Division of labor—sequential ATP hydrolysis drives assembly of a DNA polymerase sliding clamp around DNA

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The β sliding clamp encircles DNA and enables processive replication of the *Escherichia coli* genome by DNA polymerase III holoenzyme. The clamp loader, γ complex, assembles β around DNA in an ATP-fueled reaction. Previous studies have shown that γ complex opens the β ring and also interacts with DNA on binding ATP. Here, a rapid kinetic analysis demonstrates that γ complex hydrolyzes two ATP molecules sequentially when placing β around DNA. The first ATP is hydrolyzed fast, at 25–30 s⁻¹, while the second ATP hydrolysis is limited to the steady-state rate of 2 s⁻¹. This step-wise reaction depends on both primed DNA and β . DNA alone promotes rapid hydrolysis of two ATP molecules, while β alone permits hydrolysis of only one ATP. These results suggest that β inserts a slow step between the two ATP hydrolysis events in clamp assembly, during which the clamp loader may perform work on the clamp. Moreover, one ATP hydrolysis is sufficient for release of β from the γ complex. This implies that DNA-dependent hydrolysis of the other ATP is coupled to a separate function, perhaps involving work on DNA. A model is presented in which sequential ATP hydrolysis drives distinct events in the clamp-assembly pathway. We also discuss underlying principles of this step-wise mechanism that may apply to the workings of other ATP-fueled biological machines.

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Introduction

Chromosomal DNA is replicated by multi-protein polymerases that function as rapid and highly processive machines on DNA. The high processivity of these replicases is rooted in a ring-shaped clamp protein that completely encircles DNA. The clamp also binds DNA polymerase and acts as a mobile tether, enabling the polymerase to replicate DNA without dissociation from the template. This sliding clamp mechanism is utilized by DNA polymerases from a variety of organisms ranging from bacteriophage T4 to humans (reviewed in Baker and Bell, 1998). In *Escherichia coli*, for example, the

replicative DNA polymerase III holoenzyme is composed of two core polymerases and numerous accessory proteins, including the β sliding clamp. The core polymerase can extend a primer by only ten or so nucleotides before falling off DNA (Fay *et al.*, 1981). In contrast, the holoenzyme, tethered to the template by β , extends DNA by several thousand nucleotides without dissociation, facilitating efficient duplication of the *E.coli* genome (Fay *et al.*, 1981; reviewed in Kelman and O'Donnell, 1995).

Sliding clamps are typically multimeric, ring-shaped proteins with a central cavity large enough to accommodate double-stranded DNA (Kong *et al.*, 1992; Krishna *et al.*, 1994; Gulbis *et al.*, 1996; reviewed in Kuriyan and O'Donnell, 1994). The β sliding clamp is a circular dimer, formed by the head-to-tail interaction of two semicircle-shaped protomers (35 Å inner diameter; Kong *et al.*, 1992). For a clamp to function as a polymerase tether, it must be assembled at a primed site on DNA where it can bind the polymerase (Stukenberg *et al.*, 1991). This task is performed by a clamp loader, such as the *E.coli* γ complex, which opens the clamp and facilitates its closure around DNA (Turner *et al.*, 1999). The γ complex, as well as clamp loaders from other organisms, power these actions with energy from ATP binding and hydrolysis (reviewed in Sancar and Hearst, 1993; Stillman, 1994; Kelman and O'Donnell, 1995).

The γ complex is a multi-protein assembly of two to four γ subunits, and one each of the δ , δ' , χ and ψ subunits (Maki and Kornberg, 1988; Dallmann and McHenry, 1995; Onrust *et al.*, 1995a). Only the γ subunit binds and hydrolyzes ATP; therefore, it serves as the 'motor' component of the clamp-loading machine (Lee and Walker, 1987; Tsuchihashi and Kornberg, 1989; Xiao *et al.*, 1995). The δ subunit binds and opens the β clamp, serving as the 'clamp opener' component (Naktinis *et al.*, 1995; Turner *et al.*, 1999). The δ' subunit is a 'modulator' that permits or inhibits interaction between δ and β (Turner *et al.*, 1999). The other two subunits, χ and ψ , are not directly involved in clamp loading (Onrust *et al.*, 1991; Xiao *et al.*, 1993). χ binds SSB and facilitates displacement of primase from RNA primers prior to clamp assembly (Kelman *et al.*, 1998; Yuzhakov *et al.*, 1999). The function of ψ is not yet precisely defined.

Our current knowledge of the clamp-assembly mechanism is illustrated in Figure 1A. Briefly, γ complex brings together β and DNA and forms a topological link between the two in an ATP-coupled process. In the absence of ATP, the δ' subunit binds δ within γ complex and blocks its interaction with β . ATP binding changes γ complex conformation, resulting in removal of the δ' block from δ and allowing the clamp opener free access to the clamp (Naktinis *et al.*, 1995; Hingorani and O'Donnell, 1998; Turner *et al.*, 1999). Upon binding β , δ can open the clamp at the dimer interface by itself. Thus, clamp

Fig. 1. Pathway of γ complex-catalyzed β assembly on DNA leading to processive DNA replication. (A) On binding two ATP molecules, the γ complex undergoes a conformational change that promotes its interaction with both β and primed DNA. The δ subunit in γ complex opens the clamp and ATP hydrolysis completes clamp assembly, releasing β and DNA topologically linked to each other. Note, the χ and ψ subunits of γ complex are omitted in this scheme for clarity. (B) DNA synthesis by the core DNA polymerase III on a 30-nt/105-nt primed DNA is processive and yields full-length product only when the polymerase can use a β clamp assembled on the DNA by γ complex.

opening requires no further energy input (e.g. from ATP hydrolysis); ATP binding is necessary and sufficient to promote clamp opening via interaction between the two proteins. Prior to ATP hydrolysis, the proteins also bind primed DNA with high affinity to form the γ complex-open β clamp-DNA composite (Hingorani and O'Donnell, 1998). Thus, ATP binding is all that is needed for γ complex to bring DNA and the open clamp together, apparently taking the clamp-assembly process almost to completion. What then is the role of ATP hydrolysis?

Earlier studies have shown that ATP hydrolysis is necessary to form the final β -DNA product (i.e. a closed β ring with DNA passing through its center), that supports processive DNA replication (Hingorani *et al.*, 1998; Stukenberg *et al.*, 1991). Overall, two to three ATP molecules are hydrolyzed for each clamp loaded onto DNA (Turner *et al.*, 1999). Previous measurements of γ complex ATPase activity under steady-state conditions indicate that the ATPase rate is altered substantially by β and primed DNA (Onrust *et al.*, 1991; Turner *et al.*, 1999). β binding to γ complex suppresses the ATPase rate, presumably stabilizing the ATP-bound γ complex- β for interaction with DNA. Primed DNA stimulates the ATPase rate, and somehow ATP hydrolysis results in β and DNA linked topologically to each other. Here, we aim to

elucidate the kinetic mechanism of the γ complex ATPase, to understand how hydrolysis of two or three ATP molecules drives assembly of β around DNA.

One striking finding of this study is that the γ subunits in γ complex hydrolyze ATP sequentially when loading β onto DNA. The first ATP hydrolysis occurs quickly, at a rate of 25–30 s⁻¹, while the second hydrolysis is limited to the slower steady-state rate of 2 s⁻¹. This two-step ATPase activity is dependent on both β and primed DNA. β induces disparity within the γ subunits of γ complex- β , such that one γ hydrolyzes ATP and one does not. In contrast, primed DNA stimulates rapid ATP hydrolysis at both γ subunits in γ complex-DNA. Thus, during clamp assembly, primed DNA increases the ATP-hydrolysis rate and β imposes functional asymmetry in the γ complex such that only one ATP is hydrolyzed at a time, resulting in rapid but sequential ATP hydrolysis.

Our elucidation of the clamp-loader ATPase mechanism raises several questions. For example, why does γ complex hydrolyze ATP at two different rates within one catalytic cycle of clamp assembly? Perhaps the separate ATP-hydrolysis steps power different functions in the clamp-assembly pathway? What might these different functions be, and how do they result in β clamped around DNA? Our study provides answers to these questions, and sheds

new light on the mechanism of action of the clamp loader as well as other ATP-fueled biological machines.

Results

Several experiments in this report examine how γ complex binds and hydrolyzes ATP in one catalytic cycle of β assembly at a primed site on DNA. The DNA used in these experiments is a synthetic 30-nucleotide (nt) primer annealed to a 105-nt template. Primer-extension assays using this DNA show that the core polymerase by itself is distributive and yields only a ladder of short DNA products, even after 2 min of reaction (Figure 1B, lanes 6–10). Inclusion of γ complex, β and ATP in the reaction facilitates processive DNA synthesis, resulting in full-length DNA within a few seconds (lanes 1–5). A control reaction performed without γ complex does not yield any full-length DNA (data not shown). Thus, the short, primed DNA serves well as a substrate for γ complex-catalyzed β assembly, which leads to processive DNA replication.

The γ complex hydrolyzes ATP in two separate steps during clamp assembly

In the presence of β and DNA, the γ complex binds at least two ATP molecules with high affinity as shown by nitrocellulose membrane binding assays (Table I). The ATP is hydrolyzed at a steady-state rate (k_{cat}) of 1.8–2 s⁻¹ (Table II). The k_{cat} reflects the slowest step in the reaction, and therefore provides very limited information on the kinetic mechanism of ATP hydrolysis. Elucidation of the ATPase mechanism requires answers to questions such as, how fast does the γ complex bind ATP? What is the rate of ATP hydrolysis? Does ATP hydrolysis occur before or after the rate-limiting step in the reaction? To address these questions, we performed ATPase assays in the millisecond time-scale, which allowed us to observe one catalytic cycle of ATP binding and hydrolysis in real time. First, a rapid quench experiment was performed to measure the rate at which γ complex hydrolyzes ATP during clamp assembly. γ complex and β

were mixed quickly (~2 ms) with [α -³²P]ATP and primed DNA for a specified time using a rapid quench-flow instrument (see scheme in Figure 2A). Formic acid was then added to quench the reaction instantaneously, and the products were analyzed by thin-layer chromatography (TLC). The experiment was repeated at varying incubation times and the amount of [α -³²P]ADP produced was plotted versus reaction time.

The data show an initial rapid burst of ATP hydrolysis in the first turnover, followed by a slower, linear phase that reflects continuing ATPase activity in subsequent turnovers (Figure 2A; a slight lag preceding the burst phase is discussed below). This result demonstrates that ATP is hydrolyzed before the rate-limiting step in the reaction. The burst phase occurs at an apparent rate of 5 s⁻¹ and has an amplitude of 3 μ M (Table II). The stoichiometry of γ subunits in the γ complex is estimated at two to four γ per γ complex. If the γ complex composition is $\gamma_2\delta_1\delta'_1\chi_1\psi_1$, its concentration in the reaction is 3 μ M, and complete ATP hydrolysis should yield a burst of 6 μ M—equivalent to two active γ subunits. If the composition of γ complex is $\gamma_4\delta_1\delta'_1\chi_1\psi_1$, the reactions contain 2.5 μ M γ complex and complete ATP hydrolysis should yield a burst of 10 μ M—equivalent to four active γ subunits. In either case, the observed amplitude of 3 μ M indicates that only one γ subunit (2.5 or 3 μ M) hydrolyzes ATP in the burst phase. This implies that only a fraction of the γ subunits in the reaction are active for ATP binding and hydrolysis. Alternately, the γ complex may rapidly hydrolyze only a fraction of the ATP bound to it in the initial burst phase. The following experiments distinguish between these possibilities.

A pulse–chase assay was performed to measure the rate of ATP binding to γ complex, and to determine what happens to any ATP that is bound but not hydrolyzed in the initial rapid phase. Is this ATP hydrolyzed slowly or does it dissociate unhydrolyzed from γ complex? The γ complex (+ β) was again mixed rapidly with [α -³²P]ATP (+ primed DNA), but instead of the acid quench, excess non-radiolabeled ATP was added to the reactions after varying times (see scheme in Figure 2B). The reactions were continued for an additional 5 s (sufficient time for 10 turnovers) before they were quenched with acid. In the time period following initiation of the chase, γ complex can continue to hydrolyze any bound ³²P-labeled ATP and release it as ³²P-labeled ADP. Alternately, the γ complex can release the unhydrolyzed ³²P-labeled ATP back into solution. Once released, the ³²P-labeled ATP is lost to γ complex amid excess non-radiolabeled ATP in the reaction, and thus remains unhydrolyzed.

Table I. ATP binding to γ complex

Clamp loader species	ATP bound per γ complex ($\gamma_2\delta_1\delta'_1\chi_1\psi_1$)	K_d (μ M)
γ complex- β -DNA	1.7 $\pm$ 0.1	4.3 $\pm$ 1.0
γ complex	2.1 $\pm$ 0.1	6.5 $\pm$ 0.8
γ complex- β	2.4 $\pm$ 0.2	4.9 $\pm$ 1.0
γ complex-DNA	2.1 $\pm$ 0.1	4.5 $\pm$ 1.1

Table II. Kinetic parameters for the ATPase activity of γ complex

Clamp loader species	Steady-state ATPase activity		Pre-steady-state ATPase activity			
	k_{cat} (s ⁻¹)	K_m (μ M)	Rapid quench		Pulse–chase	
			Burst amplitude (μ M)	Burst rate (s ⁻¹)	Burst amplitude (μ M)	Burst rate (s ⁻¹)
γ -complex- β -DNA	1.85 $\pm$ 0.08	22 $\pm$ 4	3.0 $\pm$ 0.3	4.7 $\pm$ 1	5.9 $\pm$ 0.2	158 $\pm$ 17
γ complex	0.07 $\pm$ 0.007	9.3 $\pm$ 3.0	0	0.078 ^a	0	0.06 ^a
γ complex- β	0.012 $\pm$ 0.001	11 $\pm$ 3.0	0	0.01 ^a	2.5 $\pm$ 0.1	141 $\pm$ 25
γ complex-DNA	0.21 $\pm$ 0.01	4.5 $\pm$ 2	5.9 $\pm$ 0.1	2.0 $\pm$ 0.1	5.3 $\pm$ 0.3	130 $\pm$ 20

^aIndicates the linear rate of ATP hydrolysis in the absence of a burst phase in the reaction.

Fig. 2. γ complex hydrolyzes ATP in two separate steps during clamp assembly. **(A)** The rapid quench assay for ATP hydrolysis indicates that γ complex hydrolyzes only one ATP rapidly in the first turnover, in the presence of β and primed DNA. An exponential + linear fit of the data yields a burst amplitude of $3.0 \pm 0.3 \mu\text{M}$ (one ATP hydrolyzed per γ complex), a burst rate of $\sim 5 \text{ s}^{-1}$, and a steady-state ATPase rate of $1.7 \pm 0.2 \text{ s}^{-1}$. **(B)** In the pulse-chase assay, γ complex is allowed sufficient time to convert any bound ATP to ADP. Here, the initial burst phase shows that the γ complex binds two ATP ($5.9 \pm 0.2 \mu\text{M}$) at a rate of $158 \pm 17 \text{ s}^{-1}$ in the first turnover and that both ATP are hydrolyzed. Subsequent turnovers occur at the steady-state rate of $2 \pm 0.1 \text{ s}^{-1}$. Together, the kinetic data suggest that γ complex binds two ATP molecules and hydrolyzes them one at a time, as depicted in the results scheme below.

The results in Figure 2B show a burst of ADP production followed by the linear phase of the reaction. The burst rate of 160 s^{-1} reflects the rate at which ATP binds to the γ complex (once bound, one ATP is hydrolyzed at the $\sim 5 \text{ s}^{-1}$ rate during the chase period). The burst amplitude of $6 \mu\text{M}$ reflects the number of bound ATP molecules hydrolyzed to ADP. Interestingly, this amplitude is twice that observed in the rapid quench experiment (i.e. $3 \mu\text{M}$; Figure 2A), indicating that a second ATP molecule is hydrolyzed within the first catalytic turnover. Thus, the γ complex binds at least two ATP molecules; the rapid quench assay demonstrates that one ATP is hydrolyzed rapidly, and the pulse-chase assay demonstrates that a second ATP is also hydrolyzed, but at a slower rate than the first.

The second ATP is hydrolyzed after a slow step in the reaction

How different are these two ATP hydrolysis events? The linear phase of the rapid quench assay (after the first ATP is hydrolyzed) has a slope of $5.6 \mu\text{M s}^{-1}$. This slope, divided by the $3 \mu\text{M}$ burst amplitude, yields the steady-state k_{cat} of 1.7 s^{-1} (Figure 2A; Table II). The linear phase of the pulse-chase assay similarly yields the steady-state rate at 2 s^{-1} (i.e. $6 \mu\text{M s}^{-1}$ divided by $3 \mu\text{M}$). Subsequent ATPase turnovers at the first γ subunit are constrained to this slow rate by a rate-limiting step in the ATPase pathway. What about ATP hydrolysis at the second γ subunit? There

are two possible ways in which the second γ might hydrolyze ATP in this reaction: (i) if the γ subunits work independently, the two ATP hydrolysis events are not connected and the second ATP may be hydrolyzed at any rate slower than the observed burst rate; however, (ii) if the γ subunits work in a co-ordinated fashion (e.g. by a sequential mechanism), the second ATP will be hydrolyzed at 2 s^{-1} , the slow rate defined by a rate-limiting step after the first ATP hydrolysis.

To distinguish between these two possibilities, we designed an experiment that follows both the first and second ATP hydrolysis steps in real time. The γ complex (+ β) and [α - ^{32}P]ATP (+ primed DNA) were pre-incubated for sufficient time to allow ATP binding but not ATP hydrolysis (0.08 s; see Figure 2). Following incubation, excess non-radiolabeled ATP was added and the reaction was continued for varying times. [α - ^{32}P]ADP production during this 'chase time' reveals how fast the bound [α - ^{32}P]ATP molecules are hydrolyzed. The data in Figure 3A show that the first ATP is hydrolyzed at least 10-fold faster than the second, at $27 \pm 7 \text{ s}^{-1}$ versus $1.7 \pm 0.5 \text{ s}^{-1}$, respectively. Moreover, hydrolysis of the second ATP occurs at a rate similar to the steady-state rate measured earlier ($k_{\text{cat}} = 1.8\text{--}2 \text{ s}^{-1}$; Table II). These results demonstrate that the two γ subunits function in a co-ordinated manner, such that a slow step in the reaction after the first ATP hydrolysis defines the rate at which the second ATP is hydrolyzed.

Fig. 3. Two γ subunits hydrolyze ATP one after another. (A) A chase-time assay detects in real time the fate of the two ATP molecules bound to γ complex. After γ complex binds two ^{32}P -labeled ATP (0.08 s), addition of 140-fold excess unlabeled trap ATP prevents binding of any more ^{32}P -labeled ATP. During the chase time, one ATP is hydrolyzed at $27 \pm 7 \text{ s}^{-1}$ and the other ATP is hydrolyzed at $1.7 \pm 0.5 \text{ s}^{-1}$, after a slow step in the pathway. (B) Sequential ATPase activity continues after the first turnover of ATP hydrolysis. When the ATP trap is added after γ complex hydrolyzes ^{32}P -labeled ATP for 2 s, only one ^{32}P -labeled ATP is hydrolyzed ($2.8 \pm 0.4 \mu\text{M}$) at $2.2 \pm 0.2 \text{ s}^{-1}$ during the chase time. This confirms that most of the γ complex is bound to one ATP molecule at any given time under steady-state conditions.

We also considered the possibility that this two-step ATP hydrolysis occurs only in the first turnover, an anomalous reaction when γ complex is initially presented with ATP, β and DNA. The ‘chase time’ experiment was performed for a second time to examine ATP hydrolysis after several turnovers. This time, the γ complex was incubated with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ for 2 s (to reach steady-state) before non-radiolabeled ATP was added to the reaction. If the second ATP hydrolysis is always limited to a slow 2 s^{-1} rate, γ complex bound to one $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ should be the predominant species under steady-state conditions. Consequently, after addition of the excess ATP, we should observe hydrolysis of only that ATP molecule. Figure 3B shows that one $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ is hydrolyzed at a rate of $2.2 \pm 0.2 \text{ s}^{-1}$ before the reaction plateaus. Thus, sequential ATP hydrolysis continues after the first turnover, and hydrolysis of the second ATP remains constrained by a rate-limiting step in the reaction.

Kinetic mechanism of the γ complex ATPase

The experiments presented thus far reveal a minimal mechanism by which γ complex hydrolyzes ATP as it assembles a circular clamp around DNA (Figure 4A). Simulations of this mechanism are shown in Figure 4B (solid lines). Step 1 shows binding of two ATP molecules to the γ complex. The exponential rate of ATP binding (measured by the pulse-chase assay of Figure 2B) increases linearly with ATP concentration and yields a bimolecular rate constant of $4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for the interaction (data not shown). Step 2 shows the ATP-induced conformational change in γ complex that leads to its interaction with β (Naktinis *et al.*, 1995). According to previous studies, this step occurs before ATP hydrolysis (Hingorani and O’Donnell, 1998). The γ complex also binds primed DNA rapidly, at about 100 s^{-1} , to form the ATP-bound γ complex- β -DNA composite (B.Ason and L.B.Bloom, unpublished results; Bloom *et al.*, 1996; Hingorani *et al.*, 1998). Next, there is a slow step in the reaction (step 3), before γ complex hydrolyzes the first ATP molecule. This step is revealed by a small lag phase that occurs just before the burst of ATP hydrolysis in the rapid quench experiment (open squares, Figures 2A and 4B). When a 12 s^{-1} step is incorporated into the model

mechanism, after ATP binding but before ATP hydrolysis (step 3), the simulated curve in Figure 4B also shows a similar lag (solid line along open squares). Interestingly, an earlier study of clamp-assembly kinetics indicates that γ complex opens the β clamp at $12 \pm 2 \text{ s}^{-1}$ before loading it onto DNA (Bloom *et al.*, 1996). Together, these data are consistent with reports that γ complex binds and opens the clamp after binding ATP and prior to ATP hydrolysis (Hingorani and O’Donnell, 1998; Turner *et al.*, 1999). Therefore, we have assigned the 12 s^{-1} step in the ATPase pathway to a β -opening event.

In the next step, γ complex hydrolyzes the first ATP molecule at a rate of 27 s^{-1} (step 4). The following step is the slowest in the pathway at 2 s^{-1} (step 5), and therefore it defines the steady-state ATPase rate of the γ complex. The second ATP hydrolysis occurs at a 2 s^{-1} rate, and thus may be the rate-limiting step in the reaction. Alternately, the second hydrolysis may occur after a rate-limiting step, such as dissociation of the ADP and P_i products from the first ATP hydrolysis. Finally, at or after the second ATP hydrolysis event, γ complex completes its reaction by releasing β clamped around DNA, and ADP + P_i , and returns to its original conformation.

The γ complex is an inefficient ATPase without β and DNA

The experiments described above were performed with γ complex in the presence of its substrates, β and DNA. Under these conditions, the γ complex binds two ATP molecules rapidly and hydrolyzes them one at a time. The goal now is to understand how γ complex utilizes the two ATP-hydrolysis events for clamp assembly. Our discovery of this step-wise ATPase mechanism was rather unexpected, since not many enzymes are known to hydrolyze ATP sequentially. Therefore, we first explored the ATPase reaction further to understand why the γ subunits in γ complex hydrolyze ATP at different rates. The 10-fold rate difference indicates a functional disparity between the γ subunits, and we asked if this property is intrinsic to the γ complex, or if β and DNA influence it during the process of clamp assembly.

A rapid quench experiment was performed with γ complex alone to measure the rate of ATP hydrolysis. Figure 5A

Fig. 4. Mechanism of sequential ATP hydrolysis by the γ complex. (A) A minimal mechanism of the γ complex ATPase activity is shown, along with the rate constants that define the pathway of ATP hydrolysis. Key elements of the mechanism include rapid binding of two ATP to γ complex, followed by interaction with β and DNA; next, a slow β opening step, followed by rapid hydrolysis of one ATP molecule; finally, hydrolysis of the second ATP molecule, product release, and γ complex turnover. (B) Simulations of pulse-chase and rapid quench kinetics (solid lines), created using the above mechanism and rate constants, are compared with the experimental data from Figure 2 (pulse-chase, closed circles; rapid quench, open squares). Both simulations match the data closely, thus verifying the kinetic mechanism shown in (A).

shows that the γ complex produces no burst of ATP hydrolysis in the absence of β and DNA. Instead, the data lie in a straight line (squares, Figure 5A) and yield a rate equal to the k_{cat} of 0.08 s^{-1} (Table II). Thus, even in the first turnover, ADP is formed at the slowest rate in the reaction, indicating that ATP hydrolysis or an event preceding it is the rate-limiting step in the pathway. This is quite different from the ATPase reaction under clamp-loading conditions, where one ATP is hydrolyzed much faster than the rate-limiting step (Figure 3A). Thus, one clear outcome of β and DNA binding to the γ complex is a 330-fold increase in the ATP-hydrolysis rate, from 0.08 to 27 s^{-1} . Do β and DNA influence the ATP binding step also? We examined the rate of ATP binding to γ complex in a pulse-chase experiment (circles, Figure 5A). The data again show no burst phase, only a straight line with a rate of 0.06 s^{-1} , similar to the k_{cat} . This result is surprising as it implies that ATP binding to γ complex is excruciatingly slow, 0.06 s^{-1} compared with 160 s^{-1} in the presence of β and DNA. A more likely possibility is that ATP binding is fast, but the γ complex is not committed to hydrolyzing every ATP it binds (i.e. ATP dissociation competes effectively with ATP hydrolysis, when β and DNA are absent). The γ complex binds ATP with high affinity ($K_d = 6 \mu\text{M}$; Table I), but the kinetic data reveal that it is an exceedingly inefficient ATPase, reluctant to hydrolyze ATP in the absence of β and DNA.

The clamp induces functional asymmetry within the γ subunits and DNA stimulates ATP hydrolysis

The above results indicate that either β , or DNA, or both substrates are required for rapid and sequential ATP

hydrolysis by the γ complex. This connection between β /DNA and the γ complex ATPase activity suggests that the two ATP-hydrolysis steps are tightly coupled to the β loading mechanism. Possibly, each ATP hydrolysis fulfills a different function, such as manipulation of β and/or DNA to form the topological link. If this is the case, β and DNA probably have very characteristic effects on the ATPase activity. To test this hypothesis, we performed the ATPase assays in the presence of β alone (Figure 5B) and in the presence of primed DNA alone (Figure 5C).

The results are quite interesting in both cases. The rapid quench experiment performed with γ complex and β shows no burst of ATP hydrolysis, indicating that ADP production or a step preceding it is still rate-limiting in the reaction (squares, Figure 5B). In fact, the rate of ATP hydrolysis is reduced significantly, from 0.08 s^{-1} rate for γ complex alone to 0.01 s^{-1} for γ complex- β (Table II). These data confirm our earlier hypothesis that interaction between γ complex and β blocks ATP hydrolysis, resulting in a stable ATP-bound γ complex- β composite.

The pulse-chase experiment with γ complex and β reveals an asymmetry in the ability of the γ subunits to bind and hydrolyze ATP (circles, Figure 5B). The burst phase shows that the rate of ATP binding (140 s^{-1}) is similar to that in the presence of both β and DNA (160 s^{-1} , Figure 2B). However, the amplitude of the burst is only $2.5 \mu\text{M}$ (versus $6 \mu\text{M}$ with β and DNA), which is equivalent to binding and hydrolysis of one ATP molecule per γ complex in the reaction. No further ATP was hydrolyzed, even though the chase was continued

Fig. 5. β induces functional asymmetry in the γ subunits of γ complex. Pre-steady-state ATPase assays were performed with γ complex and ATP under various conditions. (A) with γ complex alone, ATP hydrolysis occurs at $0.08 \pm 0.01 \text{ s}^{-1}$ (squares), and absence of a burst during pulse-chase (circles) indicates that ATP tends to dissociate from the γ complex unhydrolyzed. (B) In the presence of β , ATP hydrolysis is inhibited to 0.01 s^{-1} (squares), and a burst of one ADP per γ complex during pulse-chase (circles) indicates that only one of the γ subunits in γ complex: β is active for ATP hydrolysis. (C) In the presence of DNA, both γ subunits bind ATP rapidly (circles), and hydrolyze ATP at 2 s^{-1} (squares). The steady-state rate is slower, at 0.2 s^{-1} .

for >10 ATPase turnovers. The γ complex does bind at least two ATPs with high affinity in the presence of β ($K_d = 2\text{--}5 \mu\text{M}$, Table I; Hingorani and O'Donnell, 1998). Presumably, γ complex: β either holds on to the second ATP without hydrolyzing it, or releases it back into solution, unhydrolyzed (recent experiments suggest that the second γ subunit in γ complex: β retains ATP without hydrolyzing it while the first subunit undergoes multiple turnovers; M.M.Hingorani and M.O'Donnell, unpublished results). This disparity in the ATPase activity of the γ subunits may actually form the basis for sequential ATP hydrolysis during clamp assembly. Moreover, the clamp-dependent nature of the disparity is consistent with the idea that step-wise ATP hydrolysis is integral to the mechanism of clamp assembly on DNA.

The above experiments indicate that β induces func-

tional asymmetry in γ complex by committing only one γ subunit to ATP hydrolysis, but it does not induce fast ATP hydrolysis as observed during β assembly on DNA. The next rapid quench experiment, performed in the presence of DNA, shows a burst of ATP hydrolysis at 2 s^{-1} , indicating that γ complex: DNA hydrolyzes ATP more quickly than the rate-limiting step in the reaction (squares, Figure 5B). In fact, the burst amplitude of $6 \mu\text{M}$ indicates that primed DNA stimulates hydrolysis of both ATP molecules bound to γ complex. This result is substantiated by the pulse-chase experiment which shows that two ATP molecules bind to γ complex: DNA (130 s^{-1}), and are hydrolyzed rapidly within the first turnover (circles). Both DNA and β appear to stabilize ATP binding to the γ complex, but the effect of primed DNA on ATP hydrolysis is almost exactly opposite to the effect of β

on ATP hydrolysis. Whereas the clamp suppresses ATP hydrolysis and induces disparity within the γ subunits, DNA enhances ATP hydrolysis and does not support any disparity. Thus, it appears that the rapid and sequential ATPase activity of γ complex during clamp assembly reflects the stimulatory effect of DNA as well as the functional asymmetry caused by β .

One ATP hydrolysis triggers release of the clamp from the clamp loader

The clamp loader binds primed DNA in the presence of ATP and the nonhydrolyzable analog ATP γ S, but not in the presence of ADP, suggesting that ATP hydrolysis may be coupled to dissociation of DNA from the γ complex (B.Ason and L.B.Bloom, unpublished results; Bertram *et al.*, 1998; Hingorani *et al.*, 1998). Presumably, the rapid ATP hydrolysis triggered by DNA facilitates its release from γ complex during clamp assembly. Similarly, the γ complex can bind β in the presence of ATP (or ATP γ S) but not in the presence of ADP (Hingorani and O'Donnell, 1998). Therefore, ATP hydrolysis may also be coupled to release of β from the γ complex. These data suggest that the γ complex utilizes an ATP/ADP switch for transition between conformations that are active/inactive for binding β and DNA. However, the switch model does not address the intriguing question of why γ complex hydrolyzes ATP sequentially. How does the γ complex utilize energy from two discrete ATP-hydrolysis events for clamp assembly? We addressed this question by examining what happens when only one ATP is hydrolyzed, as is the case when γ complex is bound to β .

A 'kinase-protection' experiment was designed to test whether one ATP-hydrolysis event is sufficient for release of β from the γ complex. The assay uses a modified β clamp (β^{PK}) that contains a protein kinase recognition motif at the C-terminus (Naktinis *et al.*, 1996). When β^{PK} is free in solution, the catalytic subunit of cAMP-dependent protein kinase radiolabels the clamp using [γ - 32 P]ATP. When γ complex binds β^{PK} , however, the kinase motif is occluded and the protein kinase cannot 32 P-label the clamp. In the kinase-protection assay, the β^{PK} is first bound by γ complex in the presence of ATP, and then a 20-fold excess of wild-type β (with the kinase motif absent) is added to the reaction as a trap. After varying times, aliquots of the reaction are treated with protein kinase and [γ - 32 P]ATP for 7 s (see scheme in Figure 6). As β^{PK} is released, excess wild-type β takes its place on the γ complex; therefore, β^{PK} remains free in solution and becomes 32 P-labeled by the kinase. The [32 P] β^{PK} is then separated from 32 P-labeled ATP on an SDS-polyacrylamide gel and quantitated. Figure 6A shows that initially all β^{PK} is bound by γ complex and is not labeled by the protein kinase (lane 1). After addition of excess wildtype β , increasing amounts of β^{PK} are labeled over time at an apparent rate of $0.014 \pm 0.003 \text{ s}^{-1}$ (Figure 6B). This release rate essentially matches the rate at which γ complex hydrolyzes ATP in the presence of β (0.01 – 0.012 s^{-1} ; Figure 5B and Table II). Note that only one ATP is converted to ADP when γ complex is bound to β ; thus, one ATP-hydrolysis event is sufficient to trigger dissociation of the clamp from the clamp loader. This result implies that hydrolysis of the other ATP molecule during clamp assembly may drive a different mechanical action. The potential roles

for the second ATP-hydrolysis event are presented in the discussion.

Discussion

Key elements of the ATP-driven mechanism of clamp assembly

DNA polymerase III holoenzyme catalyzes coordinated leading- and lagging-strand replication of the *E.coli* genome. It performs this task with high efficiency, using two core polymerases ($\alpha\epsilon\theta$) that are tethered to DNA by sliding clamps (β). The two cores are connected by the τ protein dimer, which also binds the clamp loader (γ complex), and thus holds together all the components of this complex catalytic machine (reviewed in Kelman and O'Donnell, 1995).

The γ complex is an assembly of five different proteins that work together to load β at primed sites on DNA, for use by the DNA polymerase. On the lagging strand, a clamp must be assembled approximately once every second onto new RNA primers for processive synthesis of Okazaki fragments (Kornberg and Baker, 1992). Thus, γ complex must bind β , open the clamp, recognize primed DNA, place the clamp on DNA, close the clamp and release it around DNA at least once every second. Clearly, the clamp loader component of the holoenzyme is itself an intricate machine with a rather complicated mechanism of action. In this study we focus on how ATP fuels the workings of this fascinating enzyme.

Earlier reports have provided substantial information on the structure and function of the individual γ complex subunits. As illustrated in Figure 7, the γ and δ' proteins are 'C'-shaped with a top and bottom domain joined by a small hinge domain (Guenther *et al.*, 1997). The ATP site is positioned near the hinge in γ , and ATP binding and hydrolysis are predicted to perturb the top and bottom domains relative to each other. Clamp assembly begins when the γ subunits in γ complex bind ATP. The resulting conformational changes in γ and δ' are likely to modulate exposure of the δ clamp-opener subunit for interaction with β (Naktinis *et al.*, 1995; Hingorani and O'Donnell, 1998). δ opens the clamp at the dimer interface (Turner *et al.*, 1999), and the ATP-bound γ complex-open β clamp composite recognizes and binds primed DNA with high affinity (Hingorani and O'Donnell, 1998). Thus, upon binding ATP, the γ complex brings together two molecules, β and DNA, that it must now make one with a topological link.

Once the γ complex-open β clamp-DNA intermediate is formed, the minimal requirements for completion of clamp assembly are: (i) placement of DNA within the open clamp; (ii) closure of the clamp around DNA; and (iii) release of the clamp and DNA from the clamp loader. Of these, placement of DNA in the clamp must come first, while the remaining steps may occur in any order, as separate steps or combined in one concerted reaction. During these ATP hydrolysis-powered rearrangements, at the very least the γ complex must hold on to both β and DNA until the topological link is formed. In a more complex mechanism, the clamp-loader subunits may actively manipulate β and DNA to form the link.

Fig. 6. Hydrolysis of one ATP triggers release of β from γ complex. Dissociation of β from γ complex was detected by ^{32}P -labeling of the β^{PK} released into solution from a pre-formed γ complex: β^{PK} composite. (A) Labeled β^{PK} was analyzed by SDS-PAGE and quantitated. At zero time, all β^{PK} is bound to γ complex and none is labeled (lane 1). Over time, an increasing amount of β^{PK} is labeled, and (B) a plot of ^{32}P -labeled β^{PK} versus time yields the apparent rate of β release from γ complex. An exponential fit of the data shows that β^{PK} release occurs at $0.014 \pm 0.003 \text{ s}^{-1}$, similar to the rate at which one ATP is hydrolyzed by γ complex: β ($0.01\text{--}0.012 \text{ s}^{-1}$, Figure 5B).

A model for sequential ATP hydrolysis coupled to clamp assembly on DNA

Figure 7 illustrates a putative mechanism for how ATP binding and sequential ATP hydrolysis are coupled to clamp assembly, based on the results of this study and data from previous reports. The initial steps leading to formation of ATP-bound γ complex-open β clamp-DNA were described above. Once this intermediate species is formed, γ complex hydrolyzes two ATP molecules, one after another.

We propose that each ATP-hydrolysis step is coupled to a functionally distinct event in the clamp-assembly pathway. The first hydrolysis step may trigger release of β onto the DNA (Figure 7, path A). In this reaction, the DNA must be positioned in the center of the clamp before ATP hydrolysis (this could simply occur on interaction between γ complex-open β and primed DNA). The second ATP hydrolysis may then release DNA (with the clamp linked around it) to complete clamp assembly. Alternately, the first ATP-hydrolysis step could release DNA, presumably to position it in the center of the open clamp (Figure 7, path B). The second ATP hydrolysis would then release β (with the DNA passing through it). The second hydrolysis step could also drive turnover of the clamp loader, with the final dissociation of $\text{ADP} + \text{P}_i$ resetting the clamp loader for the next cycle of clamp assembly.

Why the sequential mechanism of ATP hydrolysis and a step-wise mechanism for clamp assembly? The ATPase activity of γ complex under different conditions reveals that DNA stimulates ATP hydrolysis, while β slows down the reaction and appears to induce disparity within the γ subunits. Interestingly, both γ subunits hydrolyze ATP at the same rate in the presence of primed DNA. This result implies that there is no intrinsic disparity within the γ subunits (in the absence of β), or even if there is, it can be overcome on primed DNA binding to γ complex. In the presence of β , however, the two γ subunits hydrolyze ATP at different rates. Thus, β appears specifically to impose a rate-limiting step between the two ATP-hydrolysis events, implying that this slow step involves work performed by γ complex on β during clamp assembly. This interpretation favors path B in the model mechanism, where β remains associated with γ complex during the slow step, and is released after the second ATP-hydrolysis event.

The γ complex mechanism can be viewed as a dynamic relationship between three components (the ATPase activity, protein conformational changes and protein-protein or protein-DNA interactions) that result in β clamped around DNA. This report defines the ATPase component and predicts some intermediate protein-DNA conformations and interactions that may occur en route to

Fig. 7. Assembly of the β clamp on DNA powered by sequential ATP hydrolysis. A model mechanism of ATP hydrolysis-coupled clamp assembly shows that at first two γ subunits in γ complex rapidly bind ATP. The ensuing change in γ complex conformation promotes its interaction β and DNA, and slow opening of the β ring at one dimer interface. At this point, the DNA could be positioned inside of the ring (path A) or outside of the ring (path B). If DNA is already on the inside, sequential ATP hydrolysis can drive rearrangement and release of β and then DNA to complete clamp assembly (path A). If DNA is outside, sequential ATP hydrolysis may drive first the release of DNA into the open β ring, followed by release of β as a closed ring around the DNA. The release of β linked around DNA, and ADP + P_i , from the γ complex completes the catalytic cycle of clamp assembly.

clamp assembly. At present, the exact nature of these intermediate steps remains unknown, and continuing investigation of all three components of the clamp-assembly mechanism should provide further clarification of how the clamp loader works.

A common mechanism for energy utilization by clamp-loading machines?

The *E. coli* clamp loader utilizes a surprisingly complex mechanism of sequential ATP hydrolysis to power its clamp-loading activity. The need for more than one ATP hydrolysis step during clamp assembly is consistent with the presence of more than one γ subunit in the clamp loader. Although the actual number of γ subunits is somewhat under question, with estimates ranging from two to four γ per γ complex (Maki and Kornberg, 1988; Tsuchihashi and Kornberg, 1989; Dallmann and McHenry, 1995; Onrust *et al.*, 1995a), it makes little difference to our conclusion that the γ subunits function

sequentially. This study utilizes absorbance at 280 nm and molar extinction coefficients to determine protein concentration, and by this measure, $3 \mu\text{M}$ of $\gamma_2\delta'\chi\psi$ is equivalent to $2.5 \mu\text{M}$ of $\gamma_4\delta'\chi\psi$ in the reaction. Therefore, ATP hydrolysis in two steps of $3 \mu\text{M}$ each indicates that either the two γ in γ_2 complex hydrolyze ATP sequentially, or that two of the four γ in γ_4 complex hydrolyze ATP sequentially. ATPase activity of two γ subunits is consistent with an earlier report of two ATPs hydrolyzed for each processive DNA polymerase III holoenzyme assembled on DNA (Burgers and Kornberg, 1982). The stoichiometry of two γ subunits in DNA polymerase III (minus β) purified from *E. coli* also implies that *in vivo* two ATPs are hydrolyzed per clamp assembled on DNA (Onrust *et al.*, 1995b). It is important to note that the holoenzyme also contains two copies of τ , which is essentially γ with 213 additional amino acids at the C-terminus. τ dimerizes the two core polymerases (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991) and contacts DnaB during DNA replication (Kim *et al.*, 1996; Yuzhakov *et al.*, 1996). Furthermore, τ can substitute for γ to form a functional clamp loader with δ , δ' , χ , ψ (Dallmann *et al.*, 1995; Onrust *et al.*, 1995a). Although we have shown previously that τ ATPase activity is not essential for processive DNA replication (Xiao *et al.*, 1995), it is possible that additional ATP hydrolysis events catalyzed by τ may supplement γ complex clamp-loading activity.

Clamp loaders from other organisms contain multiple ATPase-active subunits, implying that like γ complex, these enzymes may also employ a sequential mechanism of ATP hydrolysis. Prominent among these are human replication factor C (hRFC) and yRFC, the human and yeast clamp loaders, respectively, as well as gp44/62, the bacteriophage T4 clamp loader. Like γ complex, the RFC complexes load circular clamps (PCNA) onto primed DNA in a reaction driven by ATP (reviewed in Stillman, 1994), and PCNA and DNA are known to stimulate hRFC ATPase activity (Tsurimoto and Stillman, 1990; Lee *et al.*, 1991; Cai *et al.*, 1997; Ellison and Stillman, 1998). The RFC complexes are composed of five different proteins, each of which contains the Walker A and B ATP binding sites (Cullman *et al.*, 1995; reviewed in O'Donnell *et al.*, 1993). A recent study of the hRFC subunits concluded that four of the five proteins contribute to the ATPase activity of hRFC (Cai *et al.*, 1998). Mutations in the ATP-binding site of each of these subunits resulted in loss of PCNA loading and inability to support processive DNA replication. All five RFC subunits share significant amino acid homology with the γ and δ' subunits of γ complex, which implies that they may have a common 'C'-shaped structure (O'Donnell *et al.*, 1993; Guenther *et al.*, 1997). These similarities between RFC and γ complex suggest that RFC may utilize the energy from ATP binding and hydrolysis in a manner similar to the γ complex.

The bacteriophage T4 clamp loader, gp44/62, has four ATPase-active gp44 subunits (Jarvis *et al.*, 1989a), which also have homology to γ and δ' (O'Donnell *et al.*, 1993). Like γ complex, gp44/62 ATPase activity is stimulated by primed DNA and the gp45 clamp (Jarvis *et al.*, 1989b). Rapid kinetic studies have shown that all four gp44 subunits in gp44/62 hydrolyze ATP when assembling the clamp onto DNA (Berdis and Benkovic, 1996; Young *et al.*, 1996). As yet there is no kinetic evidence for

sequential ATP hydrolysis by the T4 clamp loader; however, a recent report indicates that gp44/62 hydrolyzes two ATP molecules in the presence of gp45 alone and four ATP molecules in the presence of both gp45 and DNA (Sexton *et al.*, 1998). This implies that the T4 clamp loader may utilize ATP hydrolysis at two different steps in the clamp-assembly pathway, as indicated for γ complex by the results in this study.

Experiments utilizing fluorescently labeled gp45 or protein cross-linking techniques indicate that gp44/62 and gp45 undergo different conformational changes in the ATP γ S- ATP- or ADP-bound states (Sexton *et al.*, 1996; Pietroni *et al.*, 1997). One interpretation of these conformational changes is that gp44/62 opens the gp45 ring on ATP hydrolysis. Thus, Sexton *et al.* (1998) have proposed that gp44/62 hydrolyzes two ATP molecules to open the clamp and two more ATP molecules to assemble it around DNA. If this is the case, gp44/62 functions differently from the γ complex, which does not require energy from ATP hydrolysis to open β . In the *E. coli* system, β -opening assays have shown that γ complex opens the β ring simply upon binding ATP (which allows the δ subunit access to β). The δ subunit alone can open β through the energy of protein-protein interaction (Hingorani and O'Donnell, 1998; Turner *et al.*, 1999). The gp62 protein is thought to be analogous to δ , as it is essential for interaction between the T4 clamp loader and its clamp (Rush *et al.*, 1989). It would be most interesting, in comparing *E. coli* with T4, to examine gp62 for the ability to open gp45 in the absence of gp44 and ATP. The true extent to which γ complex and gp44/62 differ in their mechanism must await further studies. However, the existing data suggest that they both utilize multiple ATP molecules in a step-wise fashion. Ongoing kinetic and structure-function studies of clamp loaders should reveal whether this sequential ATPase mechanism is general to other bacterial and eukaryotic systems.

The clamp loader—molecular motor, molecular switch or a molecular machine?

ATP is the energy currency of all living organisms. Consequently, there is an incredible variety of proteins that utilize ATP (and other nucleoside triphosphates) to fuel biological processes that sustain and propagate life. To date, however, only a handful of enzymes are known to catalyze ATP hydrolysis in a sequential manner. Most of these enzymes are oligomeric molecular motors such as the kinesins, DNA helicases and the F₁-ATPase. In these motors, each catalytic cycle of ATP binding and hydrolysis powers the same mechanical action; therefore, successive ATPase events result in repetitive motor activity. For example, in the hexameric T7 helicase, sequential TTP binding and hydrolysis by individual subunits is coupled to DNA binding and release, and this iterative activity is thought to result in translocation of the helicase along DNA (Hingorani *et al.*, 1997). Similarly, subunits of the kinesin dimer appear to couple sequential ATPase activity to movement along an actin filament (Gilbert *et al.*, 1998).

The γ complex clamp loader is also a sequential ATPase, but its ATPase reaction is unusual in at least one important respect. The 'iterative' motors described above bind one ATP and hydrolyze it within one catalytic cycle, and then

repeat this cycle over and over again. In contrast, γ complex binds two ATPs, hydrolyzes one ATP rapidly and the other at or after a slow step in the reaction, all within one catalytic cycle of clamp assembly. Thus, γ complex appears to utilize ATP by a different mechanism from molecular motors such as kinesin and DNA helicases. It is possible that the γ subunit is an iterative motor, and each γ undergoes the same conformational change when ATP is hydrolyzed. However, γ subunits function in the context of the entire γ complex, analogous to motors within a molecular machine. The γ complex is an asymmetric multi-protein assembly (two to four γ and one each of δ , δ' , χ , ψ), and it works on two very different substrates, β and DNA. It releases β after one ATP hydrolysis, implying that hydrolysis of the other ATP molecule has a different role in clamp assembly. In this case, the two successive ATP hydrolysis steps are coupled to different tasks, and the γ complex functions as 'differential' motor rather than an iterative one. As more ATP-fueled biological machines are examined in detail, it is likely that more enzymes that utilize ATP in the same way as the clamp loader will be found.

Recently, several ATP-fueled enzymes have been described as 'molecular ATP-ADP switches', including the transposase activator MuB (Yamauchi and Baker, 1998), the mismatch repair proteins hMSH2-hMSH6 (Gradia *et al.*, 1997) and the chromosomal partitioning protein ParA (Bouet and Funnell, 1999). The term 'switch protein', originally defined for G proteins, describes their ability to propagate different signals depending on the state of the bound nucleotide (reviewed in Sprang, 1997). Extensive studies of G proteins have shown that GTP- and GDP-bound complexes define the 'on' and 'off' states, respectively. The process of GTP hydrolysis merely regulates the rate at which G proteins transit from one state to the other. The γ complex does switch from a high-affinity to low-affinity β and DNA binding state in the presence of ATP or ADP, respectively (Hingorani and O'Donnell, 1998). However, the sequential ATPase mechanism of γ complex revealed by the kinetic analysis suggests that it functions as more than a two-state 'ATP/on' and 'ADP/off' switch.

A switch model for the γ subunits in γ complex only assigns functional roles to the ATP and ADP-bound forms of the enzyme. However, since the γ complex binds two ATP molecules and hydrolyzes one ATP at a time, several other intermediate species may exist, including but not limited to the ATP+ADP-P_i, ATP+ADP, and the single ATP- or ADP-bound forms of γ complex. Any or all of these species may be coupled to conformational changes in β and DNA that result in clamp assembly. Therefore, if the γ complex mechanism were to be reduced to a switch protein model, the definition of a switch would have to be expanded to include more than two functional states. The oligomeric switch proteins mentioned earlier (MuB, hMSH2-hMSH6 and ParA) also use ATP to drive dynamic interactions with DNA and other proteins. Given how the clamp loader uses a step-wise ATPase mechanism to modulate interactions with DNA and β , it would be interesting to know whether any of these switch proteins hydrolyze ATP sequentially.

The *Saccharomyces cerevisiae* DNA topoisomerase II is the only other enzyme known to bind two ATP molecules

and hydrolyze them one after another, like the γ complex (Harkins and Lindsley, 1998; Harkins *et al.*, 1998). It is not yet clear how the topo II dimer utilizes energy from ATP hydrolysis to catenate/decatenate DNA, but, like the assembly of clamps around DNA, the topo II reaction involves several intermediate steps. The enzyme must bind and break a DNA duplex, pass another duplex through the break, seal the break, release DNA and finally reset to its original conformation. Perhaps, for biological machines that perform several complex actions within one catalytic cycle, a sequential ATPase mechanism is most effective in driving concerted transitions through multiple conformations required for activity. So far, two very different enzymes, γ complex and topoisomerase II, have been discovered that hydrolyze ATP in a similar fashion. How many other biological machines might use this methodical process to get the job done?

Materials and methods

Proteins

Proteins were overexpressed and purified as described previously: γ (Studwell and O'Donnell, 1990), β and β^{PK} (Kong *et al.*, 1992), δ and δ' (Dong *et al.*, 1993), χ and ψ (Xiao *et al.*, 1993), SSB (Studwell and O'Donnell, 1990). γ complex and core DNA polymerase were reconstituted from individual subunits (Studwell-Vaughan and O'Donnell, 1991; Onrust *et al.*, 1995a). Protein concentrations were determined by absorbance at 280 nm of 8 M urea or 8 M guanidine-HCl-denatured proteins, using their calculated molar extinction coefficients (β , $17\,900\text{ M}^{-1}\text{ cm}^{-1}$; $\gamma_2\delta\delta'\chi\psi$, $202\,830\text{ M}^{-1}\text{ cm}^{-1}$ and $\gamma_4\delta\delta'\chi\psi$, $244\,710\text{ M}^{-1}\text{ cm}^{-1}$). T4 polynucleotide kinase was purchased from US Biochemical Corp. The catalytic subunit of cAMP-dependent protein kinase produced in *E.coli* was a gift from Dr Susan Taylor (University of California, San Diego, CA).

Nucleotides and other reagents

ATP was purchased from Sigma Chemicals Co., and ^{32}P -labeled ATP (3000 Ci/mmol) was purchased from NEN Life Science Products. Oligodeoxynucleotides were synthesized using β -cyanoethyl phosphoramidite chemistry on an Applied Biosystems synthesizer (model 392 RNA-DNA) and purified by denaturing polyacrylamide gel electrophoresis (Bloom *et al.*, 1996). The sequences of the synthetic DNA were: 105-nt template 5'-GAG CGT CAA AAT GTA GGT ATT TCC ATG AGC GTT TTT CCT GTT GCA ATG GCT GGC GGT AAT ATT GTT CTG GAT ATT ACC AGC AAG GCC GAT AGT TTG AGT TCT TCT-3' and 30-nt primer 5'-CTG GTA ATA TCC AGA ACA ATA TTA CCG CCA-3'. DNA concentrations were determined by absorbance at 260 nm using the millimolar extinction coefficients 1005.5 and 298.7 for template and primer, respectively. Primed DNA was prepared by mixing the primer and template in a 1.2:1 ratio in 300 mM NaCl, followed by heating at 95°C for 5 min and cooling the mixture over 2–3 h. Polyethyleneimine (PEI)-cellulose TLC plates were purchased from EM Separations and nitrocellulose membranes (BA-85) were purchased from Schleicher and Schuell. Extension buffer: 20 mM Tris-HCl pH 7.5, 40 $\mu\text{g}/\text{ml}$ BSA, 8 mM MgCl_2 and 5 mM DTT. Reaction buffer: 20 mM Tris-HCl pH 7.5, 10 mM MgCl_2 , 50 mM NaCl and 2 mM DTT. SDS buffer: 50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% Bromophenol Blue and 10% glycerol.

Primer extension assays

The primer was 5' end-labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase, and annealed to the template as described previously (Bloom *et al.*, 1996). Primed DNA was pre-incubated for 2 min at 37°C with 40 nM SSB in extension buffer, and then for 5 min with 20 nM β + 3.5 nM γ complex + 0.5 mM ATP (when in the reaction). Primer extension reactions were initiated by mixing 2 nM DNA with 18 nM core polymerase in extension buffer containing 4% glycerol, 0.5 mM ATP, and 60 μM of dATP, dGTP, dCTP and dTTP (20 μl final volume). Reactions were quenched at varying times with 20 mM EDTA in 95% formamide (40 μl). The products were separated on a 10% denaturing gel and visualized on a PhosphorImager (Molecular Dynamics).

Nucleotide binding experiments

ATP binding to γ complex was measured at 4°C by nitrocellulose filter binding experiments. A constant amount of γ complex was titrated with increasing amounts of radiolabeled ATP under various conditions. The γ complex alone (2.5 μM), with β (2.5 μM), with primed DNA (2.5 μM) or with β + primed DNA (2.5 μM each) was incubated for 30 s with ATP (0–80 μM) + [α - ^{32}P]ATP, in reaction buffer and then assayed as described (Hingorani and O'Donnell, 1998). The molar amount of ATP bound to γ complex was plotted versus ATP concentration. The binding isotherms were fitted to a hyperbola to determine the maximum ATP bound per γ complex and the K_d for the interaction.

Steady-state ATPase assays

Steady-state ATPase rates were measured at 20°C with 0.1 μM of γ complex alone, γ complex and 5 μM β , γ complex and 5 μM primed DNA, or γ complex and 5 μM β + primed DNA. The proteins were mixed with ATP (10–800 μM) + [α - ^{32}P]ATP (2 μCi) in reaction buffer (total volume 50 μl). Aliquots (5 μl) were removed at different times and quenched with 5 μl of 0.5 M formic acid. Aliquots (1 μl) of the quenched solutions were spotted immediately onto PEI-cellulose TLC plates and developed in 0.6 M potassium phosphate buffer pH 3.4. The [α - ^{32}P]ATP and [α - ^{32}P]ADP were quantitated on a PhosphorImager. The molar amount of ADP produced was plotted versus reaction time to obtain initial velocities of ATP hydrolysis (at each ATP concentration). A plot of reaction velocity ($\mu\text{mol}/\text{s}$) versus ATP concentration (μM) yielded the Michaelis-Menten constant, K_m , and the rate constant, k_{cat} for the ATPase activity.

Pre-steady-state ATPase assays

The ATPase assays were performed on a rapid chemical quench-flow instrument (KinTek Corp., Austin TX; reviewed in Johnson, 1992). A 15- μl volume of 6 μM γ complex (from one syringe of the instrument) was mixed with a 15- μl volume of ATP (final concentrations: 100–500 μM) and [α - ^{32}P]ATP (3 μCi per reaction) from the second syringe, for varying times of reaction. β (8 μM) and primed DNA (7 μM) (when present in the reaction) were added to γ complex and ATP, respectively. All reactions were performed at 20°C in reaction buffer. Reactions were quenched with 0.5 M formic acid, mixed in from the third syringe. Aliquots (1 μl) were spotted immediately onto TLC plates and analyzed as described above. The burst rate for ATP hydrolysis was maximal (4–6 s^{-1}) at all ATP concentrations tested, therefore only the data set at 400 μM ATP is shown for all the pre-steady-state assays.

In pulse-chase experiments, the reactants were mixed for varying times, followed by addition of excess nonradiolabeled ATP (10 mM ATP + 10 mM MgCl_2 in reaction buffer) from the third syringe. After a chase time equivalent to 6–10 turnovers, the reactions were quenched with 70 μl of 0.5 M formic acid and analyzed as described above (chase times were 80 s for γ complex, 15 min for γ complex- β , 40 s for γ complex-DNA and 5 s for γ complex- β -DNA, based on steady-state rates). The 'chase-time' assay was performed by mixing equal volumes of γ complex (6 μM) + β (8 μM) with ATP (final concentration: 400 μM) + [α - ^{32}P]ATP (3 μCi per reaction) + primed DNA (7 μM), for 0.08 s. This was followed by addition of excess nonradiolabeled ATP (10 mM ATP + 10 mM MgCl_2) from the third syringe. The reactions were continued for varying times from 0–5 s and then quenched with 70 μl of 0.5 M formic acid. The amount of [α - ^{32}P]ADP formed over the chase time was quantitated as described above. The steady-state chase-time assay was performed similarly except that the initial incubation time was 2 s, and after addition of excess ATP the reactions were continued for 0–10 s.

Data analysis

The data from rapid quench and pulse-chase assays were fit to a single exponential followed by a linear phase, using non-linear regression analysis (KaleidaGraph; Synergy Software):

$$y = A(1 - e^{-\lambda t}) + kt \quad (1)$$

where A and λ are the amplitude and rate of the burst phase, respectively, k is the linear rate and t is the reaction time. The linear rate divided by the rapid quench burst amplitude, or γ complex concentration (in the absence of a burst phase), yields the steady-state k_{cat} . The data were also simulated using HopKINSIM (Wachstock and Pollard, 1994) and the proposed kinetic mechanism is shown in Figure 4A. Rate constants for ATP binding and hydrolysis obtained from the pre-steady-state assays were used as initial estimates in the simulations. The simulated curves

were compared with experimental data to refine the parameters of the reaction mechanism.

Data from the 'chase-time' assay were fit to a double exponential:

$$y = A_1(1 - e^{-\lambda_1 t}) + A_2(1 - e^{-\lambda_2 t}) \quad (2)$$

where A_1 and A_2 are the amplitudes of the first and second hydrolysis phase and λ_1 and λ_2 are the hydrolysis rates, respectively. The steady-state chase-time data were fit to a single exponential.

Clamp-release assay

Release of β from γ complex was assayed by mixing β^{PK} (1 μM), which has a kinase recognition site, with γ complex (2 μM) and ATP (200 μM) in reaction buffer (total volume 120 μl) for 1 min at 20°C. The incubation was followed by addition of 20 μM β (with no kinase tag) as a trap. At varying times, 10 μl aliquots of the reaction were mixed with [$\gamma\text{-}^{32}\text{P}$]ATP (0.2 μCi) and the catalytic subunit of the cAMP-dependent protein kinase (0.4 μg), to allow radiolabeling of any β^{PK} released from γ complex during the chase period. The reactions were quenched after 7 s with 5 μl EDTA (0.5 M) and 5 μl SDS buffer, and analyzed on a 12% gel by SDS-PAGE. Radiolabeled β^{PK} was visualized and quantitated on a PhosphorImager. The fraction of β^{PK} labeled at each time point (compared with total β^{PK} labeled after 20 min chase) was plotted versus chase time, and the data fitted to a single exponential to yield the rate of β release from γ complex. The amount of β^{PK} labeled at zero chase time was determined by assaying a 10 μl aliquot of the reaction as described above, but prior to addition of excess β trap.

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Note added in proof

The 'chase time' experiment (i.e. Figure 3A) was also performed by preincubating γ complex with β and ATP for 2 s (to ensure that all γ complex binds β and opens the ring), prior to adding DNA. The data were identical to Figure 3A, with two ATP molecules hydrolyzed in two distinct steps.