The β-Sliding Clamp Closes Around DNA Prior to Release by the Escherichia coli Clamp Loader γ complex

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*Running title: β-Clamp Closing and Release on DNA

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Background: The γ complex loads the ring-shaped β sliding clamp onto DNA.

Results: The rate of β-clamp closing is faster than the rate of β release on DNA, and the first turnover of ATP hydrolysis is faster than β closing.

Conclusion: Clamp closing occurs before clamp release but after a burst of ATP hydrolysis.

Significance: These results demonstrate that clamp release around DNA is a two-step process.

SUMMARY

Escherichia coli γ complex clamp loader functions to load the β sliding clamp onto sites of DNA replication and repair. The clamp loader uses the energy of ATP binding and hydrolysis to drive conformational changes allowing for β binding and opening, DNA binding, and then release of the β-DNA complex. Although much work has been done studying the sliding clamp and clamp loader mechanism, kinetic analysis of the events following β•γ complex•DNA formation is not complete. Using fluorescent clamp closing and release assays, we show that β closing is faster than β release, indicating that γ complex closes β before releasing it around DNA. Using a fluorescent ATP hydrolysis assay, we show that there is a burst of ATP hydrolysis before β closing, and that β release may be the rate-limiting step in the overall clamp loading reaction. The combined use of these fluorescent assays provides a unique perspective into the E. coli clamp loader by providing a measure of the relative timing of different events in the clamp loading reaction, helping to elucidate the complicated clamp loading mechanism.

Sliding clamps and clamp loaders are present in all kingdoms of life performing vital functions in both DNA replication and repair (reviewed in (1)). Sliding clamps are ring-shaped proteins that encircle DNA to secure proteins to and coordinate enzyme activity at a specific region of DNA (2, 3). The main function of the sliding clamp is to secure DNA polymerase to the replication fork, increasing the processivity of nucleotide polymerization from tens of nucleotides per polymerase binding event to thousands of nucleotides per polymerase binding event (reviewed in (4, 5)). The sliding clamp also plays a role in coordinating proteins involved in Okazaki fragment maturation, translesion synthesis, as well as many other functions in DNA metabolism.

The sliding clamp in E. coli, β, is a homodimer composed of three globular domains in each monomer. The monomers are arranged head to tail creating a conserved toroidal structure with an interior hole large enough to fit duplex DNA (2, 3). The two faces of β are asymmetric and proteins, including the clamp loader and DNA polymerase, bind to a hydrophobic pocket on one face of β via a conserved sequence motif (6-8). Multiple lines of evidence support a closed conformation for the β clamp in solution. In numerous crystal structures, the β clamp is in a...
closed conformation (2, 3, 9-11). Molecular dynamics simulations demonstrate that β is most stable in its closed conformation (12). β has a high half-life on closed circular DNA of approximately 72 min, indicating that transient β opening allowing dissociation from DNA occurs infrequently (13).

Due to the stable closed ring structure of the β clamp, a clamp loader is required to open β and place around DNA in the appropriate conformation as well as direct it to the appropriate location for DNA replication or repair. The clamp loader, γ complex, is a member AAA + family of ATPase enzymes, which are characterized by the use of ATP binding and hydrolysis to drive reactions that typically relocate or rearrange macromolecules (reviewed in (14-16)). The γ complex, is heteromeric, composed of three copies of the γ subunit and one copy each of δ, δ', χ, and ψ, with the three γ subunits binding and hydrolyzing ATP (17, 18). The clamp loader core (γδδ') forms a highly conserved cap-like structure with subunits arranged in a circular fashion and a gap between the δ and δ' subunits (18). The γ complex loads β on primed template junctions with a 3' recessed end. The duplex region of DNA sits inside the cap, stabilized by positively charged residues, and the single-stranded overhang bends out of the cap through the gap between δ and δ' (19).

The β clamp is estimated to be loaded every two to three seconds during lagging strand synthesis, one clamp for every Okazaki fragment. This reaction must happen in a timely and ordered fashion, so that lagging strand synthesis does not become uncoupled from leading strand synthesis, causing replication delays and increasing the probability of replication fork collapse. Therefore, to maximize efficiency, an ordered mechanism for γ complex loading β likely exists that is driven by conformational changes associated with ATP binding and hydrolysis and interactions with β and DNA. In general, ATP binding by γ complex drives β binding, opening and DNA binding (20-22). DNA binding triggers ATP hydrolysis and results in release of β•DNA (22-24). Pre-steady-state kinetic experiments have given a more detailed view of the clamp loading cycle. β binding occurs before β opening, indicating γ complex does not simply trap open clamps, but instead opens clamps (11). γ complex binding to β occurs at a rate limited by diffusion whereas binding to DNA is slower and limited by a conformational change in γ complex (25). A burst of ATP hydrolysis occurs before γ complex releases β, and DNA release occurs before β release (24, 26, 27).

The high stability of clamps in the closed conformation suggests the need for clamp loaders to open and stabilize the open clamps long enough to allow loading around DNA instead of simply capturing open clamps (11). The high stability of the β clamp in a closed ring conformation, with a Kd in the pM range and a half-life on DNA of over an hour (13, 28), also implies that clamp loaders are not needed to close the clamps around DNA. Instead, open clamps could be released first and “snap shut” after release as implied by computational studies which indicate that replication factor C simply stabilizes the open conformation of PCNA (29). This scenario could potentially result in an open clamp slipping away from DNA before it snaps shut, resulting in an unsuccessful attempt at loading the clamp, which would reduce the overall efficiency of clamp loading. In contrast, molecular dynamics simulations propose that closed β molecules are under “spring tension” which facilitates opening by the clamp loader (12). If this were the case, then this would also suggest that the β-clamp could not simply snap shut after being released and that γ complex would need to exert energy to close the clamp around DNA.

If required, energy to promote clamp closing could potentially come from hydrolysis of some or all of the bound molecules of ATP, which raises the question of the relative timing of ATP hydrolysis and β closing. A recent structural study with the T4 bacteriophage clamp loader suggested that hydrolysis of one ATP molecule causes a conformational change in the clamp loader•clamp complex that closes the clamp (30) and studies with the E. coli replisome suggest that hydrolysis of one ATP molecule is sufficient to form initiation complexes but hydrolysis of three molecules accelerates the process (31, 32). Studies have shown that ATP hydrolysis is required for clamp release (24, 26, 33), but the question here is whether ATP hydrolysis is
required for clamp closing or whether binding of the clamp loader-clamp complex to DNA is sufficient to promote clamp closing. To address the timing of β closing, and how it correlates with the other steps in the clamp loading cycle, most notably ATP hydrolysis and β release, a fluorescent β closing assay was used to measure clamp closing rates for comparison with rates of other events.

**EXPERIMENTAL PROCEDURES**

**Buffers, proteins and DNA** - Final buffer concentrations in the reactions are 20 mM Tris•HCl pH 7.5, 50 mM sodium chloride, 8 mM magnesium chloride, 0.5 mM EDTA, 4% glycerol and 2 mM DTT. Storage buffer for β is 20 mM Tris•HCl pH 7.5, 10% glycerol, 0.5 mM EDTA and 2 mM DTT. Storage buffer for γ complex is the same as for β, except that 50 mM sodium chloride is added. γ complex subunits (γ, δ, δ', χ and ψ) were purified and reconstituted as described previously (27, 34-37). Wild type β was purified as described previously (38). Purification and labeling of the β clamp mutants for the β closing and release assays are discussed in more detail below.

Synthetic oligonucleotides (Integrated DNA Technology) were purified using 10-12% denaturing polyacrylamide gel electrophoresis. Two different p/t-DNA substrates were used. The first substrate is made by annealing two 60mers to create a symmetrical structure with a 30-nucleotide duplex region and two 30-nucleotide 5’-single-stranded overhangs (Figs. 1 - 4). The second p/t-DNA substrate consisted of a 30-nucleotide primer annealed to a 60-nucleotide template to create 30-nucleotide duplex region and two 30-nucleotide 5’-single-stranded overhang (Fig. 5).

**Pre-steady state stopped flow** - Pre-steady-state reactions were performed using an Applied Photophysics SX20MV stopped-flow at 20 °C with 3.72 nm band pass. Assays were performed in sequential mix mode in which a solution of γ complex was mixed with a solution of β and ATP and incubated for 1 - 4 s before adding a solution of DNA and ATP.

**Clamp closing and release assays** - The β closing assay uses clamps labeled with two Alexa Fluor 488 (AF488) molecules covalently attached to Cys-103 and Cys-305 on either side of the monomer interfaces (11). The β release assay uses clamps covalently labeled with pyrene (PY) at Cys-299 on the surface of the clamp to which γ complex bind (25). The FRET based β release assay uses γ complex labeled with AF488 on the δ’ subunit and a β clamp labeled with the non-fluorescent quencher QSY9 (27). Pre-steady-state clamp closing and release reactions were limited to a single turnover by including an unlabeled β trap with the DNA and ATP solution. The final concentrations in these reactions were: 20 nM γ complex, 20 nM β, 40 nM DNA (when present), 0.5 mM ATP, and 200 nM unlabeled β unless otherwise noted. β-PY was excited at 345 nm and emission was measured with a 365 nm cut-off filter. β-AF488_2 and γ complex-AF488 were excited at 490 nm and emission was measured with a 515 nm cut-on filter. Observed rates were calculated for β closing reactions using a single exponential decay equation (Eq. 1), and observed rates for β release reactions were calculated using a double exponential decay equation (Eq. 2). Observed rates for FRET β release assays were calculated using a double exponential increase equation (Eq. 3).

\[
\begin{align*}
\gamma &= a(e^{-k_{\text{obs}}t}) + c \\
\gamma &= a_{\text{fast}}(e^{-k_{\text{fast}}t}) + a_{\text{slow}}(e^{-k_{\text{slow}}t}) + c \\
\gamma &= a_{\text{fast}}(1 - e^{-k_{\text{fast}}t}) + a_{\text{slow}}(1 - e^{-k_{\text{slow}}t}) + c
\end{align*}
\]

**ATP hydrolysis assay** - The ATP hydrolysis assay uses phosphate binding protein labeled with MDCC (PBP-MDCC, Invitrogen) to quantify the inorganic phosphate product of ATP hydrolysis (39, 40). PBP-MDCC was included in the DNA and ATP solution along with an excess of ATPγS as a trap. Final concentrations were: 200 nM γ complex, 200 nM β, 400 nM DNA, 0.2 mM ATP, 2 μM ATPγS and 2 μM PBP-MDCC. MDCC was excited at 425 nm and emission was measured with a 455 nm cut-on filter. Steady-state rates of ATP hydrolysis were calculated by first converting the fluorescent signal into the concentration of inorganic phosphate released. The steady-state phase was fit to a line by linear regression.
RESULTS

\textbf{\( \beta \) clamps are closed before they are released} - In order to determine the relative timing of \( \beta \) closing and release, fluorescence-based assays were used to report on these aspects of the clamp loading cycle. \( \beta \) closing was measured using a \( \beta -c \) clamp labeled on each monomer at the interface with Alexa Fluor 488 (AF488). When the clamp, \( \beta \)-AF488, is open, the AF488 fluorophores are far enough apart that there is no interaction and they fluoresce, but when the clamp closes, the fluorophores return to a position in which they are close enough to self-quench, decreasing the signal (11). \( \beta \) release is measured using a clamp that is covalently labeled with pyrene (PY) on the surface to which the clamp loader binds. When \( \beta \)-PY is bound by \( \gamma \) complex, PY is about 2 times more fluorescent than when the clamp is released and unbound (25). Reactions were initiated by adding a solution of \( \gamma \) complex, labeled \( \beta \), and ATP to a solution of DNA, ATP, and a 10-fold excess unlabeled \( \beta \) (Fig 1A). The unlabeled \( \beta \) limited reactions to a single-turnover. Experiments were done three separate times, and a representative time course for \( \beta \) closing is shown in Fig. 1B (black trace). In the first approximately 40 ms of each \( \beta \) closing time course, there is a small increase in fluorescence signal (see Fig. 3B for an expanded time scale). It is unclear what causes this increase, but it is unlikely to arise from the interaction with DNA because it is also present in experiments in which DNA is omitted (data not shown). It is more pronounced when protein concentrations are higher (Fig. 4B). Because a unique fit to the rate of the small rise was difficult to obtain, the time course was fit to a single exponential decay to calculate an observed rate of release. The average rate calculated from three separate closing reactions was 6.2 ± 1.3 s\(^{-1}\).

Time courses for \( \beta \) release gave biphasic decreases in PY fluorescence (Fig 1B, gray trace). Fits of the data to a double exponential decay gave an initial rate of 6.3 ± 0.3 s\(^{-1}\), and a second rate of 0.85 ± 0.04 s\(^{-1}\). The rapid decrease in PY fluorescence occurs at the same rate as closing measured in \( \beta \)-AF488 assays and is interpreted as being associated with a conformational change in the clamp loader•clamp complex that allows the clamp to close. The second, slower rate of 0.85 s\(^{-1}\) is interpreted as reflecting the rate of \( \beta \) release by the clamp loader. This interpretation of the biphasic decrease in PY fluorescence is consistent with previous equilibrium binding studies that show the fluorescence of PY is sensitive to the \( \gamma \) complex•\( \beta \) conformation. In the absence of ATP, \( \gamma \) complex can bind but not open \( \beta \) (11, 21, 22). In this closed conformation of \( \gamma \) complex•\( \beta \), PY fluorescence is weaker (i.e. lower quantum yield) than PY fluorescence in an open clamp loader•clamp complex (11, 25). Similarly, PY fluorescence is lower in complexes of arginine finger mutants of \( \gamma \) complex and \( \beta \), where the \( \gamma \) complex mutants are defective in \( \beta \) opening even in the presence of ATP. The amplitudes of the two phases in the \( \beta \)-PY release assay are consistent with the relative quantum yields of PY in open and closed clamp loader•clamp complexes.

The biphasic release rates are not likely to represent two populations of \( \gamma \) complex, because amplitudes of the two rates are consistent between multiple preparations of \( \gamma \) complex. If there were two populations of \( \gamma \) complex, one that reacted more rapidly than the second, then two phases would also be expected in clamp closing (AF488) assays and in other assays, but this was not observed.

An alternative interpretation of the biphasic nature of the \( \beta \)-PY release traces is that the first rate reflects \( \beta \)-PY release, and the second rate reflects loss of the interaction between \( \beta \)-PY and DNA as the clamp slides off the short linear p/t-DNA substrates. To test this possibility, two experiments were performed. In the first experiment, \( \beta \) closing and release were measured on p/t-DNA bound by SSB to block the ends and prevent \( \beta \) from sliding off the DNA. In vitro DNA replication assays demonstrated that a ss DNA overhang of 25-nt is of sufficient length to bind SSB and stabilize \( \beta \) on DNA to form a pre-initiation complex to which the core polymerase can bind (41). In assays with SSB-bound p/t-DNA, the decrease in PY fluorescence remained biphasic (Fig. 2A). Moreover, the calculated closing rates measured in the \( \beta \)-AF488 assay and closing and release rates measured in the \( \beta \)-PY assay were about the same as measured in the absence of SSB (Table 1). If the slower rate measured in the \( \beta \)-PY assay had corresponded to \( \beta \)-PY sliding off DNA, this rate should have
closing and release rates are much slower than for DNA (Fig. 3A). When DNA is omitted, both the release assays were performed in the absence of by which dissociates from the clamp loader, closing and release when the clamp passively determine whether clamp closing is also faster than clamp release when the clamp passively dissociation is the only mechanism between

reaction reflecting the equilibrium interaction released on DNA, and a passive dissociation productive clamp loading reaction in which 5

complex is labeled with a fluorescent AF488 donor, and the β clamp is labeled with a non-fluorescent QSY9 quencher (27). When β-QSY9 is bound to γ complex-AF488, the quencher is in close proximity to the AF488 fluorophore, so the fluorescent signal is quenched, but when the clamp is released, the QSY9 is no longer close enough to quench the AF488 signal. This reaction was performed as illustrated in Fig. 1A by incubating γ complex-AF488 with β-QSY9 for 4 s before the addition of DNA and excess unlabeled β. Time courses for this reaction were also biphasic, with an initial calculated rate of $4.1 \pm 0.2 \text{ s}^{-1}$ and a second calculated rate of $0.82 \pm 0.06 \text{ s}^{-1}$ (Fig 2B). Again, the rapid increase is interpreted as clamp closing which moves AF488 farther from the QSY9 quencher, and the slower increase is interpreted as dissociation of β. Because β-QSY9 is not fluorescent, interactions between β and DNA cannot be responsible the slower phase of the reaction. Taken together, these results show that the rapid decrease in fluorescence in β-PY assays reflects β closing and the slow decrease reflects β release.

β closing before release requires DNA-dependent ATP hydrolysis – Clamps can dissociate from the γ complex by two mechanisms, a productive clamp loading reaction in which β is released on DNA, and a passive dissociation reaction reflecting the equilibrium interaction between β and the γ complex. In the absence of DNA, passive dissociation is the only mechanism by which β can dissociate from the γ complex. To determine whether clamp closing is also faster than clamp release when the clamp passively dissociates from the clamp loader, closing and release assays were performed in the absence of DNA (Fig. 3A). When DNA is omitted, both the closing and release rates are much slower than for

a productive clamp loading reaction on DNA. The closing rate is about 200 times slower in assays without DNA (Table 1). Interestingly, rates calculated for β closing and release in the absence of DNA are the same within experimental error. In addition, the clamp (β-PY) release trace was no longer biphasic, but could be fit by a single rate. These data show that clamp closing is not faster than release in the passive dissociation reaction. β closing and release may be simultaneous, or more likely the clamp may rapidly close on its own after dissociation from the γ complex.

These data show that DNA binding promotes β closing prior to β release. Given that DNA binding is a bimolecular reaction, the rates of clamp closing and release may increase with increasing DNA concentrations. To determine how clamp closing and release rates are influenced by DNA concentration, both reactions were measured at DNA concentrations of 20, 40, and 100 nM (Figs. 3B and C). Changing the DNA concentration had no effect on rates of clamp closing or release showing that these rates must be limited by the rate of some intramolecular reaction such as a DNA-induced conformational change in the clamp loader-clamp complex or ATP hydrolysis.

DNA binding triggers ATP hydrolysis by the γ complex to promote release of β on DNA in a productive clamp loading reaction (24, 26). To determine whether DNA binding alone is required to activate clamp closing prior to release or whether ATP hydrolysis is required, β-AF488₂ closing and β-PY release were measured in assays in which the non-hydrolyzable analog, ATPγS, was substituted for ATP. Time courses for reactions with ATPγS were similar to time courses for reactions lacking DNA. Calculated rates for clamp closing were about the same in reactions with ATPγS as in reactions lacking DNA (Table 1) and the rate of clamp closing was the same as the rate of clamp release. Most likely, clamp closing and release occur via a passive dissociation mechanism rather than an active clamp loading mechanism in reactions with ATPγS (44).

Together, these data show that DNA-dependent ATP hydrolysis is required for β to close prior to release by the γ complex and that the rate of clamp closing is limited by an intramolecular reaction in the ternary clamp
A burst of ATP hydrolysis occurs before the β clamp closes - A fluorescent ATPase assay was used to measure the timing of ATP hydrolysis relative to β closing. ATP hydrolysis is quantified using phosphate-binding protein (PBP) labeled with MDCC, which increases in fluorescence when the inorganic phosphate product of ATP hydrolysis binds to MDCC-PBP (39, 45). ATPase assays were done using a sequential mixing scheme in which a solution of γ complex and ATP were incubated for 1 s before adding a solution of p/t-DNA, PBP-MDCC, and excess ATPγS (Fig 4A). ATP hydrolysis reactions were limited to a single turnover by inclusion of a trap, unlabeled β or ATPγS. In multiple turnover reactions on the short linear p/t-DNA substrates, clamps slide off DNA after being loaded and are reloaded by the clamp loader setting up a steady-state cycle (41). To determine how reaction kinetics change when the clamp loaders catalyze multiple rounds of clamp loading, pre-steady-state kinetic assays were repeated in the absence of these trapping agents (Fig 5). These clamp closing and ATP hydrolysis reactions contained the same concentrations of substrates as in Fig. 4B. Multiple turnover ATPase time courses (black) have the same basic shape as the single-turnover experiments, but reach a limiting fluorescence intensity at earlier times as the reaction reaches steady-state. The multiple-turnover ATPase time courses (gray) contain three phases, a sigmoidal shaped burst of hydrolysis present in the single-turnover experiments, and a lag that is followed by a linear increase in fluorescence as the reaction reaches steady-state. Both β-closing and ATP hydrolysis time courses transition into the steady-state phase over the same time period between about 0.4 and 0.6 s. The burst amplitude in the multiple-turnover ATPase reactions is the same as in the single-turnover reactions showing that the amplitude of the burst phase in single-turnover ATPase reactions is not reduced by rapid exchange of ATP for ATPγS. Steady-state rates of ATP hydrolysis determined previously yielded $k_{cat}$ values of approximately 2 s⁻¹ (22, 24, 47), which agrees with the value of 1.8 ± 0.2 s⁻¹ calculated from the ATP hydrolysis reactions performed here. When this $k_{cat}$ value is corrected for the number of active ATP sites based on the burst amplitudes, a $k_{cat}$ of about 1 s⁻¹ per active ATP site is obtained. This $k_{cat}$ value is about the same as the rate of release of the β clamp suggesting that clamp release may be the rate-limiting step in the clamp loading reaction cycle.
DISCUSSION

The *E. coli* clamp loader, γ complex, performs the vital function of loading the β sliding clamp onto DNA for various DNA replication and repair functions. In order to load clamps, the γ complex must modulate its affinity for multiple substrates, first by having a high affinity for β and DNA to bring these macromolecules together, and then by decreasing its affinity for β and DNA to release them. The γ complex uses ATP binding and hydrolysis to regulate the different affinities by driving conformational changes that make interactions with β and DNA more or less favorable. Clamp loading must be quick and efficient to complete these steps in the timetable required for DNA replication so there is an ordered mechanism to prevent unproductive clamp loading events.

This work uses fluorescent kinetic assays reporting on individual interactions and reaction steps in the clamp loading cycle to measure the temporal correlation of events catalyzed by the *E. coli* γ complex. The main focus was on the relative timing of clamp closing and release. Given the stability of the ring-shaped β dimer in solution and the requirement for the clamp loader to stabilize the clamp in an open conformation to be loaded onto DNA, it was quite possible that the clamp simply shut rapidly upon release by the clamp loader. However, in single turnover fluorescent β closing and release assays, β closing is faster than β release showing that the β-clamp closes while still bound to the γ complex (Fig 6). This order of events may ensure that β clamps are closed around DNA and reduce the possibility that DNA slips out of an open clamp before closure. A two-step clamp closing/release reaction is unique to the situation in which clamps are loaded on DNA. In the passive clamp dissociation reaction in the absence of DNA, clamp closing and release rates are the same albeit much slower than in the clamp loading reaction. Similarly, in assays with nonhydrolyzable ATPγS, clamp closing and release rates are slower but the same. In these reactions, either clamp closing and release occur at the same time or the clamp may rapidly “snap shut” on release.

There are several possible mechanisms that would facilitate β closing prior to release from the γ complex; two possibilities are described below.

The first is that an ATP hydrolysis-induced conformational change in the γ complex forces the clamp closed. This type of mechanism is supported by molecular dynamics simulations on closed β clamps showing that β is held closed under spring tension. This spring tension would facilitate clamp opening by the δ subunit of the clamp loader, but would require energy to close the clamp (12, 48). It is possible that it takes more effort for the γ complex to close the β clamp than to open it. On the other hand, while the δ subunit can transiently open clamps to unload clamps from DNA (12), the δ subunit alone does not stabilize the clamp in an open conformation in solution sufficiently to produce a measurable population of open clamps (11). Formation of open clamps in solution requires intact clamp loaders (11). Therefore, a second possible mechanism for β closure is that ATP hydrolysis-induced conformational changes in the γ complex remove clamp loader•clamp interactions that stabilize an open conformation of the clamp, without destabilizing the complex enough to promote clamp dissociation. This type of mechanism is supported by the high stability of the closed β conformation and the requirement for binding to the clamp loader to maintain a relatively large fraction of clamps in an open conformation (11). This model is supported by molecular dynamics simulations with the eukaryotic clamp loader, replication factor C, and PCNA clamp that show that clamp loader•clamp interactions simply stabilize the open conformation of the clamp (29). And the corollary to this is that removing these interactions would destabilize the open conformation and allow clamp closure.

DNA binding triggers a burst of ATP hydrolysis by the clamp loader (24, 26). Here, we show that clamp closing is slower than ATP hydrolysis, supporting the idea that ATP hydrolysis-induced conformational changes in the clamp loader are required for clamp closure regardless of whether the reactions are measured in single or multiple turnover situations (Figs. 4 and 5). It will be interesting to determine the relative timing of DNA release, clamp closure, and hydrolysis of ATP molecules at individual sites. Previous work suggested that DNA may be released prior to clamp release (27). Do the same conformational changes that allow clamp closure
also promote release of DNA or do clamp closure and DNA release occur sequentially possibly regulated by ATP hydrolysis at individual clamp loader sites? In a recent crystal structure of the bacteriophage T4 clamp loader bound to a closed clamp, ADP was bound at one of the sites while ATP was bound to the others suggesting that hydrolysis of ATP at one site promotes clamp closure (30). This opens the possibility that sequential clamp closure and DNA release could be regulated by sequential ATP hydrolysis.

Clamp release following DNA-dependent ATP hydrolysis (active clamp loading) is on the order of 10–20 times faster than in the absence of DNA-dependent ATP hydrolysis (passive dissociation). Structural studies yield an explanation for this result. If γ complex bound to closed β resembles the structures for both the closed bacteriophage T4 and S. cerevisiae clamp loader•clamp complexes, then γ complex would have a lower affinity for closed β simply because there are fewer contacts between γ complex subunits and the clamp than are present in the open clamp loader•clamp complex (30, 49).

Passive dissociation of the open clamp loader•clamp complex may be slower because it would have to break more contacts.

The clamp loading reaction cycle is complex and composed of multiple steps driven by ATP binding and hydrolysis at multiple sites and interactions with two other ligands, the β clamp and DNA. These interactions with the clamp, DNA and ATP likely promote conformational changes in the clamp loader that facilitate the next step in the reaction cycle to generate an ordered clamp loading mechanism that ensures β is loaded quickly, in the correct position, and with as little wasted effort as possible. This type of mechanism could potentially give the clamp loading reaction the efficiency required to keep pace with the moving replication fork. This work, through the use of unique fluorescent assays, helps to fill in the gaps of the known γ complex clamp loading mechanism, and give a better understanding of how this remarkable enzyme, as well as the highly conserved clamp loaders from other organisms, function in the cell.

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**FOOTNOTES**

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2 Abbreviations: AF488, Alexa Fluor 488; MDCC, 7-Diethylamino-3-(((2-Maleimidyl)ethyl)amino)carbonyl)coumarin; p/t-DNA, primed template DNA; PY, pyrene.

**FIGURE LEGENDS**

**FIGURE 1.** Time courses for β-sliding clamp closing and release onto DNA. *A*, A cartoon diagram of the sequential mix stopped-flow reaction scheme is shown. *B*, β closing and release reactions were performed with final concentrations of 20 nM γ complex, 20 nM labeled β, 40 nM p/t-DNA, 0.5 mM ATP and 200 nM unlabeled β trap. Symmetrical p/t-DNA substrates were made by annealing two 60mers to create a 30-nt duplex and two 30-nt 5’ template overhangs. Representative reaction time courses are shown for clamp closing measured in reactions with β-AF4882 (black) and for clamp release measured with β-PY (gray).

**FIGURE 2.** Time courses for β release measured in reactions with SSB and measured by a FRET assay. *A*, β closing and release reactions from Figure 1 were repeated except that 400 nM SSB was added to block the single-stranded DNA ends. Representative time courses are shown for β closing measured in reactions with β-AF4882 (black) and for β release measured with β-PY (gray). *B*, β release was measured with an alternative FRET-based assay, where γ complex is labeled with the fluorophore and β is labeled with a non-fluorescent quencher. Release reactions were performed with final concentrations of 20 nM γ complex-AF488, 20 nM β-QSY9, 40 nM p/t-DNA, 0.5 mM ATP and 200 nM unlabeled β trap. The inset graph shows the residuals of both single and double exponential fits of the FRET based β release experiment.

**FIGURE 3.** Time courses for β closing and release in the absence of DNA and as a function of DNA concentration. *A*, β closing and release assays were performed omitting DNA, using the same sequential mixing scheme outlined in Fig 1A. Final reactions contained 20 nM γ complex, 20 nM labeled β, 0.5 mM ATP and 200 nM unlabeled β trap. Representative reaction time courses for clamp closing are shown in black and for clamp release are shown in gray. β closing (B) and β release (C) were measured in clamp loading reactions with varying concentrations of DNA. Representative traces for β closing (B) and release (C) with 20 nM (black), 40 nM (gray), and 100 nM DNA (light gray) are shown.

**FIGURE 4.** Temporal correlation of β closing and ATP hydrolysis in single-turnover reactions. *A*, A cartoon diagram of the sequential mix stopped-flow reaction scheme for measuring ATP hydrolysis is shown. Final reactions contained 200 nM γ complex, 200 nM β, 400 nM p/t-DNA, 0.2 mM ATP, 2µM MDCC-PBP and 2 mM ATPγS. *B*, A representative trace of ATP hydrolysis as measured by MDCC fluorescence and converted to concentration of inorganic phosphate released (gray) was overlaid with a
trace of β closing (black) at a final concentration of: 200 nM γ complex, 200 nM labeled β, 400 nM p/t-DNA, 0.5 mM ATP and 2 μM unlabeled β trap.

**FIGURE 5. Temporal order of β closing and ATP hydrolysis in multiple-turnover reactions.** Multiple turnover β closing (black) and ATP hydrolysis (gray) representative traces are shown. All reactions were performed using the stopped flow sequential mixing scheme illustrated in Fig. 1A and 3A but omitting the unlabeled β and ATPγS chases. Final concentrations are: 200 nM γ complex, 200 nM labeled β, 400 nM p/t-DNA, and 0.5 mM ATP for the β closing reactions and are the same for ATPase assays except for the inclusion of 2 μM PBP-MDCC and 200 nM unlabeled β in place of the labeled β.

**FIGURE 6. Model for the temporal order of events in loading the clamp on DNA.** On the left, γ complex, with ATP, forms a ternary complex composed of β in an open conformation and DNA. DNA binding triggers hydrolysis of ATP, followed by closing of β around DNA. On the right, once β is closed, γ complex releases the β•DNA complex resulting in a loaded clamp and freeing γ complex to load another clamp.

**TABLE 1. Rate constants calculated for β closing and release reactions.**

| Substrates               | $k_{obs, closing}$ (s$^{-1}$) | $k_{obs, release, 1}$ (s$^{-1}$) | $k_{obs, release, 2}$ (s$^{-1}$) |
|--------------------------|-------------------------------|---------------------------------|---------------------------------|
| p/t-DNA                  | 6.2 ± 1.3                     | 6.3 ± 0.3                       | 0.85 ± 0.04                     |
| p/t-DNA + SSB            | 8.7 ± 0.6                     | 5.2 ± 1.3                       | 0.5 ± 0.3                       |
| No DNA                   | 0.03 ± 0.01                   | 0.04 ± 0.02                     | N/A                             |
| p/t-DNA + ATPγS$^b$      | 0.078 ± 0.002                 | 0.055 ± 0.007                   | N/A                             |

$^a$Standard deviations are given for rate constants calculated from three independent experiments.  
$^b$ATPγS was substituted for ATP.
A. Sequential Mixing Scheme

Syringe 1: 

incubate 4s 

cuvette

Syringe 2: + ATP

Syringe 3: + ATP + excess unlabeled β

B. 

\[ \text{Relative AF488 intensity} \]

\[ \text{Relative pyrene intensity} \]

Time (s)

β closing

β release
A. Relative AF488 intensity

- β closing
- +SSB
- β release

Relative release intensity

Time (s)

B. Relative AF488 intensity

- Single exponential fit
- Double exponential fit

Residuals

Time (s)
Fig. 3

A. 

Initial AF488 intensity: 1.05

Relative AF488 intensity vs. time (s)

- **no DNA**
- **β release**

B. 

Relative AF488 intensity vs. time (s)

- 20nM DNA
- 40nM DNA
- 100nM DNA

C. 

Relative pyrene intensity vs. time (s)

- 20nM DNA
- 40nM DNA
- 100nM DNA
A. Sequential Mixing Scheme

- Syringe 1:
- Syringe 2: + ATP
- Syringe 3: + ATPγS + MDCC-PBP

B. Normalized AF488 intensity

Normalized AF488 intensity vs. Time (s)

- ATP hydrolysis
- P release (nM)

Fig. 4
A.

Normalized intensity vs. time (s).

- **β closing**
- **ATP hydrolysis**

Fig. 5
The β-Sliding Clamp Closes Around DNA Prior to Release by the *Escherichia coli* Clamp Loader γ complex
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