Mechanism of Loading the *Escherichia coli* DNA polymerase III β Sliding Clamp on DNA:

*BONA FIDE* PRIMER/TEMPLATES PREFERENTIALLY TRIGGER THE γ COMPLEX TO HYDROLYZE ATP AND LOAD THE CLAMP

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SUMMARY

The *Escherichia coli* DNA polymerase III $\gamma$ complex clamp loader assembles the ring-shaped $\beta$ sliding clamp onto DNA. The core polymerase is tethered to the template by $\beta$, enabling processive replication of the genome. Here we investigate the DNA substrate specificity of the clamp loading reaction by measuring the pre-steady-state kinetics of DNA binding and ATP hydrolysis using elongation-proficient and -deficient primer/template DNA. The ATP-bound clamp loader binds both elongation-proficient and -deficient DNA substrates either in the presence or absence of $\beta$. However, elongation-proficient DNA preferentially triggers $\gamma$ complex to release $\beta$ onto DNA with concomitant hydrolysis of ATP. Binding to elongation-proficient DNA converts the $\gamma$ complex from a high affinity ATP-bound state to an ADP-bound state having $10^5$-fold lower affinity for DNA. Steady-state binding assays are misleading suggesting that $\gamma$ complex binds much more avidly to non-extendable primer-template DNA because recycling to the high affinity binding state is rate-limiting. Pre-steady-state rotational anisotropy data reveal a dynamic association-dissociation of $\gamma$ complex with extendable primer-templates leading to the diametrically opposite conclusion. The strongly favored dynamic recognition of extendable DNA does not require the presence of $\beta$. Thus, the $\gamma$ complex uses ATP binding and hydrolysis as a mechanism for modulating its interaction with DNA, where the ATP-bound form binds with high affinity to DNA, but elongation-proficient DNA substrates preferentially trigger hydrolysis of ATP and conversion to a low-affinity state.
In the absence of processivity proteins, synthesis by DNA polymerases is relatively inefficient due to repeated DNA dissociation and rebinding events. Sliding clamps alleviate this problem by encircling DNA and tethering polymerases to the DNA templates to dramatically enhance the processivity of synthesis. The *Escherichia coli* β sliding clamp is composed of two identical crescent-shaped subunits that form a ring in solution (1,2). The clamp does not spontaneously load onto DNA, but requires the activity of a clamp loader for assembly. Clamp loaders perform the mechanical task of opening clamps and depositing them on DNA templates (3). Binding and hydrolysis of ATP drive this assembly reaction.

The *E. coli* clamp loader is composed of five different subunits, three copies of the dnaX gene product (γ and/or τ), δ, δ, χ, and ψ (4-7). The dnaX gene produces two polypeptides, a full-length gene product, τ, and a truncated gene product, γ, about two-thirds the length of τ (8-10). The C-terminal region of τ interacts with the α subunit of the polymerase and supports the formation of a DNA polymerase III holoenzyme complex *in vivo* that contains two core polymerases and a clamp loader consisting of τ<sup>2</sup>γδδχψ (11-13). Both τ and γ contain ATP binding sites and are capable of functioning in fully active clamp loading complexes, referred to as the τ (τ<sup>3</sup>δδχψ) and γ (γ<sup>3</sup>δδχψ) complexes, respectively (5,14). ATP binding by the clamp loader induces a conformational change that exposes binding sites for the β clamp (15) and DNA (16). Hydrolysis of ATP is coupled to the release of the clamp on DNA and most likely produces a conformational change that masks binding sites for β and DNA (17,18). Thus, ATP binding and hydrolysis modulate γ complex”β and γ complex”DNA interactions during the clamp loading cycle.

This paper addresses a key question about the *E. coli* clamp loading reaction. How does
the *E. coli* clamp loader target the β clamps to the correct sites on DNA? Ideally, DNA replication would be most efficient if the clamp loader assembled clamps onto primed templates at primer 3’ ends. This DNA substrate specificity could be achieved if the clamp loader had a high affinity for ss/ds\(^1\) junctions at primer 3’ ends. However, the DNA polymerase must also bind to these ss/ds junctions so the clamp loader would compete with the polymerase for binding and reduce the efficiency of DNA synthesis. This competition would be particularly detrimental on the lagging strand where clamps must be loaded for every one to two kb Okazaki fragment that is synthesized. The clamp loader does in fact have a high affinity for ss/ds DNA junctions at primer 3 ends, but these sites trigger a change in the \(\gamma\) complex causing it to release DNA (16). The clamp loader then exists in a state with reduced affinity for DNA. This DNA-induced decrease in affinity provides a mechanism that prevents the clamp loader from competing with the polymerase for loaded clamps. It also could provide a dynamic mechanism for recognition of sites for loading β where only the appropriate sites trigger the loading reaction. In this paper, we investigate the DNA structural features required to trigger \(\gamma\) complex to release the clamp and DNA and show that the presence of an elongation-proficient p/t DNA substrate is absolutely necessary to trigger \(\gamma\) complex to hydrolyze ATP and release the clamp.

**EXPERIMENTAL PROCEDURES**

*Enzymes* - All DNA polymerase III proteins were purified and \(\gamma\) complex was reconstituted as described (1,5), and stored in 20 mM Tris·HCl pH 7.5, 2 mM DTT, 0.5 mM EDTA, and 10% glycerol. Assay buffers for all experiments contained 8 mM MgCl\(_2\) in 20 mM Tris·HCl pH 7.5, 50 mM NaCl, 5 mM DTT, and 40 \(\mu\)g/ml BSA.
Oligonucleotides  Synthetic oligonucleotides were made on an ABI 392 DNA synthesizer using standard β-cyanoethylphosphoramidite chemistry and reagents from Glen Research (Sterling, VA). DNA was purified by denaturing polyacrylamide gel electrophoresis. A 105-nt template with the sequence shown below was used in all experiments.

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’

Amino modifiers were incorporated into this template to allow for site-specific labeling with X-rhodamine isothiocyanate (Molecular Probes, cat. #X-491) as described (19,20). These amino modifiers were positioned at three separate sites for control experiments to demonstrate that binding results were not affected by the position of the RhX probe. Two modifiers were located on T’s at positions 26 and 39 (Amino modifier C2dT, Glen Research, Sterling, VA), indicated by lower case letters, and the third modifier, was located on the terminal 5’ hydroxyl (Amino modifier C6, Glen Research, Sterling, VA).

Three 30-nt primers complementary different sites on this 105mer (see Fig. 1) were annealed separately by incubating 1.2 equivalents of primer and with 1 equivalent of template in 20 mM Tris•HCl pH 7.5 and 50 mM NaCl at 80° C for 5 min and then slowly cooling to room temperature. Annealed p/t’s were used without further purification because we previously demonstrated that a small excess of primer had no effect on loading reactions (16).

Steady-State Fluorescence Anisotropy Measurements – Steady state anisotropy
measurements were taken using a QuantaMaster QM-1 fluorometer (Photon Technology International, London, Ontario) as described (16). Titration experiments were performed by addition of a constant volume of γ complex solution to a cuvette containing a solution of RhX-labeled DNA and assay buffer. Assays contained variable amounts of γ complex and 50 nM RhX-labeled DNA, and 0.5 mM ATP in assay buffer. Binding experiments were performed with RhX probes located at three different sites and results were not influenced by the site of labeling (data not shown).

*Competition binding assays*  Binding of γ complex to a mixture of labeled and unlabeled DNA was initiated by the addition of ATP as described (16). After addition of ATP, solutions contained 50 nM RhX-labeled ss 50mer, 400 nM γ complex, 0 500 nM unlabeled competitor DNA, and 0.5 mM ATP in assay buffer.

*Pre-steady-state fluorescence anisotropy measurements*  Assays were performed using a Biologic SFM-4 stopped-flow (Molecular Kinetics, Pullman, WA) equipped with four independently driven reagent syringes and a 30-µl cuvette (model FC-15) with a 1.5 mm pathlength as described (16). One stopped-flow syringe was loaded with 480 nM γ complex, 0.5 mM ATP, and 1 µM β dimer (when present) in assay buffer. The second syringe was loaded with 100 nM DNA and 0.5 mM ATP in assay buffer. Reactions were initiated by mixing 80 µl of γ complex solution with 80 µl of DNA solution at a flow rate of 10 ml/s at 20° C. The reaction dead time under these mixing conditions was 3.7 ± 0.7 ms determined using the method described by (21). Vertically and horizontally polarized emission intensities were measured at 1 ms intervals and 16 to 24 stopped-flow runs were signal averaged. Raw anisotropy data were fit to sums of exponentials and used in kinetics simulations. Data shown in Fig. 3 has been
smoothed over 3 data points. As a control, pre-steady-state experiments with the 3’ center p/t were done with the probe at three different sites to demonstrate that the results were not influenced by the site of labeling (data not shown).

*Pre-steady-state ATPase assays* - *E. coli* phosphate binding protein covalently labeled at Cys-197 with N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide was used to quantitate inorganic phosphate released by ATP hydrolysis in real time as described (17). An Applied Photophysics SX.18MV stopped-flow was used. A “three-syringe” experiment was performed by loading one syringe with 0.97 µM γ complex and 1.2 µM β dimer (when present), a second syringe with 20 µM MDCC-PBP and 800 µM ATP, and a third syringe with 0.6 µM unlabeled DNA. Contents of the first two syringes were mixed to preincubate γ complex and β when present with ATP for 1 s prior to adding DNA from the third syringe. The computer calculated dead time was 1.5 ms.

**Combined analysis of DNA binding and ATP hydrolysis kinetics** Kinetics of ATP hydrolysis and DNA binding were measured separately in reactions containing 260 nM γ complex and 450 nM DNA in assay buffer. ATPase assays contained 200 µM ATP and 5 µM MDCC-PBP and anisotropy binding assays contained 500 µM ATP. Both experiments were done in the Applied Photophysics stopped-flow using a mixing scheme where γ complex was preincubated with ATP for 1 s prior to adding DNA (and MDCC-PBP in ATPase assays).

These reaction kinetics were simulated by the model shown in Fig. 5C using KINSIM (22). The same model and associated rate constants were used to simulate both data sets and output the concentration of PBP-MDCC bound to P_i and the anisotropy of RhX on DNA. The observed anisotropy, r_{obs}, at any time, t, was calculated using eq. 1 where r_f and r_b represent the
anisotropy of free and bound DNA, respectively, and $x_f$ and $x_b$ represent the fraction of free and bound DNA as a function of reaction time.

$$r_{\text{obs}}(t) = r_f x_f(t) + r_b x_b(t)$$  \hspace{1cm} (1)

The experimentally determined anisotropy for free DNA ($r_f = 0.19$) was used. An anisotropy of 0.32 was used for bound DNA ($r_b$). This value represents the increase in anisotropy observed when DNA is saturated with either β and γ complex or with single-stranded binding protein.

**RESULTS**

*Binding of γ complex to DNA substrates with primers located at different sites* The anisotropy of X-rhodamine (RhX) covalently attached to DNA substrates was used to report on binding interactions with the γ complex. In these experiments, the increase in anisotropy of RhX in the presence of increasing concentrations of γ complex was measured under steady-state binding conditions. Three partially duplex DNA substrates were made by annealing three separate primers, 30 nucleotides (nt) in length, to different sites on a 105 nt long template (see Fig. 1). Each of these DNA’s were covalently labeled with RhX at the 5 position of a template T located 39 nt from the 5’ end. Two substrates, the 5’ blunt p/t and center p/t, have 5’ template overhangs and support primer extension by a DNA polymerase (elongation-proficient). The third substrate, 3’ blunt p/t, positions the primer 3’ end at a blunt duplex end and cannot be extended by a DNA polymerase (elongation-deficient).

When γ complex (0 - 1600 nM) was titrated into a solution containing 50 nM DNA substrate and 500 µM ATP, a relatively large increase in anisotropy was observed for the 3 blunt
p/t substrate whereas smaller increases were observed for the 5 blunt and center p/t substrates (Fig. 1). These results seem to indicate that the apparent affinity of γ complex is greater for the 3 blunt p/t that is not extendable by DNA polymerases. However, earlier work with the center p/t substrate revealed that γ complex does in fact bind with high affinity to this substrate under pre-steady-state conditions (16). This interaction with the center p/t substrate converts γ complex from a high-affinity to a low-affinity binding state so that only a small population of DNA is bound at steady-state.

**Competition binding assays** Competition binding assays were used to measure the ability of each of the p/t substrates to compete with a single-stranded DNA substrate for γ complex binding. Increasing concentrations of unlabeled p/t competitor were added to a solution of γ complex (400 nM) and ss 50mer DNA (50 nM) covalently labeled with RhX. As the concentration of competitor DNA was increased, the fraction of γ complex bound to the ss 50mer decreased in each case (Fig. 2). However, the 5 blunt and center p/ts more effectively competed with the ss 50mer for γ complex than the 3 blunt p/t. These results apparently contradict those of direct binding assays (Fig. 1) which suggested that γ complex binds the 5 blunt and center p/ts more weakly. Together, these steady-state binding assays indicate that the γ complex interacts with the 5 blunt and center p/t substrates in the similar manner, but interacts differently with the 3 blunt substrate.

**Kinetics of γ complex binding to different DNA substrates** Pre-steady-state anisotropy measurements were made to examine the dynamic nature of the binding interactions between γ complex and various p/t DNA substrates including ss DNA. In the absence of β, initial rapid increases in anisotropy indicated that γ complex bound rapidly to each of the four DNA
substrates. However, two different types of binding kinetics were observed for elongation-proficient and -deficient substrates. For the elongation-proficient 5 blunt p/t (Fig. 3A, -β) and center p/t (Fig. 3B, -β) substrates, a rapid increase in anisotropy over the first 50 milliseconds was followed by a slower decrease in anisotropy over about the next 250 ms. At steady-state in the absence of β clamp, the anisotropy was barely above that of free DNA (light gray) indicating that only an extremely small fraction of DNA was bound. These binding kinetics are consistent with a reaction cycle in which an activated γ complex rapidly binds DNA, the interaction with DNA inactivates γ complex converting it to a state with reduced affinity for DNA, and the γ complex slowly reverts back to the activated DNA binding state (16).

In contrast, time courses for binding reactions with the elongation-deficient 3’ blunt p/t (Fig. 3C, -β) and ss 105mer DNA (Fig. 3D, -β) showed a rapid increase in anisotropy, but little or no decrease in anisotropy. The reaction with the 3 blunt p/t showed a slight decrease in anisotropy, but not nearly the magnitude observed for the 5 blunt or center p/t DNA. The higher anisotropy values seen in the steady-state regime of these reactions indicate a greater fraction of DNA was bound by γ complex. These DNA substrates do not seem to efficiently trigger the γ complex to release DNA and convert to a low affinity DNA binding state.

This dynamic interaction with elongation-proficient DNA substrates is not an artifact of performing assays in the absence of β but part of the clamp loading reaction. Similar results were obtained in loading reactions where the β clamp was present. In assays containing β and the elongation-proficient 5 blunt (Fig. 3A, +β) and center (Fig. 3B, +β) p/t DNA, a rapid increase in anisotropy due to β’γ complex binding followed by a slower decrease due to release of the clamp on DNA and dissociation of γ complex was observed. Because the clamp has been
loaded onto DNA, anisotropy values remain higher at steady-state than in reactions without the clamp. In reactions with the 3 blunt p/t (Fig. 3C, +β) and ss 105mer (Fig. 3D, +β), a rapid increase in anisotropy to a constant value was observed which is consistent with an equilibrium binding interaction of the β”γ complex with these DNA substrates rather than DNA-triggered release of the clamp on DNA. Time-courses for reactions containing elongation-deficient DNA substrates and ATP (Fig. 3C, D) resemble those done previously with elongation-proficient DNA substrates in the presence of nonhydrolyzable ATPγS where clamp release does not occur (23). The magnitude of the increase in anisotropy was greater in reactions with β than without for all four DNA substrates. This difference in amplitudes is consistent with the model presented below where an equilibrium population of about 40% of the γ complex is present in a conformation that is active for DNA binding in the absence of β.

*Kinetics of ATP hydrolysis in the presence of different DNA substrates* The anisotropy data show that DNA substrates suitable for polymerase extension induce a change in γ complex that reduces its affinity for DNA whereas elongation-deficient DNA substrates do not. Kinetics of ATP hydrolysis in assays with each of the four DNA substrates were measured to determine whether they also differed for extendable DNA substrates compared to non-extendable substrates. A real time fluorescence-based assay was used to quantitate the concentration of inorganic phosphate released on hydrolysis of ATP (24). This assay uses *E.coli* phosphate binding protein, PBP, which avidly binds inorganic phosphate. When PBP is covalently labeled with a coumarin fluorophore (MDCC-PBP), phosphate binding produces an increase in fluorescence of the probe.

DNA substrates capable of supporting synthesis by a DNA polymerase gave different time...
courses for ATP hydrolysis than those that cannot. In assays without the β clamp, the first
turnover of ATP was rapid relative to the steady-state turnover for the extendable 5 blunt and
center p/ts (Fig. 4A). The first turnover was biphasic with the combined amplitudes indicating
that three ATP molecules were hydrolyzed for every molecule of γ complex present. Kinetics of
ATP hydrolysis in assays with the non-extendable 3 blunt p/t and ss 105mer differed
significantly; they lacked a burst of ATP hydrolysis and showed only a linear increase in P_i
release at a rate of 0.92 µM/s.

Addition of β to assays containing the extendable 5 blunt and center p/ts increased the
overall rate of the first ATP turnover by γ complex (Fig. 4B). Three molecules of ATP (3.2
based on amplitudes) per molecule of γ complex were hydrolyzed in a single rapid phase taking
about 150–200 ms to complete as compared to 600–700 ms in the absence of β (Fig. 4A). The
increased rate was attributable primarily to the disappearance of the slower second pre-steady-
state phase seen in assays with γ complex only. No burst and a linear increase in P_i release was
observed at rates of 1.2 and 1.0 µM/s for the elongation-deficient 3 blunt p/t and ss DNA,
respectively, in assays with γ complex and β. The presence of β did not significantly increase the
rate of ATP hydrolysis for non-extendable substrates suggesting that hydrolysis is not coupled to
a productive loading reaction (17).

Combined analysis of ATP hydrolysis and DNA binding and release kinetics – To
determine whether the burst of ATP hydrolysis seen in the reaction with the center p/t was
associated with the release of DNA, pre-steady-state kinetics of DNA binding and ATP
hydrolysis were measured in assays under identical reaction conditions. The center rather than
5’ blunt p/t DNA was chosen for purely technical reasons to more closely examine the
relationship of ATP hydrolysis to the γ complex binding and release reactions. This center p/t is typically used in all of our assays and allows us to compare these results with those of other studies. These experiments were performed with an excess of DNA over γ complex so that every clamp loader could bind DNA and hydrolyze ATP in the first turnover.

Time courses for ATP hydrolysis and DNA binding showed similar features as those in Figs. 3 and 4. Rapid biphasic hydrolysis of ATP followed by a slower steady-state hydrolysis was observed in the ATPase assay. A concomitant rapid increase in anisotropy followed by a decrease to the level of free DNA was observed in the anisotropy binding assay (Fig. 5A, B). Combined analysis of these two data sets allows us to determine the timing of ATP hydrolysis relative to DNA binding and release; DNA binding occurs prior to ATP hydrolysis but ATP hydrolysis occurs prior to DNA release. A kinetic model for the binding of γ complex, hydrolysis of ATP and release of the extendable p/t DNA, including rate constants for each reaction (Fig. 5C) was used to simulate pre-steady-state anisotropy binding and ATP hydrolysis data (Fig. 5A, B). The solid lines through the data points were obtained from simulation of model using KINSIM (22) (Fig. 5C).

The rapid biphasic release of P₁ by γ complex could be due to the presence of nonequivalent sites where individual γ subunits hydrolyze ATP at two different rates. Alternatively, the biphasic kinetics could result from two populations of γ complex in equilibrium where one population exists in a conformation that is active for DNA binding and rapidly hydrolyzes all three molecules of ATP at the same rate. The second population is inactive for DNA binding and ATP hydrolysis but can slowly convert to the active form which gives rise to the slow phase of P₁ release. Our model assumes the second case that γ complex
exists as an equilibrium mixture of two different conformational states and is supported by unpublished data (Williams, et al., in preparation). Following ATP hydrolysis by the active form, the ADP-bound form of γ complex rapidly releases DNA.

The kinetic simulation starts with an equilibrium mixture of γ complex that is active (40% $\gamma_c^*"T""T"T$) and inactive for DNA binding (60% $\gamma_c"T""T""T$). The exact nature of these two species is not defined by this experiment, and it is assumed that they both contain three molecules of ATP. However, it is also formally possible that binding one or more ATP molecules converts the inactive to active state. Under these experimental conditions where saturating ATP concentrations were used, we cannot distinguish between these two possibilities.

The active state of γ complex ($\gamma_c^*"T""T"T$) binds DNA and hydrolyzes all three molecules of ATP sequentially at the same rate before rapidly dissociating from DNA. The inactive form of γ complex slowly (2.7 $s^{-1}$) converts to the active form where it binds DNA and hydrolyzes its ATP prior to releasing DNA.

These assays (Fig. 5) provide information about the kinetics of the first turnover but less about subsequent turnovers where the rates of ADP release from γ complex and ATP binding come in to play. For this reason, we have combined these steps into one first-order reaction where the ADP-bound form of γ complex is converted to the ATP-bound form and used apparent rate constants to model the steady-state portion of the reaction. This first-order approximation is a reasonable approach since the ATP concentration is saturating, and less than 1% of the ATP is converted to ADP over the time course of the reaction so that the ATP concentration effectively remains constant. Finally, since $P_i$ release could not be observed until
it bound MDCC-PBP, we included rate constants for this $P_i$ binding step (24) in our model.

**DISCUSSION**

The *E. coli* clamp loader accomplishes the mechanical task of assembling the ring-shaped $\beta$ clamp onto DNA. Our results show that the clamp loader uses a dynamic mechanism to target clamps specifically to DNA substrates that can serve as templates for synthesis by DNA polymerases (Fig. 6). Primed template DNA substrates that contain a ss/ds junction with the correct polarity for extension by a DNA polymerase, that is 3 primer end with a 5 template overhang, efficiently trigger hydrolysis of ATP and release of DNA by the clamp loader (Figs. 3A, B and 4A, B). The $\gamma$ complex is also able to bind non-extendable 3 blunt-end p/t DNA and ss DNA in either the presence or absence of $\beta$, but, in contrast, fails to release $\beta$ on the DNA (Fig. 3C, D) and fails to exhibit a pre-steady-state burst of ATP hydrolysis (Fig. 4 A, B). These data are completely consistent with our previous data showing that ATP hydrolysis is also associated with release of the clamp (17). Thus, the appropriate sites on DNA for clamp assembly trigger the clamp loader to hydrolyze ATP and release the clamp on DNA. These studies provide a mechanistic explanation for earlier observations that $\gamma$ complex selectively loads clamps at ss/ds junctions with a 5 ss overhang (25).

A key result is that steady-state measurements of direct binding interactions between $\gamma$ complex and different DNA substrates can be misleading by apparently demonstrating that the $\gamma$ complex binds with greater affinity to DNA substrates that are *not* extendable by DNA polymerase. Real time anisotropy binding assays were key to uncovering the dynamic nature of the interaction of $\gamma$ complex with DNA constructs that can serve as substrates for DNA
polymerases. These assays show a rapid increase in anisotropy followed by a slower decrease when γ complex binds to extendable DNA substrates containing ss/ds junctions with a 5 ss overhang (Fig. 3A, B). A simple equilibrium binding reaction of γ complex to DNA would have shown an increase in anisotropy to a value that represented the equilibrium population of DNA bound by γ complex (i.e. a simple exponential rise) as observed in assays with ss DNA alone and 3 blunt p/t DNA containing β (Fig. 3C, D). The decrease in anisotropy in assays with elongation-proficient p/t substrates having a 5 ss overhang indicated that a new state of γ complex with reduced affinity for DNA forms during the binding reaction. Because the rate-limiting step in the steady-state reaction is recycling of this low-affinity DNA binding state to the high-affinity state (Fig. 5C), very little DNA is bound giving the appearance that the affinity of γ complex for elongation-proficient DNA is low. DNA p/ts that are not extendable do not efficiently trigger conversion of γ complex to the low-affinity state and thus give the appearance that they are bound with greater affinity in steady-state assays. It is likely that this low-affinity DNA binding state is an ADP-bound form of γ complex based on results from ATPase assays. This dynamic DNA binding interaction is also likely to occur with the eukaryotic RFC clamp loader. Stronger binding interactions with an extendable p/t were seen in steady-state assays with nonhydrolyzable ATPγS than in assays with ATP (26).

The γ complex has long been known to have DNA-dependent ATPase activity. More recently, the ATP requirements for individual steps in the clamp loading reaction have been defined. ATP binding but not hydrolysis is required for DNA binding activity (16,20) and clamp binding activity (15,27) of the γ complex. A conformational change in the clamp loader that exposes sites for binding both DNA and the clamp is most likely produced by ATP binding.
Hydrolysis of ATP is required for release of the clamp on DNA (17,18,23). Here, we show that hydrolysis of ATP is dependent on the structural features of the DNA substrate. DNA structures that can be extended by a DNA polymerase, the 5’ blunt and center p/t’s, efficiently trigger a pre-steady-state burst of ATP hydrolysis whereas those that cannot be extended, the 3’ blunt and ss DNA, do not (Fig. 4).

Combined analysis of DNA binding and ATP hydrolysis assays done under identical conditions in the absence of the β clamp revealed that γ complex initially binds DNA prior to ATP hydrolysis and subsequently hydrolyzes ATP before releasing DNA. This same sequence of events occurs in assays containing β that result in a productive loading reaction (17). Our results are consistent with a model (Fig. 5C) in which the γ complex cycles through a reaction where it has a high affinity for DNA, binds DNA, then converts to a lower affinity state and releases DNA. The high-affinity DNA binding state is an ATP-bound form of γ complex and the low-affinity state is an ADP-bound form. The kinetic parameters derived from this model suggest that the ATP-bound form of γ complex binds DNA with an affinity that is $10^5$ times greater (2 nM) than the ADP-bound form (100 µM). Thus, the clamp loader uses ATP binding and hydrolysis as a means for modulating its interaction with DNA so that it has a high affinity before clamp loading and a low affinity afterwards.

Taken together, these results demonstrate the γ complex uses a dynamic mechanism for targeting the β sliding clamp to template sites where DNA synthesis is slated to begin. The clamp loader binds DNA with high affinity and those sites with an ss/ds junction of the correct polarity to be extended by a DNA polymerase efficiently trigger the clamp loader to hydrolyze ATP and release the clamp on DNA (Fig. 6). DNA-triggered hydrolysis of ATP converts the γ
complex to a state, most likely ADP-bound, with lower affinity for DNA, thus providing a mechanism for targeting the clamp to the proper sites on DNA and for preventing the clamp loader from competing with the polymerase for primer/template ends. Coordination between clamp loader and polymerase is critical for DNA synthesis on the lagging strand where a clamp must be loaded for each of the 1-2 kb Okazaki fragments synthesized every 1-2 s by the polymerase. Pre-steady-state kinetic data show that the γ complex-catalyzed β clamp loading and release reactions occur rapidly (~12 s⁻¹ (20)) compared to the time scale for Okazaki fragment synthesis, and are therefore not rate limiting for Okazaki fragment synthesis. ATP binding and hydrolysis modulate the affinity of the clamp loader for DNA and the clamp so that it has a high affinity for both before the clamp is loaded and a low affinity for both after the clamp is loaded. This prevents competition between the clamp loader and core polymerase for clamps that have just been loaded on DNA. The dynamic nature of γ complex"DNA interactions is likely to be a common theme in DNA replication where many enzymes are required to work at the replication fork and each must have access to the DNA at the appropriate time.
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FOOTNOTES

1 Abbreviations: ss and ds, single- and double-stranded; nt, nucleotide; p/t, primer/template; kb, kilobase, PBP, *E. coli* phosphate binding protein, *phoS* gene product, MDCC-PBP, PBP covalently labeled at Cys-197 with N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide; RhX, X-rhodamine.

2 The two-state model for the biphasic kinetics of ATP hydrolysis by γ complex in the absence of β provides a reasonable explanation for the effects of β on the kinetics of DNA binding and ATP hydrolysis. Our model assumes β, like DNA, only binds with high affinity to the active state of γ complex. Preincubation of β with γ complex and ATP would trap nearly all the γ complex in the active DNA binding conformation by forming a β”γ complex. This active β”γ complex then rapidly binds DNA and hydrolyzes ATP. Because nearly all the γ complex is present in the active β”γ complex, the amplitude of the anisotropy rise in reactions with β increases relative to reactions in the without β where a fraction (60%) of γ complex is initially in the inactive conformation. For the same reasons, reactions with β result in a single rapid phase of P₁ release.
**FIGURE LEGENDS**

**Fig. 1.** Steady-state anisotropy binding assays measuring the affinity of the clamp loader for DNA substrates primed at different sites. Each of the DNA substrates (50 nM) containing ss/ds junctions was titrated with γ complex (0 - 1600 nM). The increase in anisotropy for the RhX probe on DNA is plotted as a function of γ complex concentration for the 5 blunt p/t substrate (filled triangles), center p/t substrate (open squares), 3’ blunt substrate (filled circles). The DNA template is labeled with RhX on T at position 39. Assay buffer contained 500 µM ATP and 8 mM MgCl₂ in 20 mM Tris·HCl pH 7.5, 50 mM NaCl, 40 µg/ml BSA, and 5 mM DTT.

**Fig. 2.** Ability of partially duplex DNA substrates to compete with a single-stranded 50mer for γ complex binding. Binding of γ complex (400 nM) to a ss 50mer (50 nM) that was 5end-labeled with RhX was measured in the presence of increasing concentrations (0 - 400 nM) of unlabeled competitor DNA. The fraction of labeled 50mer that remained bound by γ complex was plotted as a function of concentration of unlabeled 5 blunt p/t (filled triangles), center p/t (open squares), and 3 blunt p/t (filled circles) competitors. Assay buffer contained 500 µM ATP and 8 mM MgCl₂ in 20 mM Tris·HCl pH 7.5, 50 mM NaCl, 40 µg/ml BSA, and 5 mM DTT.

**Fig. 3.** Kinetics of γ complex binding to DNA and loading β clamps on DNA. Solutions of γ complex were preincubated with ATP in the presence or absence of the β clamp prior to addition of each of the three p/t DNA substrates and a ss 105mer substrate. Binding kinetics are plotted in separate graphs for A, the 5’ blunt p/t, B, the center p/t, C, the 3’ blunt p/t, and D, the ss 105mer where each graph shows three plots of anisotropy as a function of time. In each case,
the dark gray plot showing the greatest increase in anisotropy is the kinetics of loading of β onto DNA, the dark gray plot showing a small increase in anisotropy is the kinetics γ complex binding DNA, and the light gray plot showing no change in anisotropy is free a DNA control generated by the addition of buffer only. Smooth black lines through the data represent empirical fits to the sums of exponentials. Final concentrations were 240 nM γ complex, 50 nM DNA, 500 µM ATP, 8 mM MgCl₂, and 500 nM β clamp when present in 20 mM Tris"HCl pH 7.5, 50 mM NaCl, 40 µg/ml BSA, and 5 mM DTT. The three p/t substrates (A-C) were labeled with RhX on the template on T at position 26 and the ss 105mer was 5-end-labeled with RhX.

Fig. 4. Kinetics of ATP hydrolysis by γ complex in the presence and absence of the β clamp. The concentration of MDCC-PBP bound to inorganic phosphate released by hydrolysis of ATP is plotted as a function of time. A, Reactions with γ complex alone. B, Reactions with γ complex in the presence of β. The dotted gray line in each graph represents the concentration of $P_i$ expected for the hydrolysis of three molecules of ATP per molecule of γ complex. Reactions done in both the presence and absence of β show a rapid increase in $P_i$ release followed by a slow steady-state release for 5 blunt (black) and center (gray) p/t substrates whereas reactions with the 3 blunt p/t (black) and ss DNA (gray) show a linear steady-state release of $P_i$. Assays were performed by preincubating a solution of γ complex (240 nM), ATP (200 µM), MgCl₂ (8 mM), and β (300 nM, when present) for 1 s prior to adding a solution of DNA (300 nM) and MDCC-PBP (5 µM) to give final concentrations indicated in 20 mM Tris"HCl pH 7.5, 50 mM NaCl, 40 µg/ml BSA, and 5 mM DTT.
Fig. 5. Correlation between hydrolysis of ATP and release of DNA by γ complex. ATPase and DNA binding assays were performed by preincubating γ complex and ATP for 1 s prior to adding DNA (and MDCC-PBP in ATPase assays). Final concentrations were 0.26 µM γ complex, 0.45 µM center p/t DNA (5-end-labeled with RhX on the template), 200 µM ATP, and 8 mM MgCl₂, and 5 µM MDCC-PBP (in ATPase assays only) in assay buffer. The concentration of MDCC-PBP bound to inorganic phosphate (left axis) and the anisotropy of RhX on DNA (right axis) are plotted as a function of time. Data over a time range of 1.5 s is shown in A and on an expanded scale of 0.6 s is shown in B. Solid lines through the data were obtained from simulation of the kinetic model shown in C. In this model, \( \gamma_c^{"T""T""T} \) represents γ complex bound to 3 molecules of ATP (T) that is inactive for binding DNA, \( \gamma_c^{"T"T"T} \) represents γ complex bound to 3 ATPs that is active for DNA binding, N represents DNA, D represents ADP, and P represents inorganic phosphate. Rate constants used in the simulation are indicated for each step. Experimentally determined rate constants for inorganic phosphate binding to and dissociating from PBP-MDCC (24) were included.

Figure 6. γ complex uses a dynamic mechanism for targeting clamps to sites where DNA synthesis is slated to begin. In both the presence and absence of the β clamp, ATP-bound γ complex binds with high affinity to extendable and non-extendable DNA substrates. However, extendable DNA substrates trigger γ complex to hydrolyze ATP and release the clamp on DNA. Thus, γ complex uses a dynamic mechanism for recognition of extendable primers where those sites preferentially trigger the loading reaction.
A

\[
\gamma_c \cdot T \cdot T \cdot T \xrightarrow{k_1 = 4.05 \text{ s}^{-1}} \gamma_c' \cdot T \cdot T \cdot T + N \xrightarrow{k_2 = 0.8 \text{ s}^{-1}} \gamma_c' \cdot T \cdot T \cdot N \xrightarrow{k_3 = 0.1 \mu M^{-1}\text{s}^{-1}} \gamma_c' \cdot T \cdot T \cdot T
\]

B

\[
\gamma_c' \cdot T \cdot D \cdot N + P \xrightarrow{k_4 = 65 \text{ s}^{-1}} \gamma_c' \cdot T \cdot D \cdot N + P \xrightarrow{k_5 = 0.1 \mu M^{-1}\text{s}^{-1}} \gamma_c' \cdot T \cdot T \cdot T
\]

C

\[
\gamma_c' \cdot D \cdot D \cdot N + P \xrightarrow{k_6 = 1000 \text{ s}^{-1}} \gamma_c' \cdot D \cdot D \cdot N + P \xrightarrow{k_7 = 0.22 \text{ s}^{-1}} \gamma_c' \cdot T \cdot T \cdot T
\]

\[
P + PBP \xrightarrow{k_{on} = 136 \mu M^{-1}\text{s}^{-1}} P \cdot PBP \xrightarrow{k_{off} = 13.6 \text{ s}^{-1}} P \cdot PBP
\]
Dynamic Binding Interaction with Extendable p/t's

ATP $\rightarrow$ ATP $\rightarrow$ ADP $\rightarrow$ ADP

Binding Interaction with Non-extendable p/t's

ATP $\rightarrow$ ATP
Mechanism of loading the Escherichia coli DNA polymerase III β sliding clamp: Bonafide primer/templates preferentially trigger the γ complex to hydrolyze ATP and load the clamp

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