activated, although both DNA and protein cofactor are required for this large rate enhancement (Berdis & Benkovic, 1996). One can easily envision that the ATPase stimulation of the 44/62 protein is enhanced by the physical interaction with the 45 protein, similar to that of the molecular motor systems interacting with their respective filament partner. Furthermore, transient kinetic measurements of the ATP hydrolysis reaction indicate that myosin (Sadhu & Taylor, 1992), kinesin (Hackney et al., 1989), and the 44/62 protein (Berdis & Benkovic, 1996) all display biphasic kinetic behavior in which there is a rapid burst in ATP consumption followed by a slower steady state rate of consumption. The principal difference between myosin and kinesin is that the rate-limiting step for kinesin is release of ADP whereas for myosin the step is a conformational change before product release. The 44/62 protein appears more similar to myosin in this regard since the release of ADP from the enzyme appears to be partially rate-limiting during turnover. Aluminum tetraflouride binds very tightly to both the 44/62 protein (vide supra) and kinesin (Romberg & Vale, 1993), presumably forming a stable transition-state mimic resulting from the slow(er) release of ADP from the enzyme.

Although the precise molecular events of the mechanochemical cycle are not completely understood, force production in both myosin and kinesin is proposed to be coupled with product release following ATP hydrolysis (Johnson & Gilbert, 1995). With these enzymes, movement of the filaments as a result of ATP hydrolysis has been directly demonstrated (Malik et al., 1994) and in some cases visualized (Svoboda et al., 1993; Svoboda & Block, 1994). We have performed analogous experiments which clearly demonstrate that ATP hydrolysis by the 44/62 protein is absolutely required for the assembly of the holoenzyme complex. Although the dynamics of ring opening and/or closing of the 45 protein are also currently undefined, we propose that the 44/62 protein may catalyze ring opening and/or closing by a mechanism similar to the "power stroke" exhibited by kinesin. We envision that the 44/62 protein acts as a "molecular lever" to open and/or close the ring-shaped 45 protein. The 44/62 protein is a tightly associated complex, containing one 62 protein and four 44 subunits (Jarvis et al., 1989a) which contain the ATP binding and hydrolysis domains. While the function of the 62 protein is presently unclear, it is known that the 62 protein is absolutely required for interactions with the 45 protein (Rush et al., 1989). It is tempting to speculate that the 62 protein acts as the "fulcrum" to physically open the closed, circular 45 protein and that activation (or movement) of the "fulcrum" is derived from the energy of ATP hydrolysis by each 44 subunit. Indeed, we have recently demonstrated that the 44/62 protein and 45 protein form a tight complex in the presence of ATP but in the absence of DNA (Sexton et al., 1996). This model may explain why four ATPs are consumed by the 44/62 protein per each loading event of the 45 protein (Berdis & Benkovic, 1996). Although not

conclusive proof, the similarities in the nature of the ATP-dependent processes (force-generation) as well as in the overall kinetics and mechanism of ATP hydrolysis suggest that the 44/62 protein indeed acts as a molecular motor in the process of clamp loading.

ADDED IN PROOF

A recent paper published by the von Hippel laboratory (Young et al., 1996) has confirmed several of our results and mechanistic interpretations. These authors have established that ADP binds very tightly to the 44/62 protein ($K_d = 14 \pm 7$ μM), identical to our reported K_i value of 20 ± 5 μM, and that phosphate does not inhibit the enzyme up to a concentration of 23 mM. They have also demonstrated that ATP hydrolysis is irreversible, confirming our positional isotope exchange measurements. Their conclusion that the "internal" equilibrium constant for ATP hydrolysis is very large and in favor of product formation is consistent with our model of the clamp loader acting as a molecular motor during loading of the 45 protein onto duplex DNA.

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