ATP Binding to the *Escherichia coli* Clamp Loader Powers Opening of the Ring-shaped Clamp of DNA Polymerase III Holoenzyme*  

(Received for publication, May 13, 1998, and in revised form, July 16, 1998)

Manju M. Hingorani‡ and Mike O’Donnell§

*From the ‡Rockefeller University, the §Rockefeller University and Howard Hughes Medical Institute, New York, New York 10021

The *Escherichia coli* γ complex serves as a clamp loader, catalyzing ATP-dependent assembly of β protein clamps onto primed DNA templates during DNA replication. These ring-shaped clamps tether DNA polymerase III holoenzyme to the template, facilitating rapid and processive DNA synthesis. This report focuses on the role of ATP binding and hydrolysis catalyzed by the γ complex during clamp loading. We show that the energy from ATP binding to γ complex powers several initial events in the clamp loading pathway. The γ complex (γ∗,βδγ∗φδ) binds two ATP molecules (one per γ subunit in the complex) with high affinity (Kd = 1–2.5 × 10⁻⁶ M) or two adenosine 5’-O-(3-thiotriphosphate)(ATP•S) molecules with slightly lower affinity (Kd = 5–6.5 × 10⁻⁶ M). Experiments performed prior to the first ATP turnover (kcat = 4 × 10⁻⁶ s⁻¹ at 4 °C), or in the presence of ATP•S (kcat = 1 × 10⁻⁶ s⁻¹ at 37 °C), demonstrate that upon interaction with ATP the γ complex undergoes a change in conformation. This ATP-bound γ complex binds β and opens the ring at the dimer interface. Still prior to ATP hydrolysis, the composite of γ complex and the open β ring binds with high affinity to primer-template DNA. Thus ATP binding powers all the steps in the clamp loading pathway leading up to the assembly of a γ complex-open β ring-DNA intermediate, setting the stage for ring closing and turnover of the clamp loader, steps that may be linked to subsequent hydrolysis of ATP.

Rapid and efficient duplication of genomic DNA depends on biomolecular machines known as DNA replisomes. In diverse organisms these multicomponent biological machines exhibit varying degrees of complexity, including substantial differences in subunit composition. It is apparent, however, that certain mechanisms of action and the associated protein tools are conserved through evolution. One prominent example is the mechanism for processive DNA replication common among replicases from bacteriophage T4, *Escherichia coli*, yeast, and humans. Briefly, a clamp loader uses energy from ATP to assemble a circular protein clamp around DNA; the clamp, now topologically linked to DNA, tethers DNA polymerase to the template, and by sliding freely on duplex DNA it allows continuous replication of several thousand nucleotides without a dissociation event (reviewed in Refs. 1–3).

The *E. coli* clamp loader, γ complex, is a composite of five different proteins, γ, δ, δ’, χ, and ψ, of which γ is the ATP-binding subunit essential for clamp loading (4–7). The clamp, β, is a dimeric ring with a 35-Å inner diameter, large enough to encircle DNA (8). β and γ complex form part of DNA polymerase III holoenzyme, the replicative DNA polymerase of *E. coli*. The holoenzyme assembly also includes a core polymerase (αβδɛ), composed of α, the DNA polymerase (9), ε, the proofreading exonuclease (10, 11), and θ of unknown function (12, 13), as well as τ, a dimeric protein that holds together two polymerase cores and binds one γ complex (14–16). The core polymerase is a nonprocessive, inefficient DNA polymerase that extends a primer by only 10–20 nucleotides before dissociating from the template (17). When the β clamp tethers the core polymerase to template DNA, however, the enzyme develops high processivity and extends DNA by several thousand nucleotides per binding event (18, 19).

In the current model for *E. coli* DNA replication, after the assembly of an initiation complex in which the polymerase III holoenzyme is tethered to a primed DNA template by circular protein clamps, the two core polymerases in the holoenzyme synthesize leading and lagging strand DNA synchronously (20–23). Synthesis of the leading strand occurs in the direction of replication fork movement, but the lagging strand is synthesized in the opposite direction in discrete 1–2-kilobase pair-long Okazaki fragments. Therefore on the leading strand, after one clamp loading event and initiation complex formation, the polymerase can extend an RNA primer for several thousand nucleotides. On the lagging strand, however, the polymerase must initiate synthesis at new primers every few seconds, and a protein clamp must be loaded at each primer for initiation complex formation (24). Thus, clamp loader action is required continuously through the entire process of genome replication.

The clamp loader belongs to the category of proteins known as molecular matchmakers. These proteins, by definition, use their ATPase activity to promote assembly of a stable complex between target macromolecules (25), as the clamp loader uses ATP to assemble a complex between a clamp and DNA. Recent studies of the *E. coli* clamp loader have revealed substantial information about its subunit composition and its mechanism of action. The γ complex contains one each of δ, δ’, χ, and ψ subunits, and two or three γ subunits (6, 26). δ is the only subunit that binds β (27); γ binds and hydrolyzes ATP (5, 28); δ’ appears to bury δ within the γ complex and block its interaction with β; χ interacts with SSB,² facilitating DNA chain

---

*This work was supported by National Institutes of Health Grant GM 38389 (to M. O’D.) and was also made possible in part by funds granted by the Charles H. Revson Foundation (to M. M. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 212-327-7251; Fax: 212-327-7254; E-mail: odonnell@rockvax.rockefeller.edu; manju@mod.rockefeller.edu.

§ The abbreviations used are: SSB, *E. coli* single-stranded DNA-binding protein; ATP•S, adenosine 5’-O-(3-thiotriphosphate); AMP-
extension at physiological ionic strength, and the function of ψ is unknown (29, 30). Initial insight into how the clamp loader works was provided by studies showing that the free δ subunit binds β in the absence of ATP, but when δ is in the γ complex ATP is required for its interaction with β. It has been postulated that in the presence of ATP a conformational change in γ complex presents δ for interaction with β (27). Once this interaction is established, the clamp loader must assemble the β ring around DNA, and it must release the β-DNA complex to complete its job as a molecular matchmaker. At present there is not a clear understanding of how these steps occur and how the ATPase activity of γ complex powers β assembly on DNA.

An earlier study with an ATPase mutant γ complex demonstrated that inhibition of ATP hydrolysis blocks DNA replication, and this effect was attributed to the inability of the mutant complex to assemble a β clamp on DNA (7). In the present study we have continued to investigate how the clamp loader uses ATP, and we were surprised to find that ATP binding powers the clamp loading process almost to completion. Initially, quantitative nucleotide binding assays were used to characterize γ complex interaction with ATP and its slowly hydrolyzing analog, ATPβS. Partial proteolysis assays, DNA binding experiments, and a novel ring-opening assay were performed prior to and subsequent to ATP/ATPβS hydrolysis to separate the role of ATP binding from hydrolysis in clamp loading. We show here that ATP binding induces a conformational change in the γ complex, promotes its interaction with β, and powers β ring opening. The ATP-bound γ complex-β complex binds primer-template DNA leading to formation of a stable γ complex-open β ring-DNA composite that is an important intermediate in the clamp loading pathway (Fig. 10).

**EXPERIMENTAL PROCEDURES**

**Proteins**—Proteins were overexpressed and purified as described: γ (31), β and L273C-β (32), δ and δ' (33), χ and φ (34), and K51R-γ (7). γ complex (with γ or K51R-γ) was constituted from purified component subunits as described (26), and core polymerase (αβδ) was prepared as described (16). Protein concentrations were determined by absorbance measurements (280 nm) of the proteins denatured in 8 M urea, using their calculated molar extinction coefficients (26). T4 polynucleotide kinase was purchased from New England Biolabs; Pronase E and trypsin were purchased from Sigma; Phe, Tyr, and L273C-γ (32), and K51R-γ (7), were prepared as described (36), to a specific activity of 104 cpm/pmol (dimer), and β (1 μM dimer) in the presence of 1 μM ATP or ATPβS, for 5 min at 23 °C in Reaction buffer A (30 μl final volume). Core polymerase (αβδ) was added (0.25 μl), and following incubation for another 1 min the reaction was shifted to 37 °C and replication initiated by addition of 500 μM [α-32P]dATTP. At various times the reaction was quenched by mixing 7 μl of the reaction mix with 7 μl of 1% SDS + 100 μM EDTA. Aliquots were spotted onto DE81 filters (Whatman), and free [α-32P]dATTP was rinsed away with 0.3 M ammonium formate + 0.01 M pyrophosphate. The molar amount of [α-32P]dATTP incorporated into DNA was quantitated by liquid scintillation counting and plotted versus reaction time.

**Measurement of ATPase/ATPβSase Activity**—The steady-state ATPase activity of γ complex (γ or K51R-γ) was measured at 4 or 37 °C by monitoring hydrolysis of [α-32P]ATP to [α-32P]AMP + γP. γ complex (γ or K51R-γ, 1 μM) was mixed with ATP + [α-32P]ATP (1 μM) and β (1 μM dimer) in the presence of 1 μM primer-template DNA in Reaction buffer B. Aliquots were removed after varying time intervals and quenched with an equal volume of 1 N HCl, followed by chloroform denaturation of protein and neutralization with base (1 N NaOH + 0.25 M Tris-base). The quenched solutions were analyzed by polyacrylamide gel electrophoresis, thin layer chromatography in 0.6 M potassium phosphate buffer, pH 3.4. [α-32P]ATP and [α-32P]ADP were quanititated on a PhosphorImager (Molecular Dynamics), and the molar amount of ADP formed was plotted versus reaction time. ATPase rate constants were calculated from initial velocities of ADP formation and γ complex concentration.

**Quantitative Nucleotide Binding**—ATP and ATPβS binding to γ complex was measured by monitoring the incorporation of [γ-32S]ATPβS to ADP + [γ-32S]βiphosphinate. The assays were performed at 37 °C with γ complex (5 μM), [γ-32S]ATPβS (500 μM), with or without β (5 μM) and primer-template DNA (5 μM), as described above for ATPase activity. ATPase activity of the γ subunit (5 μM) was also measured by similar assays performed at 37 °C in the absence of β and DNA.

**Nitrocellulose Membrane Binding Assays**—Bovine serum albumin, DTT, diithothreitol; Me3SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; FCNA, proliferating cell nuclear antigen; RF, replication factor C.

PNP, 5′-adenylylimidodiphosphate; nt, nucleotide; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; pol, polymerase; BSA, bovine serum albumin; DTT, diithothreitol; Me3SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; FCNA, proliferating cell nuclear antigen; RF, replication factor C.
protein concentration; \( K_m \) in an earlier report (27). Briefly, 100 mM NaCl. ATP was added to initiate the reaction, and after 5 s

\[ {MLM}_T = K_p (K + L_p) \]

(Eq. 2)

where \( ML \) is the amount of ligand bound to protein; \( M_p \) is the total protein concentration; \( K_1 \) and \( K_2 \) are association constants; \( L_p \) is the free ligand concentration (ATP or ATPγS); \( n \) is the Hill coefficient; \( K \) is a dissociation constant; and \( {MLM}_T \) is the fraction of ligand bound per γ complex, or ligand bound per subunit in the γ complex (in the Hill equation). The binding curves were fit by a nonlinear least squares fitting program using KaleidaGraph (Synergy Software).

**Partial Tryptic Digestion of γ Subunit and γ Complex—**Tryptic digestion of γ was performed at 37 °C in the absence and in the presence of various nucleotides. The reaction contained γ subunit (10 μM) and no nucleotide, ATP, ADP, or ATPγS (2 mM) in Reaction buffer B in a total volume of 12 μl. Trypsin was added to a final concentration of 0.05 μg/mL. After 10 min at 37 °C the reaction was quenched with 10 μl of SDS loading buffer and boiled for 5 min. The proteolytic products were analyzed on a 12% gel by SDS-PAGE and visualized by staining with Coomassie Blue.

Tryptic digestion of the γ complex was performed with 5 μg γ complex in Reaction buffer B in the presence or in the absence of ATP, ADP, ATPγS (500 μM) in a total reaction volume of 10 μl. Trypsin was added to a final concentration of 0.8 μg/mL, and following incubation for 10 min at 37 °C the reaction was quenched with an equal volume of SDS loading buffer. The products were analyzed on a 12% polyacrylamide gel as described above. Some of the proteolytic fragments were N-terminally sequenced at the Rockefeller University Protein/DNA Technology Center.

**Ring-opening Assay—**A modified version of β, with Leu-273 (at the dimer interface) changed to cysteine and Cys-333 (at the protein surface) changed to serine, was prepared and tested for activity as described.1 L273C-β activity in ATPase assays, DNA replication assays, and clamp loading assays was identical to that of wild-type β, ring opening was detected by measuring the labeling of Cys-273 with eosin-5-maleimide. A mixture of L273C-β (2 μM) and γ complex (2 μM) in 20 mM Tris-HCl, pH 7.5, + 10 mM MgCl₂ (10 μM final value) was incubated for 2 min at 4 °C for 1 h. After 2 min ATPγS (0.5 mM) was added, followed by 10 s by addition of 1 μl of eosin-5-maleimide stock solution (5 mM in Me2SO). Control reactions were performed with L273C-β alone and with γ complex + L273C-β in the absence of nucleotides. The reactions were quenched after 30 s with an equal volume of 0.5 M DTT + SDS loading buffer; the samples were boiled for 2 min and subjected to SDS-PAGE on a 10% gel. Fluorescence-labeled β was visualized on a Fluor-S MultiImager (Bio-Rad). The ring-opening assay was performed similarly in the presence of 3 μM primer-template DNA. The proteins were incubated with primer-template DNA in the presence of ATPγS (1 min at 23 °C), and the labeling reaction and analysis were performed as described above.

RESULTS

The E. coli clamp loader uses ATP to convert the inefficient polymerase III core enzyme into a highly processive DNA replicating machine. Earlier studies have demonstrated that γ complex loads the circular clamp β onto a primed DNA template, and β tethers the polymerase to DNA, facilitating highly processive DNA synthesis. Here we investigate how γ complex uses its ATPase activity for clamp loading. To assemble β on DNA the γ complex must bind β as well as DNA, open the β ring and place it around DNA, and finally dissociate from the complex to allow polymerase access to the clamp. The question is: what powers various steps in the clamp loading pathway or, alternately, how is energy from ATP binding and hydrolysis used for clamp loading?

**ATP Hydrolysis Requirement for Clamp Loading: an ATPase Mutant Study—**Initially we used an ATP-ase-inactive γ complex to investigate the role of ATP in clamp loading. Mutants of the clamp loader subunits δ and γ had been prepared previously to determine which subunit harbors the ATPase activity essential for clamp loading (40). According to data from UV cross-linking experiments, the δ subunit may interact with ATP (41); however, mutation of Lys-225 to Ala in the near-consensus nucleotide-binding site in the δ subunit did not inhibit the ATPase activity or the clamp loading activity of γ complex reconstituted with mutant δ (40). On the other hand, mutation of Lys-51 to Ala in the nucleotide-binding Walker A site of γ subunit inactivated the ATPase activity of γ complex. Therefore, mutations that result in the loss of ATPase activity of γ complex, indicating that γ serves as the clamp loader ATPase (7). The mutant γ complex did not coprecipitate with nicked DNA replicates, and it was hypothesized that the defect was due to a loss of clamp loading activity resulting from the loss in clamp loader ATPase activity.

In this study we tested the above hypothesis by assaying directly the clamp loading activity of a mutant ATPase γ complex. Fig. 1, panel A, shows that the mutant K51R-γ complex...
Bio-Gel A-15m gel filtration column, [3H]M under the same conditions K51R-assembles [3H]gel-filtered as described in the "Experimental Procedures." Circular M13 ssDNA (400 fmol) for 5 min at 37 °C. The reactions were loading clamps. These data together with the earlier results plotted panel B SSB-coated, primed M13mp18 DNA (Fig. 1, a + 500 fmol of [35S]ATP (1 mM), primer-template DNA (1 μM), β (1 μM), and γ complex (1 μM), as described under "Experimental Procedures." Molar amount of ADP formed is plotted versus time of reaction (seconds), and the linear slope yields \( k_{cat} = 5 \text{s}^{-1} \) for wild-type γ complex and \( k_{cat} = 6.9 \times 10^{-2} \text{s}^{-1} \) for K51R-γ complex (panel A). Clamp loading activity was assayed by incubating 500 fmol of γ complex (wild-type, with 1 mM ATP, ○; K51R, with 1 mM ATP, ⬤; K51R, with 1 mM ATP, ○; with [3H]β(500 fmol) and SSB-coated circular M13 ssDNA (400 fmol) for 5 min at 37 °C. The reactions were gel-filtered as described in the "Experimental Procedures." β assembled on M13 ssDNA elutes in fractions 10–15 and free β elutes in fractions 18–35 (panel B).

has severely inhibited ATPase activity. At 37 °C, wild-type γ complex hydrolyzes ATP with a \( k_{cat} = 5 \text{s}^{-1} \) in the presence of β and a primed DNA template, whereas the ATPase activity of K51R-γ complex is much slower with a \( k_{cat} = 6.9 \times 10^{-2} \text{s}^{-1} \). The clamp-loading activity of the mutant γ complex was assayed directly by following the assembly of [3H]-labeled β on SSB-coated, primed M13mp18 DNA (Fig. 1, panel B). On a Bio-Gel A-15m gel filtration column, [3H]β assembled on DNA elutes in the early fractions (fractions 10–15) and is resolved from free β protein (fractions 18–35). Wild-type γ complex assembles [3H]β around DNA in the presence of ATP, but under the same conditions K51R-γ complex is incapable of loading clamps. These data together with the earlier results confirm that ATP hydrolysis is necessary for loading β clamps on DNA and is therefore essential for processive DNA synthesis.

**ATP Hydrolysis Requirement for Clamp Loading:** an ATP-γS Study—The apparent requirement of ATP hydrolysis for the assembly of β onto DNA as well as for processive DNA synthesis was investigated further by measuring γ complex activity in the presence of ATP-γS, an ATP analog that is poorly hydrolyzed by most ATP hydrolases. First we examined the effect of ATP-γS on replication of a (dT)\(_{35}\)-primed poly(dA) template (Fig. 2, panel A). Utilization of a poly(dA) template allowed measurement of DNA synthesis in the absence of dATP which, like ATP, can support γ complex-catalyzed loading of clamps on DNA. The clamp loader, clamp, and DNA were incubated with ATP or ATP-γS to allow clamp loading, followed by addition of core polymerase (αεθ) and [α\(^{32}\)P]dTTP to initiate DNA replication. As shown in Fig. 2, panel A, [α\(^{32}\)P]dTTP is incorporated into DNA at a rate of 2.2 μm \(^{-1}\) when ATP is present in the reaction. In the presence of ATP-γS, however, DNA replication is indistinguishable from the basal level of replication in the absence of any nucleotide (0.2 μm \(^{-1}\)). We then examined the effect of ATP-γS on the clamp loading activity of γ complex. Clamp loading assays using [3H]β were performed in the presence of ATP or ATP-γS, and reactions containing ATP-γS were sampled at longer times to account for potentially slow ATP-γS hydrolysis. As shown in Fig. 2, panel B, when ATP-γS was present in the reaction, assembly of [3H]β onto DNA was barely detectable over the background, even after 1 h at 37 °C.

To determine why ATP-γS does not support clamp loading, we tested the ability of γ complex to hydrolyze [35S]ATP under various conditions. The assays were performed at high ATP-γS concentrations to measure the maximum steady-state rate of hydrolysis. Fig. 2, panel C, shows that γ complex (5 μM) hydrolyzes ATP-γS very slowly (\( k_{cat} = 1 \times 10^{-4} \text{s}^{-1} \)) both in the absence and in the presence of β. There is no burst activity at initial time points in the reaction, and the \( k_{cat} \) reflects the slowest rate in the reaction pathway; therefore one turnover of ATP-γS hydrolysis requires more than 2 h at 37 °C. There is also no stimulation of ATP-γS hydrolysis on addition of primer-template DNA, a substrate in the clamp loading reaction. Inability of the clamp loader to hydrolyze ATP-γS efficiently, coupled with its inability to load clamps or stimulate DNA synthesis in the presence of ATP-γS, is consistent with earlier evidence that ATP hydrolysis is essential for clamp loading and processive DNA replication (Ref. 7; Fig. 1).

It is possible, however, that the clamp loader cannot bind ATP-γS as it binds ATP and therefore cannot use ATP-γS to assemble β onto DNA. To address this possibility we used nitrocellulose membrane binding assays to measure and compare interactions of γ complex with ATP and ATP-γS. Fig. 3, panel A, shows data from the titration of a constant amount of γ complex and β (2 μM) with increasing concentrations of ATP, where the γ complex-β-bound ATP is plotted versus total ATP in the reaction. The binding isotherm reaches saturation at 4 μM ATP, demonstrating quite strikingly that two ATP molecules bind γ complex with high affinity. Earlier evidence from high pressure liquid chromatography and SDS-PAGE analysis of the γ complex has indicated that the E. coli clamp loader is likely composed of 2–3 γ subunits (Ref. 7; Fig. 1).3 Like γ complex the τ complex (τβδτφ) also assembles β around DNA in the presence of ATP, but unlike γ complex, it stimulates replication of the dT-primed poly(dA) template by core polymerase in the presence of ATP-γS ([α\(^{32}\)P]dTTP is incorporated into DNA at 6 μm \(^{-1}\) in the presence of ATP, 2 μm \(^{-1}\) in the presence of ATP-γS, and 0.5 μm \(^{-1}\) in the absence of nucleotides). Similar γ complex activity has also been observed in an assay measuring replication of M13 G\(_{90}\) DNA (59). Perhaps the τ complex can hydrolyze ATP-γS and use this ATP analog for clamp loading. It should be noted, however, that unlike γ, the τ subunit binds both DNA and the core DNA polymerase; therefore, the τ complex may bring the clamp, DNA, and polymerase in close proximity and facilitate their interaction even in the absence of ATP hydrolysis. See "Discussion" for details of the clamp loading pathway.

3 Like γ complex the τ complex (τβδτφ) also assembles β around DNA in the presence of ATP, but unlike γ complex, it stimulates replication of the dT-primed poly(dA) template by core polymerase in the presence of ATP-γS ([α\(^{32}\)P]dTTP is incorporated into DNA at 6 μm \(^{-1}\) in the presence of ATP, 2 μm \(^{-1}\) in the presence of ATP-γS, and 0.5 μm \(^{-1}\) in the absence of nucleotides). Similar γ complex activity has also been observed in an assay measuring replication of M13 G\(_{90}\) DNA (59). Perhaps the τ complex can hydrolyze ATP-γS and use this ATP analog for clamp loading. It should be noted, however, that unlike γ, the τ subunit binds both DNA and the core DNA polymerase; therefore, the τ complex may bring the clamp, DNA, and polymerase in close proximity and facilitate their interaction even in the absence of ATP hydrolysis. See "Discussion" for details of the clamp loading pathway.
The data were fit to an equation describing the interaction of two ligands with one macromolecule (Equation 1), and Fig. 3, panel B, shows that the two ATP molecules bind γ complex with slightly differing affinities, with dissociation constants $K_1 = 2.6 \times 10^{-6}$ M and $K_2 = 1.12 \times 10^{-6}$ M. The binding isotherm fits the Hill equation (Equation 2) with a coefficient $n = 1.5$, which also indicates slight positive cooperativity in the interaction of two ATP with γ complex; β (data not shown). The results from nitrocellulose membrane binding assays are consistent with previous equilibrium gel filtration experiments, which yielded a dissociation constant $K_d \sim 1 \times 10^{-6}$ M for ATP binding to γ complex. Interestingly, although the γ subunit is an inefficient ATPase, it binds ATP with the same affinity as the γ complex, $K_d = 1.2 \times 10^{-6}$ M. Presumably, assembly of γ into a complex with other subunits does not affect ATP binding but stimulates γ subunit ATPase activity by influencing other steps in the ATPase pathway.

The high affinity interaction between ATP and γ complex was compared with ATPγS binding. Like ATP, two ATPγS molecules bind per γ complexβ (Fig. 3, panel C). The Hill coefficient, $n = 1.4$, indicates that the two molecules bind with slight positive cooperativity (data not shown), and Fig. 3, panel D, shows that the two dissociation constants for ATPγS binding are similar $K_1 = 6.5 \times 10^{-6}$ M and $K_2 = 5 \times 10^{-6}$ M. Most notably, the data show that ATPγS binds γ complex with only 2–5-fold lower affinity than ATP; therefore, the inability of the clamp loader to use ATPγS for clamp loading is likely not due to a defect in ATPγS binding.

The data presented in Figs. 2 and 3 indicate that ATP binding is not sufficient, and ATP hydrolysis is necessary for γ complex-catalyzed assembly of circular clamps around DNA. We have also found that the E. coli clamp loader binds ATPγS but does not hydrolyze it efficiently; therefore, ATPγS can be considered a nonhydrolyzable ATP analog in experiments performed within a short time. The assays described in the following sections utilize ATPγS in conjunction with ATP to determine whether ATP binding plays an important role in clamp loading.

**ATP Binding to γ Promotes a Conformational Change in the γ Complex**—It has been postulated that ATP facilitates interaction between γ complex and β by inducing a conformational change in γ complex. This idea is supported by the observation that the Pronase digestion pattern of β subunit within the γ complex is altered in the presence of ATP (27). Does ATP binding affect a change in γ complex conformation? To address this question, a partial tryptic digest of the γ subunit was performed in the absence of ATP and in the presence of various nucleotides. The digest products were analyzed by SDS-PAGE, and the Coomassie Blue-stained gel is shown in Fig. 4, panel A. In the absence of nucleotide almost all the γ in the reaction is proteolyzed in 10 min at 37 °C (lane 2). Several proteolytic fragments range between masses 20–30 kDa, and some are the result of cleavage at Arg-208 or Arg-215 and varying C-terminal cleavage sites (data from N-terminal sequencing and mass spectroscopic analysis not shown). In the presence of ATP, the digest pattern is significantly different than in the absence of nucleotides (Fig. 4, panel A, lanes 2 and 3). The small proteolytic products observed in lane 2 are not formed when ATP is present in the reaction (lane 3), and γ appears to be relatively resistant to tryptic digest. A similar change in digest pattern is detected when ADP or ATPγS are present in the reaction (Fig. 4A, lanes 4 and 5). The γ subunit hydrolyzes ATP ($k_{cat} = 3 \times 10^{-3}$ s$^{-1}$ at 37 °C) but not ATPγS (hydrolysis undetectable; data not shown) during the tryptic digest. Both nucleotides, however, induce similar changes in the γ subunit digest pattern indicating that the change occurs on ATP binding to γ. In

---

**Fig. 2. ATPγS does not support β loading or processive DNA replication.** Panel A shows a DNA replication assay performed with a (dT)$_{50}$ poly(dA) template (120 μM total nucleotide) and 0.025 μM γ complex and β in the absence of nucleotide (□), and in the presence of ATP(●) or ATPγS (●). The molar amount of [α-$^32$P]dTMP incorporated into DNA is plotted versus reaction time (minutes), and the rate of reaction is indicated. Panel B shows clamp loading activity of γ complex (500 fmol) incorporated into DNA is plotted versus reaction time (minutes), and the rate of reaction is indicated. Panel C shows steady-state ATPγSase activity assays of γ complex (5 μM) performed at 37 °C with γ-[35S]ATPγS (500 μM) in the absence of β (Δ), in the presence of 5 μM β dimer (∙), and in the presence of β + 5 μM primer-template DNA (+) as described under “Experimental Procedures.” The molar amount of [35S]thiophosphate formed is plotted versus time of reaction (minutes), and the linear slopes yield $k_{cat} = 7 \times 10^{-5}$ s$^{-1}$.1 × 10$^{-4}$ sec$^{-1}$ for ATPγS hydrolysis.
control assays performed with other isolated proteins, it was determined that trypsin activity is generally unaffected by the presence of nucleotides (data not shown).

The proteolytic digest assay was also performed with γ complex to determine if it changes conformation on ATP binding, as does the free γ subunit. The complex was subjected to tryptic digestion in the absence and in the presence of nucleotides, and the products were analyzed by SDS-PAGE (Fig. 4, panel B). Comparison of the peptide products in lane 3 (no nucleotide in the reaction) with lanes 4 and 6 (ATP and ATPγS in the reactions, respectively) shows that the digest pattern of γ complex changes in the presence of nucleotides. Again, ATPγS is not hydrolyzed in the time scale of the assay, indicating that the change occurs on nucleotide binding. ADP binding also induces changes in both γ and γ complex, similar to those observed on ATP binding (Fig. 4, panel A, lane 4, and Fig. 4, panel B, lane 5, respectively). These data imply that after ATP binding and hydrolysis the complex may not revert back to its original conformation until after ADP dissociation.

**ATP Binding Facilitates Interaction between γ Complex and β**—Next we analyzed the role of ATP binding and hydrolysis in promoting the association of γ complex with β. In an earlier study, interaction of γ complex with β was analyzed by gel filtration on a Superose 12 column, and it was found that ATP is necessary for stable γ complex-β interaction (27). In a similar assay used here, γ complex (5 μM) was incubated with β dimer (2.5 μM) in the absence of nucleotide and in the presence of ATP or ATPγS; the mixture was analyzed by gel filtration and SDS-PAGE of the column fractions. Panels A and B in Fig. 5 confirm the earlier report that ATP is required for γ complex binding to β, and panel C shows that like ATP ATPγS supports stable interaction between γ complex and β.

A more rapid assay based on protein footprinting was used to determine whether the γ complex-β interaction occurs upon ATP binding and whether ATP hydrolysis is required for this interaction. A 32P-labeled β clamp was incubated with γ complex in the absence and in the presence of nucleotides and proteolyzed with Pronase E, and the protein footprint was examined for changes due to γ complex-β interaction. In an earlier study, a similar protein footprinting assay demonstrated that ATP-induced interaction between β and γ complex protects a C-terminal fragment of β from proteolytic digest (38). Fig. 6, panel A, shows the expected [32P]β footprint in the presence of ATP and γ complex (lane 4); the protected fragment is indicated by an arrow. ATPγS facilitates similar protection of the β C terminus, and because it is not hydrolyzed in the 2-min time scale of the assay nucleotide binding appears sufficient for γ complex interaction with β (lane 6). To confirm this observation, the assay was also performed at 4 °C within 10 s, prior to the first turnover of ATP (kcat = 4 × 10⁻⁶ s⁻¹; no presteady-state burst of hydrolysis in the absence of DNA). In the presence of ATP, β is protected by γ complex even under

![ATP binding to γ complex](image)

**ATP binding to γ complex**

**A Stoichiometry**

![Stoichiometry graph](image)

**B Binding constants**

![Binding constants graph](image)

**ATPγS binding to γ complex**

**C Stoichiometry**

![Stoichiometry graph](image)

**D Binding constants**

![Binding constants graph](image)
these conditions (Fig. 6, panel B, lane 2); therefore, ATP binding but not hydrolysis is necessary for γ complex-β interaction. The assay also shows that the β C terminus is not protected by γ complex in the presence of ADP (Fig. 6, panel A, lane 5). Therefore, although ADP binding can lead to a change in γ complex conformation, it does not support γ complex-β interaction. This result implies that after ATP is hydrolyzed, γ complex-β may dissociate, resulting in γ complex turnover and its catalytic loading activity.

**ATP Binding Facilitates Interaction between γ Complex-β and DNA**—The E. coli clamp loader must bring β and primed DNA together to assemble the clamp around DNA. Consequently, one intermediate that must form during the clamp loading process is the three-component composite of γ complex, β, and DNA. To detect and study this intermediate, we examined the interactions between γ complex, β, and DNA, as well as the effects of nucleotide ligands on these interactions. [3H]γ complex was incubated with single-stranded M13mp18 DNA, in the absence and in the presence of β and nucleotides, and analyzed by gel filtration on an agarose A-15m column (Fig. 7, panels A and B). [3H]γ complex bound to DNA elutes early (fractions 11–16) and is resolved from free protein (fractions 16–31) during gel filtration. All γ complex in the reaction binds to M13 ssDNA in the presence of β and ATPγS (panel A). β appears essential for stable complex formation with DNA, because no interaction is detectable in its absence (panel A). In a complementary assay performed with 32P-labeled β and unlabelled γ complex the clamp co-elutes with the clamp loader on DNA, although appearance of free β in the gel filtration profile suggests that the clamp may be less stable in the protein-DNA complex as compared with the clamp loader (panel C). Since the DNA binding assay is complete within 13 min and prior to ATPγS hydrolysis (refer Fig. 2, panel C), the results demonstrate that ATPγS binding is sufficient for the interaction of γ complex-β with DNA.

Interestingly, when the assay is performed using ATP instead of ATPγS only a small fraction of 3H-labeled γ complex elutes with ssDNA, and when ADP is used there is no detectable interaction between the proteins and DNA (panel B). The results with ATPγS, indicating that ATP binding increases the affinity of γ complex-β for ssDNA, coupled with the fact that ADP does not support stable DNA binding suggest that ATP binding and hydrolysis modulate interaction of γ complex and β with DNA; thus ATP binding to γ complex promotes DNA binding, and ATP hydrolysis triggers DNA release. Consequently, the ATP turnover rate likely controls turnover of the protein-DNA composite, which explains why the composite is stable to gel filtration in the presence of the slowly hydrolyzing analog ATPγS but not in the presence of ATP.

It should be noted that β can be assembled as a ring around double-stranded DNA, or RNA-DNA, e.g. a primed ssDNA template, but not around ssDNA. Furthermore, ATP hydrolysis is essential for the assembly of β around primed DNA, and this final product of the clamp loading pathway is stable during gel filtration even if no ATP is present in the column buffer (refer Fig. 2, panel B). In contrast, ATPγS must be present in the column buffer to maintain a stable interaction between γ complex, β, and single-stranded M13mp18 DNA during gel filtration. Thus the protein-DNA composite formed in the presence of ATPγS is different from the final product of the clamp loading pathway. Evidence from an earlier study also indicates that after hydrolyzing ATP and loading β around DNA, the γ com-

---

**Fig. 4. Nucleotide binding induces a conformational change in γ and γ complex.** A partial tryptic digest was performed on 10 μM γ (undigested, lane 1) with no nucleotide (lane 2) or with 2.5 mM ATP (lane 3), ADP (lane 4), or ATPγS (lane 5) in the reaction as described under “Experimental Procedures,” and the reactions were analyzed by SDS-PAGE (panel A). γ complex (5 μM) was assayed similarly by tryptic digest (undigested: lane 2), in the absence of nucleotides (lane 3), or with 0.5 mM ATP (lane 4), ADP (lane 5), or ATPγS (lane 6) in the reaction (panel B). The differences in proteolytic cleavage are indicated by asterisks, and lanes 6 (panel A) and 1 (panel B) show protein standards whose masses are indicated.

---

**Fig. 5.** Trypsin action. This result implies that after ATP is hydrolyzed, γ complex-β may dissociate, resulting in γ complex turnover and its catalytic loading activity.

---

**Fig. 6.** ATP Binding Facilitates Interaction between γ Complex-β and DNA—The E. coli clamp loader must bring β and primed DNA together to assemble the clamp around DNA. Consequently, one intermediate that must form during the clamp loading process is the three-component composite of γ complex, β, and DNA. To detect and study this intermediate, we examined the interactions between γ complex, β, and DNA, as well as the effects of nucleotide ligands on these interactions. [3H]γ complex was incubated with single-stranded M13mp18 DNA, in the absence and in the presence of β and nucleotides, and analyzed by gel filtration on an agarose A-15m column (Fig. 7, panels A and B). [3H]γ complex bound to DNA elutes early (fractions 11–16) and is resolved from free protein (fractions 16–31) during gel filtration. All γ complex in the reaction binds to M13 ssDNA in the presence of β and ATPγS (panel A). β appears essential for stable complex formation with DNA, because no interaction is detectable in its absence (panel A). In a complementary assay performed with 32P-labeled β and unlabelled γ complex the clamp co-elutes with the clamp loader on DNA, although appearance of free β in the gel filtration profile suggests that the clamp may be less stable in the protein-DNA complex as compared with the clamp loader (panel C). Since the DNA binding assay is complete within 13 min and prior to ATPγS hydrolysis (refer Fig. 2, panel C), the results demonstrate that ATPγS binding is sufficient for the interaction of γ complex-β with DNA.

Interestingly, when the assay is performed using ATP instead of ATPγS only a small fraction of 3H-labeled γ complex elutes with ssDNA, and when ADP is used there is no detectable interaction between the proteins and DNA (panel B). The results with ATPγS, indicating that ATP binding increases the affinity of γ complex-β for ssDNA, coupled with the fact that ADP does not support stable DNA binding suggest that ATP binding and hydrolysis modulate interaction of γ complex and β with DNA; thus ATP binding to γ complex promotes DNA binding, and ATP hydrolysis triggers DNA release. Consequently, the ATP turnover rate likely controls turnover of the protein-DNA composite, which explains why the composite is stable to gel filtration in the presence of the slowly hydrolyzing analog ATPγS but not in the presence of ATP.
complex dissociates from β-DNA (39). The presence of γ complex in the ATPγS-bound protein-DNA composite suggests that it is an intermediate species in the clamp loading pathway.

DNA binding properties of γ complexβ were characterized further by testing different DNA substrates. Fig. 8 shows the results of non-denaturing gel-shift assays used to detect interactions between γ complexβ and primer-template DNA, ssDNA, or dsDNA. In the presence of ATPγS, γ complexβ binds with high affinity to a 32P-labeled primer-template DNA substrate (28-nt primer annealed to a 100-nt ssDNA of random sequence), resulting in a gel-shifted complex (Fig. 8, panel A). γ complexβ also binds to a 100-nt single-stranded DNA (Fig. 8, panel B), but no gel shift can be detected with the 100-nt double-stranded DNA even at 10 μM protein concentration (Fig. 8, panel C). Binding to dsDNA was also examined by gel filtration (as described in Fig. 7) using Bluescript plasmid DNA; however, no interaction could be detected between γ complexβ and dsDNA (data not shown).

The data from experiments performed with ss- and dsDNAs suggest that γ complexβ binds predominantly the single-stranded region on primer-template DNA. The primer-template substrate used here has a 36-nt single-stranded overhang on either side of the primer. Interestingly, binding assays performed with various ssDNAs showed that a minimum length of about 70 nts is required to detect a stable gel-shift from γ complexβ binding to DNA (data not shown). Presumably on our primer-template substrate the ss/ds junction structure confers additional stability to the protein-DNA composite beyond that provided by binding to a 36-nt ssDNA alone. In an assay designed to compare γ complexβ interaction with primer-template DNA to single-stranded DNA, the protein-DNA complexes were challenged with 60-fold excess 70-nt ssDNA. As shown in Fig. 8, panel D, the composite of γ complexβ and 32P-labeled primer-template DNA remains intact following addition of the unlabeled ssDNA trap to the reaction (lane 3). The composite of γ complexβ and 32P-labeled 100-nt ssDNA, however, dissociates easily, and the proteins are trapped effectively by unlabeled DNA, whereas the 32P-labeled 100-nt ssDNA migrates as free DNA (Fig. 8, panel D, lane 6). The same results were obtained when the assay was performed by mixing 32P-labeled DNA with the trap DNA prior to incubation with proteins (data not shown). Thus γ complexβ binds primer-template DNA with both greater affinity and stability than ssDNA, which may be a result of specific interaction between γ complexβ and the ss/ds DNA junction on the primer-template.

**ATP Binding Powers γ Complex-catalyzed β Ring Opening**—As mentioned earlier, it has been hypothesized that clamp loading involves γ complex-catalyzed opening of the β ring at the dimer interface followed by assembly around a primed DNA template. To investigate this hypothesis, an assay was developed that directly measures β ring opening.1 A modified β clamp, L273C-β, was prepared by changing Leu-273, a residue buried at the dimer interface, to Cys, and by changing a reactive Cys-333 on the protein surface to Ser (note: L273C-β activity was found identical to that of wild-type β in DNA replication, clamp loading, and ATPase assays). When the ring is closed Cys-273 is buried within the L273C-β dimer interface, inaccessible to solvent, and therefore unreactive. When the ring opens, however, Cys-273 can be labeled with thiol-reactive agents. In the current study, the role of ATP binding in clamp loading was investigated further with L273C-β and eosin-5-maleimide, a fluorescent reagent that reacts covalently with Cys-273 when the ring is open.

L273C-β was incubated with eosin-5-maleimide in the absence and in the presence of γ complex and nucleotides, and the proteins were analyzed by SDS-PAGE followed by fluorescence detection on a Fluor-S Multilmage (Bio-Rad). L273C-β, alone or with γ complex and no nucleotide, reacts weakly with eosin-5-maleimide (Fig. 9, panel A, lanes 1 and 2, respectively). When both γ complex and ATP are present in the reaction, fluorescent

---

**Fig. 5.** ATP and ATPγS promote interaction between γ complex and β. γ complex (5 μM) was incubated with β (2.5 μM dimer) under various conditions, and the mixture was gel-filtered on a Superose 12 column as described under “Experimental Procedures.” Aliquots (40 μl) of the column fractions were analyzed by SDS-PAGE on 12% gels. Panel A shows results from a reaction performed in the absence of nucleotides; panels B and C show the results from reactions containing 0.3 mM ATP and ATPγS, respectively. Fraction numbers and the positions of γ complex and free β are indicated above each gel, and the last lane of each gel contains protein standards whose masses are indicated to the right.

---

**β Opening and Assembly on DNA**
labeling of L273C-β is stimulated significantly, indicating that the reactive cysteine at the β dimer interface is exposed under these conditions (lane 3). Lane 4 in Fig. 9, panel A, shows similar stimulation of β labeling in the presence of ATPγS, and this occurs prior to ATPγS hydrolysis (reaction conditions, 30 s at 4 °C). Therefore, when it binds ATP the γ complex facilitates opening of the β clamp, presumably to place DNA in the clamp through the open ring interface.

The ring opening assay was also used to examine if the clamp is open in the three-component γ complex: β-DNA composite that is formed on ATPγS binding. As demonstrated earlier, the interaction between γ complex: β and primer-template DNA is very stable in the presence of ATPγS (Fig. 8, panel D); therefore, the ring opening assay was performed in the presence of the primer-template DNA substrate. Fig. 9, panel B, shows that fluorescence labeling of β increases significantly over the background when γ complex and ATPγS are present in the reaction with primer-template DNA (lane 3). Together, the data in Fig. 9 demonstrate that ATP binding powers γ complex-catalyzed opening of the β clamp as well as formation of a stable open-clamp complex on a primed DNA template.

**DISCUSSION**

The ring-shaped β clamp does not assemble on DNA by itself. The clamp loader, γ complex, catalyzes assembly of clamps around primed template DNA for use by the core DNA polymerase. The γ complex requires ATP for clamp loading, and consistent with this observation, mutation of Lys-51 in the Walker-A ATP-binding site in γ inhibits the clamp loading activity of γ complex and blocks processive DNA replication (7).

But how does the clamp loader couple its ATPase activity to clamp loading? In this study, we have examined the effects of ATP on γ complex and β (on protein conformation and protein-protein/DNA interactions), and we demonstrate that ATP binding powers several initial steps in the clamp loading pathway (Fig. 10).

The E. coli clamp loader functions as a "protein topoisomerase," cracking open the ring-shaped β clamp to place DNA in the center of the ring. ATP binding powers changes in γ complex that lead to formation of a γ complex-open β ring-DNA composite, which is likely an important intermediate in the clamp loading pathway. This information has been incorporated into a model for the role of ATP binding in the clamp loader pathway which will be discussed in detail later (Fig. 10). Furthermore, based on the effects of ATP and ADP on γ complex activity, we have hypothesized that after ATP binding powers formation of the γ complex-open β ring-DNA composite, ATP hydrolysis may trigger placement/closing of the clamp around DNA or simply dissociate the proteins and DNA so that β is released as a ring around DNA. In rendering the model we have also engaged in some speculation, imagining that the protein topoisomerase clamp loader functions like DNA topoisomerase II (42), taking in substrate DNA through one gate and releasing it into the β ring through a second gate to form a topological link between the clamp and DNA.

**Nucleotide Binding—**Nucleotide binding and hydrolysis assays demonstrated that γ complex binds ATPγS with similar affinity as ATP, and it hydrolyzes ATPγS very slowly so that one turnover can take up to 2 h at 37 °C ($k_{cat} = 1 × 10^{-4}$ s$^{-1}$
D. J. Jeruzalmi and J. Kuriyan, unpublished data.

with no presteady-state burst of hydrolysis); thus, ATPγS can be considered nonhydrolyzable in all assays that are completed for our study of the role of ATP binding in clamp loading, or with 32P-labeled ATP. For our study of the role of ATP binding in clamp loading, oligomers break down in the presence of the clamp loader comprising two subunits (43). In various other studies using gel filtration (5), densitometry of stained SDS-polyacrylamide gels (6), or sedimentation equilibrium analysis (44), the molar ratio of γ complex has been reported as low as 2 or as high as 4 subunits per γ complex. The subunits in γ complex have also been resolved by high performance liquid chromatography under denaturing conditions and quantitated by absorbance at 280 nm (using known extinction coefficients). This analysis indicated the presence of 2–3 γ subunits and 1 each of δ, δ', χ, and ψ per γ complex (26). Recently, we have analyzed the molecular mass of γ complex by multi-angle laser light scattering, and the molar ratio of γ subunits per γ complex is 2.5, a value closer to 2 rather than 4.5. Finally, high pressure liquid chromatography analysis of polymerase III* purified from cell lysates has shown that 2.2 γ subunits are present per polymerase III* assembly (polymerase III* indicates polymerase III holoenzyme minus β; see Ref. 16). Thus, our measure of 2 ATP or ATPγS-binding sites per γ complex is consistent with earlier evidence that γ complex composition in the holoenzyme is likely γ2δδ'χψ rather than γ4δδ'χψ.

Changes in Conformation—A recent study shows that in order to forge a topological link between the β ring and DNA, γ complex acts as a protein topoisomerase rather than a “DNA topoisomerase,” cracking open the β ring to assemble it around DNA. This process likely involves interaction between the δ subunit of γ complex and β3 (27). Free δ binds β in the absence of ATP, but γ complex requires ATP to bind β, suggesting that ATP promotes a change in γ complex that induces its interaction with β (27). By using a partial proteolysis assay, we have shown that the γ subunit undergoes a change in conformation on binding ATP, both alone and in complex with other clamp loader proteins. Particularly striking are the changes in tryptic cleavage of γ at Arg-208/Arg-215 after it binds ATP (Fig. 4).

Analysis of the recently solved structure of δ provides an interesting interpretation of the proteolytic assay results. The δ subunit is a “C”-shaped protein that shares sequence identity and homology with the γ subunit (45). A three-dimensional model structure of γ created with the MODELLER program suggests that the entire δ’ structure may be a good model for the structure of γ (45–47).

Arg-215 in γ has been identified as a highly conserved residue in the “Sensor-2 Motif,” common to E. coli, bacteriophage T4, and human clamp-loader proteins. In both δ and γ, the Sensor-2 motif is in a domain that forms a “hinge” between the top and bottom domains of the C (45). This motif packs closely against the β strand-P loop-a helix structural segment containing the ATP-binding Walker-A sequence, GTRGNYGTK, in γ. The Arg-215 residue may even serve as a ligand for the nucleotide phosphates; therefore, a tryptic cleavage site in this region is likely in a position of sensitivity to nucleotide binding. Trypsin activity at this site may be directly inhibited by nucleotide binding, but perhaps, more interestingly, tryptic cleavage is blocked because of a conformational change in the Sensor-2 motif. In γ, an ATP binding-mediated change in hinge conformation could facilitate movement of top and bottom domains, possibly even an opening/closing of the “jaws” that may be coupled to opening/closing of the β ring.

ADP has the same effect on the proteolytic digest pattern of γ and γ complex as ATP does, implying that ADP binding induces similar changes in protein conformation (Fig. 4). ADP binding, however, does not facilitate γ complex-β interaction like ATP does; therefore, the ADP-bound γ complex must differ from the ATP-bound complex. Presumably the proteolytic digest assay is not sensitive enough to pick up these differences. These results do suggest that when the clamp loader changes its conformation on ATP binding, it does not revert back to its original shape until after ATP hydrolysis and dissociation of the ADP product. Another interesting observation is that while the γ subunit in γ complex is digested by trypsin, the other

Fig. 7. ATPγS promotes γ complex-β binding to DNA. Protein-DNA interactions were analyzed by gel filtration. Panel A, [3H]γ complex (0.3 μm) and M13 ssDNA (0.02 μm) were incubated with β (0.5 μm) in the absence of nucleotide (○) and in the presence of ATPγS (●) or incubated with ATPγS in the absence of β (■) for 1 min at 23 °C. The reactions were gel-filtered within 13 min over 5-ml Bio-Gel A-15m columns in column buffer A containing the appropriate nucleotide. [3H]γ complex bound to DNA elutes in fractions 11–16 and free γ complex elutes in fractions 16–31. Panel B shows the same assays performed in the presence of β and ATP (●) or ADP (▲). Panel C shows complementary assays performed with [32P]-labeled β and unlabeled γ complex, in the absence of nucleotide (□), in the presence of ATPγS (●), or with [32P]-labeled β and ATPγS in the absence of γ complex (○). 

4 H. Xiao and M. O’Donnell, unpublished data.

5 D. J. Jeruzalmi and J. Kuriyan, unpublished data.
subunits, δ, δ', χ, and ψ, remain mostly intact as if immune to proteolysis. Perhaps the two C-shaped γ subunits in the γ complex are arranged so as to limit exposure of the other subunits to trypsin. The γ complex sketch in our model clamp loading pathway reflects this speculative arrangement with all the subunits except δ ensconced deep within the complex (Fig. 10). The δ subunit is depicted as partially accessible because at high protein concentrations β can interact with γ complex even in the absence of ATP.6

Ring Opening—Experiments performed with ATPγS and with ATP (prior to and post-hydrolysis) revealed that the clamp loader interacts with β on binding ATP. Following interaction with β, and still prior to ATP hydrolysis, γ complex opens the β ring at the dimer interface. A novel assay based on labeling a reactive cysteine at the β dimer interface was used to detect opening of the β ring. β was modified by changing Leu-273 at the interface to cysteine; at the same time a surface-exposed Cys at position 333 was changed to Ser, creating a β clamp that can be labeled with a thiol-reactive reagent only when the ring is open and the interface exposed.7 As shown in Fig. 9, panel A, the clamp is opened and fluorescence-labeled with eosin-5-maleimide when γ complex binds ATP or ATPγS, and ATP hydrolysis is not necessary for ring opening.

Recent reports have suggested that the bacteriophage T4 clamp loader, gp44/62, hydrolyzes ATP to open the gp45 clamp ring (48, 49). In one detailed study, photo-cross-linking techniques were used to track changes in conformation of the gp45 clamp in various stages of the loading cycle. Conformational changes were detected in the presence of ATP, ATPγS, ADP, as well as primer-template DNA. These data were presented in a model proposing that on ATP binding there occurs a rearrangement in intersubunit contacts between gp45 and gp44/62, and on ATP hydrolysis further changes occur that lead to gp45 ring opening (48). Our direct labeling assay with eosin-5-maleimide shows that γ complex cracks open the β dimer interface on binding ATP, and ATP hydrolysis is not required for this activity. However, it remains possible that E. coli shares similarities with T4 bacteriophage in that γ complex-catalyzed ATP hydrolysis may lead to further opening of the β ring. This idea will be interesting to test in future studies in which transition state analogs of ATP, such as ADP-AlF4−, may be used to analyze clamp loading intermediates that form during ATP hydrolysis.

DNA Binding—ATP binding to the clamp loader also promotes high affinity binding of γ complex-β to both primer-template DNA and single-stranded DNA substrates. Although in our gel shift assays we do not detect interaction between γ complex alone and DNA, a more sensitive assay measuring changes in rotational anisotropy of fluorescence-labeled DNA has shown weak interaction between an 80-nt ssDNA and γ complex (50). Therefore, γ complex may bind DNA and β in random order to form the γ complex-β-DNA composite.

γ complex and β bind single-stranded DNA but not double-stranded DNA in the gel-shift assays implying that on primed

---

6 X. Hui and M. O'Donnell, unpublished data.

---

**Fig. 8.** γ complex-β binds stably to primer-template DNA and ssDNA but not dsDNA. γ complex-β binding to DNA was analyzed by nondenaturing agarose gel-shift assays. 1 μM 32P-labeled primer-template DNA (panel A), 100-nt ssDNA (panel B), or 100-nt dsDNA (panel C) was incubated with increasing concentrations of γ complex-β (0, 0.5, 1, 3, 5 μM; lanes 1–5, 10 μM in panel C, lane 6) in the presence of ATPγS (0.5 mM) and analyzed by gel electrophoresis in 1% agarose gels containing 0.1 mM ATPγS and 10 mM MgCl2. The protein-bound 32P-labeled DNA and free DNA were visualized on a PhosphorImager. Panel D shows a gel-shift assay testing relative stability of γ complex-β binding to primer-template DNA versus ssDNA. γ complex-β (5 μM) was incubated with 1 μM 32P-labeled primer-template DNA for 5 min at 26 °C (lane 2) followed by incubation with 60 μM 70-nt unlabeled ssDNA trap for 1 min at 4 °C (lane 3) and nondenaturing gel electrophoresis. A similar assay was performed with 32P-labeled 100-nt ssDNA (without ssDNA trap: lane 2; with ssDNA trap: lane 6). Lanes 1 and 4 show free primer-template and 100-nt ssDNA, respectively.

---

**Fig. 9.** γ complex binds ATP or ATPγS, ADP, as well as primer-template DNA. A more sensitive assay measuring changes in rotational anisotropy of fluorescence-labeled DNA has shown weak interaction between an 80-nt ssDNA and γ complex (50). Therefore, γ complex may bind DNA and β in random order to form the γ complex-β-DNA composite.
Fig. 9. β ring opening. Panel A shows fluorescence labeling of L273C-β (a modified β containing a cysteine residue at the dimer interface) under various conditions: L273C-β (2 μM) alone (lane 1), and incubated with γ complex (2 μM) in the absence of nucleotides (lane 2), in the presence of 0.5 mM ATP (lane 3), and 0.5 mM ATP·S (lane 4). The reaction was performed in the dark at 4 °C and quenched after 1 min with DTT + SDS, and following SDS-PAGE analysis the fluorescence-labeled proteins were visualized on the Fluor-S MultiImager. Panel B shows a similar experiment performed in the presence of DNA; L273C-β was labeled in the presence of 3 μM primer-template DNA, without γ complex (lane 1) and with γ complex in the absence of nucleotides (lane 2) or in the presence of ATP·S (lane 3). Fluorescence-labeled β is indicated by an arrow.

ssDNA these proteins may interact with only the single-stranded regions. However, a study using photoreactive primers on template DNA demonstrated that the γ and δ subunits in γ complex link covalently to the primer at position −2 (51), which indicates its close proximity to the double-stranded region and the primer-template junction. Our DNA binding experiments also show that γ complex·β binds primer-template DNA with higher affinity and stability than ssDNA (Fig. 8, panel D). Furthermore, primed ssDNA stimulates γ complex ATPase activity substantially more than other DNA substrates1 (28). Presumably the ss/ds DNA junction structure influences the interaction of γ complex and β with DNA, and the ability of the clamp loader to recognize specifically the primer-template junction may play an important role in targeting clamps to primed sites on template DNA.

Interaction between γ complex·β and DNA was not detectable in the absence of nucleotides or in the presence of ADP (Fig. 7). Given that γ complex also does not bind β in the presence of ADP, these results suggest that ATP binding and hydrolysis modulate the interaction of γ complex with both its clamp loading substrates, β and DNA. Thus in each catalytic cycle, ATP binding to γ complex brings together the DNA and proteins in preparation for clamp loading, and ATP hydrolysis leads to clamp assembly around DNA and dissociation of all the components by a mechanism that is not yet clearly understood. Concurrent with this study, the interaction of γ complex·β with DNA has been examined by fluorescence anisotropy measurements of rhodamine-labeled primer-template DNA. These data also indicate that a stable ternary complex of γ complex·β·DNA is formed on ATP binding and suggest that the complex turns over when ATP is hydrolyzed (Ref. 52 (accompanying paper)).

A γ Complex·Open β Ring-DNA Intermediate in the Clamp Loading Pathway—The β clamp in the γ complex·β·DNA composite is an open ring. In a reaction containing this stable composite, eosin-5-maleimide labels L273C-β at the dimer interface indicating the clamp loader holds the β dimeric ring open. Because all the components necessary for clamp loading are assembled at this point, our current model of the clamp loading cycle shows this ternary composite as the final intermediate before β is released onto DNA (Fig. 10D). At the start of the loading cycle, ATP binding induces a change in γ complex (Fig. 10, A → B) that facilitates its interaction with β and DNA, perhaps in random order (Fig. 10, B → C). γ complex ATPase activity is greatly stimulated by DNA (28), and the next step in the pathway reflects this property by showing a DNA-induced change in γ complex that may speed up its catalytic activity (Fig. 10, C → D). Perhaps after the γ complex·open β ring-DNA intermediate is formed (D), ATP hydrolysis powers other changes such as further opening of the β ring to place it around DNA. On the other hand, ATP hydrolysis may induce conformational changes that close the ring around DNA. Equally possible is the pathway in which ATP binding powers the work necessary for clamp loading up to and including placement of the open β ring in the right configuration around DNA; ATP hydrolysis then simply triggers dissociation of the composite, allowing the β dimer to assume its lowest free energy state, i.e. a closed ring, and the close proximity of primer-template junction ensures that the ring closes around DNA.

Although we speculate here about the effects of ATP hydrolysis, there is no detailed information available as yet that definitively explains the role of ATP hydrolysis in E. coli γ complex-catalyzed β loading. However, based on our results with ADP and the slowly hydrolyzing analog ATP·S, it does appear that turnover of ATP is essential for turnover of the γ complex·open β ring-DNA composite. This is shown in the next step of the model pathway where ATP hydrolysis and product dissociation complete the clamp loading cycle with formation of β-DNA and free γ complex (Fig. 10, panels D → E). Finally, evidence from proteolytic digestion of γ complex suggests that ADP must dissociate before γ complex returns to its original free state; therefore, the pathway shows that after ATP is hydrolyzed and ADP is released by the clamp loader, the clamp
In humans, ATP composite has been observed also in the eukaryotic DNA replication (53). The study employed an elegant strand-displacement assay that follows the formation of active T4 holoenzyme by measuring its processive DNA synthesis activity (54). The assay was performed with ATP and analogs ATPγS and AMP-PCP, and the data showed that ATP hydrolysis is absolutely necessary for processive DNA replication. The interpretation of the results was that nonhydrolyzable ATP analogs do not support processive T4 holoenzyme activity because energy from the ATP hydrolysis step is required to open the clamp and assemble it on DNA (53). Our study with the Escherichia coli clamp loader activity proposed that gp44/62 requires energy from ATP hydrolysis to physically open or close the gp45 ring around DNA (53). The study employed an elegant strand-displacement assay that follows the formation of active T4 holoenzyme by measuring its processive DNA synthesis activity (54). The assay was performed with ATP and analogs ATPγS and AMP-PCP, and the data showed that ATP hydrolysis is absolutely necessary for processive DNA replication. The interpretation of the results was that nonhydrolyzable ATP analogs do not support processive T4 holoenzyme activity because energy from the ATP hydrolysis step is required to open the clamp and assemble it on DNA (53). Our study with the E. coli clamp loader shows that the ATPγS-bound γ complex-open β ring-DNA composite is quite stable and may not break up until the the nucleotide dissociates or is hydrolyzed. The core DNA polymerase competes with the clamp loader for β (38); therefore, a stable interaction between γ complex and β can block processive DNA polymerase activity. Similarly in T4, in the presence of nonhydrolyzable ATP analogs, gp44/62 may be trapped in a complex with gp45 and DNA and block the access of T4 polymerase to the clamp, thus inhibiting DNA synthesis (earlier studies have shown that T4 polymerase and gp44/62 compete for the gp45 clamp; see Refs. 55 and 56). In the T4 bacteriophage, ATP hydrolysis may lead to ring opening, or ring closing around DNA, or simply dissociation of the clamp loader-clamp-DNA complex, and inhibition of any of these steps in the clamp loading pathway would inhibit processive DNA replication; therefore, the exact mechanistic role of ATP hydrolysis in the clamp loading pathway still remains an open question.

ATPγS-mediated formation of the clamp loader-clamp-DNA composite has been observed also in the eukaryotic replication systems of both humans and Saccharomyces cerevisiae. In humans, ATPγS supports stable interaction of the clamp loader, RFC, with its clamp, PCNA, and primed DNA template (57). The authors noted that the DNA polymerase, polδ, binds to the protein-DNA composite and catalyzes DNA synthesis only when the clamp is formed in the presence of ATP but not when it is formed in the presence of ATPγS. They also found that the ATPγS-bound RFC-PCNA-DNA composite can be activated for DNA synthesis by adding ATP to the reaction. Based on information from the E. coli clamp loading pathway, we speculate that polδ does not bind the ATPγS-bound composite because it is blocked by RFC, and when ATPγS dissociates, the subsequent turnover of RFC in the presence of ATP allows polδ access to PCNA-DNA, thereby allowing it to synthesize DNA processively.

Experiments performed with the S. cerevisiae RFC and PCNA provide yet additional information (58). As in the systems described above, in the presence of ATPγS an RFC-PCNA-DNA composite is formed, which does not bind polδ or stimulate DNA synthesis. As expected, addition of ATP to this composite allows the polymerase to synthesize DNA processively (most likely because after the composite dissociates, RFC can load PCNA on DNA and release PCNA-DNA in the presence of ATP). However, the authors also found that if they isolated ATPγS-bound RFC-PCNA-DNA by gel filtration, addition of polδ to the reaction resulted in some DNA synthesis even without addition of ATP; furthermore, addition of excess ATPγS to this isolated composite completely inhibited polδ activity. To explain these results, the authors speculated that after the ATPγS-bound composite was formed and isolated, perhaps it could be reactivated for DNA synthesis by the other nucleotides added to the reaction (dCTP, dGTP, or dTTP, but not AMP-PNP), and further addition of ATPγS to the reaction blocked this reactivation. We offer another explanation based on our study of the γ complex and presuming some similarity between the S. cerevisiae and E. coli clamp loading pathways. Perhaps in the ATPγS-bound RFC-PCNA-DNA composite the PCNA ring is open and ready for release around DNA. If this composite breaks up when ATPγS dissociates from RFC and the PCNA ring closes, it may sometimes snap closed around DNA, because of the close proximity of DNA with the clamp, resulting in stimulation of polδ activity. If there is excess ATPγS present in the reaction and it binds RFC rapidly, the composite will stay predominantly ATPγS-bound and retain its integrity, thereby blocking polδ access to PCNA and processive DNA synthesis. We were unable to isolate a stable γ complex-open β ring-DNA intermediate without having excess ATPγS present in the reaction; therefore, we could not test directly if ATPγS dissociation allows the composite to break up such that β snaps closed around DNA and can be used by core polymerase. However, the data from S. cerevisiae lends substance to the idea that ATP hydrolysis may simply trigger dissociation of the clamp loader-clamp-DNA intermediate, resulting in release of the clamp as a ring around DNA.

In conclusion, our model mechanism for clamp loading shows that ATP binding to the clamp loader powers most of the work involved in the assembly of a ring-shaped clamp on DNA. Such efficient use of ATP binding energy is reminiscent of the mechanism by which ligands activate allosteric enzymes. In multi-subunit allosteric enzymes, the energy derived from ligand binding leads to specific conformational changes, in the component subunits, that are coupled to modulation of the catalytic activity. Similarly the energy from ATP binding to the clamp loader leads to conformational changes, in the component subunits, that are coupled to the work of opening the clamp ring and holding it in close proximity to DNA. Then, ATPase activity of the clamp loader functions as a switch mechanism and changes the clamp loader from a form that
prefers binding the clamp and DNA into a form that prefers releasing the clamp and DNA, topologically linked to each other.

Acknowledgments—We are grateful to Dr. Hui Xiao for construction and purification of the ATPase mutant K51R-γ and Dr. Wenzhu Zhang and Dr. Brian Chait for mass spectroscopic analysis of γ peptides. We also thank Jennifer Turner and Zvi Kelman for informative discussions.

REFERENCES
1. Kelman, Z., and O’Donnell, M. (1995) Annu. Rev. Biochem. 64, 171–206
2. Stillman, B. (1994) Cell 78, 725–728
3. Kurtyan, J., and O’Donnell, M. (1994) J. Mol. Biol. 234, 915–925
4. Lee, S. H, and Walker, J. R. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2713–2717
5. Tsuchihashi, Z., and Kornberg, A. (1989) J. Biol. Chem. 264, 17790–17795
6. Maki, S., and Kornberg, A. (1988) J. Biol. Chem. 263, 6555–6560
7. Xiao, H., Naktinis, V., and O’Donnell, M. (1995) J. Biol. Chem. 270, 13378–13383
8. Kong, X. P., Onrust, R., O’Donnell, M., and Kuriyan, J. (1992) Cell 69, 425–437
9. Maki, H., and Kornberg, A. (1985) J. Biol. Chem. 260, 12876–12992
10. DiFrancesco, R., Bhattacharya, S. K., Brown, A., and Bessman, M. J. (1984) J. Biol. Chem. 259, 5567–5573
11. Scheuermann, R. H., and Ehols, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 81, 7747–7751
12. Studwell-Vaughan, P. S., and O’Donnell, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10168–10172
13. Scheuermann, R. H., and Echols, H. (1985) J. Mol. Biol. 181, 193–221
14. McHenry, C. S. (1991) J. Biol. Chem. 266, 13348–13357
15. Tsuchihashi, Z., and Kornberg, A. (1989) J. Biol. Chem. 264, 11328–11334
16. Sippl, M. (1993) Proteins Struct. Funct. Genet. 17, 355–362
17. Fay, P. J., Johanson, K. O., McHenry, C. S., and Bambara, R. A. (1981) J. Biol. Chem. 256, 976–983
18. Maki, S., and Kornberg, A. (1988) J. Biol. Chem. 263, 6561–6569
19. Stukenberg, P. T., Studwell-Vaughan, P. S., and O’Donnell, M. (1991) J. Biol. Chem. 266, 11328–11334
20. McHenry, C. S., and Crow, W. (1979) J. Biol. Chem. 254, 1748–1753
21. McHenry, C. S. (1991) J. Biol. Chem. 266, 19127–19130
22. Studwell-Vaughan, P. S., and O’Donnell, M. (1991) J. Biol. Chem. 266, 19833–19841
23. Onrust, R., Finkelstein, J., Turner, J., Naktinis, V., and O’Donnell, M. (1995) J. Biol. Chem. 270, 13366–13377
24. McHenry, C. S. (1991) J. Biol. Chem. 266, 13348–13357
25. McHenry, C. S. (1991) J. Biol. Chem. 266, 13348–13357
26. Onrust, R., Finkelstein, J., Naktinis, V., and O’Donnell, M. (1995) J. Biol. Chem. 270, 13358–13365
27. Naktinis, V., Onrust, R., Fang, L., and O’Donnell, M. (1995) J. Biol. Chem. 270, 13358–13365
28. Onrust, R., Stukenberg, P. T., and O’Donnell, M. (1991) J. Biol. Chem. 266, 21681–21686
29. O’Donnell, M., and Studwell, P. S. (1990) J. Biol. Chem. 265, 1179–1187
30. Kelman, Z., Yuzhakov, A., Andjelkovic, J., and O’Donnell, M. (1998) EMBO J. 17, 2436–2449
31. Studwell, P. S., and O’Donnell, M. (1990) J. Biol. Chem. 265, 1171–1178
32. Onrust, R. (1993) The Structure and Function of Accessory Proteins of the E. coli DNA Polymerase III Holoenzyme, Ph.D. thesis, pp. 198–203, Cornell University Medical College
33. Dong, Z., Onrust, R., Skangalis, M., and O’Donnell, M. (1993) J. Biol. Chem. 268, 11758–11765
34. Xiao, H., Law, K. S., and O’Donnell, M. (1993) J. Biol. Chem. 268, 11773–11778
35. Turner, J., and O’Donnell, M. (1995) Methods Enzymol. 262, 442–449
36. Kelman, Z., Naktinis, V., and O’Donnell, M. (1995) Methods Enzymol. 262, 430–442
37. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
38. Xiao, H., and O’Donnell, M. (1991) Nature 355, 1171–1178
39. Sancar, A., and Hearst, J. E. (1993) J. Biol. Chem. 268, 13348–13357
40. Xiao, H. (1996) Structural and Functional Analysis of the Clamp Loader in the E. coli DNA Polymerase III Holoenzyme, Ph.D. thesis, Cornell University Medical College
41. Biswas, S. B., and Kernberg, A. (1984) J. Biol. Chem. 259, 7990–7993
42. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) Nature 379, 225–232
43. Xiao, H., Dong, Z., and O’Donnell, M. (1993) J. Biol. Chem. 268, 11779–11784
44. Dallmann, H. G., and McHenry, C. S. (1985) J. Biol. Chem. 270, 28563–28569
45. Guenther, R., Onrust, R., Sali, A., O’Donnell, M., and Kuriyan, J. (1997) Cell 91, 335–345
46. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
47. Pietroni, P., Young, M. C., Latham, G. J., and von Hippel, P. H. (1997) J. Biol. Chem. 272, 31666–31676
48. Sexton, D. J., Carver, T. E., Berds, A. J., and Benkovic, S. J. (1996) J. Biol. Chem. 271, 22045–22051
49. Bloom, L. B., Turner, J., Kelman, Z., Beechem, J. M., O’Donnell, M., and Goodman, M. F. (1996) J. Biol. Chem. 271, 30699–30708
50. Rees, A. J., Wood, S., and McHenry, C. S. (1995) J. Biol. Chem. 270, 5606–5613
51. Bertram, J. G., Bloom, L. B., Turner, J. T., O’Donnell, M., Beechem, J. M., and Goodman, M. F. (1998) J. Biol. Chem. 273, 24564–24574
52. Berdis, A. J., and Benkovic, S. J. (1997) Biochemistry 36, 2733–2743
53. Kaboord, B. F., and Benkovic, S. J. (1995) Curr. Biol. 5, 149–157
54. Capson, T. L., Benkovic, S. J., and Nossal, N. G. (1991) EMBO J. 10, 249–258
55. Munn, M. M., and Alberts, B. M. (1991) J. Biol. Chem. 266, 20834–20844
56. Lee, S., and Hurwitz, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5672–5676
57. Burgers, P. M. J. (1991) J. Biol. Chem. 266, 22708–22706
58. Olson, M. W., Dallmann, H. G., and McHenry, C. S. (1995) J. Biol. Chem. 270, 29570–29572