The β protein, a dimeric ring-shaped clamp essential for processive DNA replication by Escherichia coli DNA polymerase III holoenzyme, is assembled onto DNA by the γ complex. This study examines the clamp loading pathway in real time, using pre-steady state fluorescent depolarization measurements to investigate the loading reaction and ATP requirements for the assembly of β onto DNA. Two β dimer interface mutants, L273A and L108A, and a nonhydrolyzable ATP analog, adenosine 5′-O-(3-thiotriphosphate) (ATPγS), have been used to show that ATP binding is required for γ complex and β to associate with DNA, but that a γ complex-catalyzed ATP hydrolysis is required for γ complex to release the β-DNA complex and complete the reaction. In the presence of ATP and γ complex, the β mutants associate with DNA as efficiently as wild type β. However, completion of the reaction is much slower with the β mutants because of decreased ATP hydrolysis by the γ complex, resulting in a much slower release of the mutants onto DNA. The effects of mutations in the dimer interface were similar to the effects of replacing ATP with ATPγS in reactions using wild type β. Thus, the assembly of β around DNA is coupled tightly to the ATPase activity of the γ complex, and completion of the assembly process requires ATP hydrolysis for turnover of the catalytic clamp loader.

Replication of genomic DNA in Escherichia coli involves the assembly of a multisubunit enzyme complex on the DNA molecule. These proteins converge in such a way as to promote processive synthesis in both leading and lagging strands. In organisms as diverse as bacteriophages, bacteria, yeasts, and humans, the components that characterize a processive replicating machine are quite similar. In the case of E. coli, the γ complex, using ATP, loads β subunit onto DNA. The β clamp, fully encircling the DNA molecule, associates with core and holds it firmly in place so extension of the primer can proceed. Because lagging strand synthesis is discontinuous, a cycling of the proteins upon completion of each Okazaki fragment to a new primer is required; therefore, periodic loading of β is essential for replication of the genome to continue.

Duplication of genomic DNA in E. coli requires processive DNA replication activity of the 10-subunit DNA polymerase III holoenzyme. The E. coli DNA polymerase III holoenzyme consists of three main functional units: the core polymerase, the β sliding clamp, and the γ complex clamp loader (reviewed in Refs. 1 and 2). The three subunits distinctive to E. coli DNA polymerase III core (3) are α, which has DNA polymerase activity (4); δ, which has a 3′ to 5′ exonuclease activity (5, 6); and ε, which, at this time, lacks a well defined function (3, 7).

The core, by itself, is not processive. It is capable of synthesizing short segments of DNA but dissociates readily from the primer/template, extending the primer only 10–20 nucleotides per binding event in vitro (8). The β sliding clamp and the γ complex are accessory proteins, the presence of which converts this nonprocessive component into an apparatus that synthesizes thousands of nucleotides per binding event (4, 9).

Analysis by x-ray diffraction has shown that the β subunit is a ring-shaped dimer that has a central hole, with an inner diameter of about 35 Å, large enough to encircle duplex DNA (10). The γ complex is a multisubunit clamp loader composed of two γ subunits and one each of the δ, δ′, χ, and ψ subunits (11, 12). Biochemical evidence has established a mechanism by which the γ complex acts to assemble β onto DNA, wherein β acts as a sliding clamp, tethering the core polymerase to the DNA molecule.

Techniques for studying the mechanism of the γ complex-catalyzed reaction have been confined to observing their assembly on large circular single-stranded DNA, such as bacteriophage templates, or by combining the components and observing their association using gel filtration and SDS-polyacrylamide gel electrophoresis. To further characterize the pathway of clamp assembly, we used fluorescence depolarization to measure the change in anisotropy of a short primer/template labeled with a fluorescent probe, rhodamine-χ. The rotational motion of the DNA is dependent on its molecular weight and is slowed when proteins are bound; therefore, the change in fluorescence anisotropy of the labeled pT1 DNA is a measure of the interactions between the γ complex, β, and DNA.

The β clamp is a stable dimer in solution due to the presence of several hydrogen bonds and hydrophobic contacts at the dimer interface between the two monomers. The γ complex must break these contacts to open the ring, and therefore, the nature of the β dimer interface likely plays an important role in the ATP-coupled process of clamp assembly on DNA. We have...
found that mutation of the hydrophobic residue Leu^{272} to Ala or mutation of Leu^{196} to Ala at the dimer interface yields β clamps that can assemble on DNA but inhibit the DNA-dependent ATPase activity of the γ complex. Steady state and pre-steady state measurements of the protein-DNA interactions indicate a corresponding inhibition in the dissociation of γ complex from the β-DNA complex. Results with ATP-γS, using wt β, substantiate the β mutant study, indicating that in the absence of ATP hydrolysis, release of γ complex from β-DNA is blocked. Thus, we show that β can be loaded onto DNA in the presence of ATP; however, ATP hydrolysis is required to release the clamp loader from the clamp completing the clamp loading process.

EXPERIMENTAL PROCEDURES

Enzymes—Subunits of the γ complex were overexpressed and purified as described: γ (13), β (8' (14), χ, and δ (15). The γ complex was reconstituted from purified subunits as described (12). Site-directed mutagenesis of the dnaN gene was achieved producing wt L273A mutant β protein and an L108A mutant β protein. Wild type β and mutant β proteins were overexpressed and purified as described (16). The enzyme reaction buffer contained 20 mM Tris-HCl, pH 7.5, 40 μg/ml bovine serum albumin, 5 mM diithiothreitol, 8 mM MgCl₂, and 50 mM NaCl.

Oligonucleotides—Oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry on an Applied Biosystems synthesizer (model 392 RNA/DNA) and purified by denaturing polyacrylamide gel electrophoresis. Fluorescence measurements were done using the following primer/template sequence:

ACG AAC ATT ACC GCC A 3' TCT TCT TGA GTC TGA TAG CGA GCCAGCCA CCA TTA TAG GTC

AA 5' CTGG AAT ATC CAG TTG TTA TAA TCG CTC GTA ACG TGG TCC TTT TGG CGA GT 5'

SEQUENCE 1

The 80-mer template was labeled at the 5'-end with rhodamine-x (Molecular probes, catalog no. X-491) to which the 30-mer primer was annealed (17).

Steady State Anisotropy Measurements—A QuantaMaster QM-1 fluorometer (Photon Technology International) with a single emission channel (L format) was used to measure steady state anisotropy. Samples were excited with vertically polarized light (Oriel dichroic sheet polarizer) at 580 nm (3-nm band pass), and both vertical and horizontal emission was monitored at 610 nm (8-nm band pass). Three individual anisotropy measurements were taken for each sample, and the values were averaged to compensate for manual rotation of the polarizer. The G factor was determined, and its value was used to calculate anisotropy.

Steady state experiments were performed in a 1-cm path cuvette by measuring the initial anisotropy of 54 nM p/t DNA (labeled with ρT and CD3) and developing with an 11:7:2 NH₄OH/isopropanol:water solution. The steadystate anisotropy was measured again. One final anisotropy measurement was taken (data not shown). Hence, all observed anisotropy changes reflect the rotational property of p/t DNA and not any other property. The diffusion rate of free DNA is greater than that of DNA bound by proteins, the fluorescence anisotropy of the DNA bound by proteins, the fluorescence anisotropy of the DNA in reaction buffer, and the second steady state anisotropy measured was 0.15 "trap DNA" reaction buffer. MiR trap DNA was used in these experiments except that ATP was replaced by ATP-γS in reaction buffer.

RESULTS

The γ complex-catalyzed reaction of loading β onto DNA was analyzed directly in solution using steady state fluorescence depolarization, a technique measuring the rotational diffusion of a fluorophore (19). In these experiments a fluorescent probe, rhodamine-x, was covalently attached to DNA, and the fluorescence anisotropy of the probe on DNA was measured. Because the diffusion rate of free DNA is greater than that of DNA bound by proteins, the fluorescence anisotropy of the DNA probe will directly report on the association of proteins with DNA (20). A decrease in the rate of rotation, due to protein binding, will result in an overall increase in steady state anisotropy. In all equilibrium and pre-steady state anisotropy experiments, the total intensity signal was examined and found to be absolutely invariant under all experimental conditions (data not shown). Hence, all observed anisotropy changes reflect the rotational property of p/t DNA and not any other photophysical effect. These results are consistent with our previous time-resolved fluorescence measurements using the same system (17). For a recent review on the use of fluorescence anisotropy for protein-DNA interactions, see Heyduk et al. (22). Interaction between protein-DNA was determined under steady state conditions for clamp loading activity and also under pre-steady state conditions to observe formation of the protein-DNA complex in real time.

For anisotropy measurements, a 30-nucleotide primer was annealed to an 80-nucleotide template labeled at the 5'-end with a fluorescent probe, rhodamine-x. The primer was placed
Loading of $\beta$ Clamp on DNA by $\gamma$ Complex

A

Wild Type $\beta$

![Diagram A](image)

B

![Diagram B](image)

**FIG. 1.** Increase in steady state anisotropy for rhodamine-x-labeled DNA when $\gamma$ complex loads wt $\beta$ on DNA. A, changes in the steady state anisotropy of an 80-nucleotide template labeled at the 5'-end with rhodamine-x annealed to a 30-nucleotide primer when wt $\beta$ and then $\gamma$ complex were added to DNA. The steady state anisotropy of the p/t DNA (DNA) in reaction buffer was measured. After a small aliquot of wt $\beta$ (Beta) was added, the anisotropy of this DNA solution was again measured. The anisotropy of the DNA solution was again taken after $\gamma$ complex (Gamma) was added. The reaction was allowed to stand for 15 min, and then another anisotropy measurement was taken (15 min). One final anisotropy measurement was taken following the addition of ATP (ATP). Final concentrations were 50 nM p/t DNA, 400 nM $\beta$, 200 nM $\gamma$ complex, and 500 $\mu$M ATP. B, changes in the steady state anisotropy of an 80-nucleotide template labeled at the 5'-end with rhodamine-x and annealed to a 30-nucleotide primer when $\gamma$ complex and then $\beta$ were added to the DNA. In this experiment, the anisotropy of p/t DNA (DNA) was measured, and after the addition of each component the anisotropy was re-measured. First, $\gamma$ complex (Gamma) was added, and then $\beta$ (Beta) was added. The reaction was allowed to stand for 15 min, and then ATP was added. The conditions were identical to those shown in A. By adding the $\gamma$ complex first, it was shown that both $\gamma$ complex and $\beta$ are required to give the large increase in anisotropy of 0.25 for the p/t DNA. A sketch of the reaction is shown at the top of each figure. This experiment was previously published in Ref. 17 and was independently repeated here to compare the results of wt $\beta$ with two $\beta$ mutants shown in Fig. 2.

in the center of the 80-mer, leaving single-stranded segments, each 25 nucleotides in length, at both ends of the template. This 30/80-nucleotide primer/template combination was shown to be the minimum size for DNA to still support processive synthesis by the core polymerase in the presence of both the $\gamma$ complex and the $\beta$ dimer (17).

Loading of wt $\beta$ and $\beta$ Mutants onto p/t DNA Under Steady State Conditions—The loading of wt $\beta$ and $\beta$ mutants onto DNA was measured under steady state conditions using titration experiments. The proteins were added to DNA in a specific order to determine how each constituent affects the fluorescence anisotropy of the DNA probe. Labeled p/t DNA (50 nM), ATP (500 $\mu$M), and buffer containing Mg$^{2+}$ were placed in a cuvette, and the anisotropy of the p/t DNA was determined. Two types of titration experiments were performed. In the first (Figs. 1A and 2, A and C) $\beta$ (400 nM) was added to p/t DNA prior to the addition of $\gamma$ complex (200 nM), and in the second (Figs. 1B and 2, B and D) $\gamma$ complex was added to DNA prior to $\beta$. Anisotropy measurements were taken following the addition of each protein. The results of these experiments were previously shown for wt $\beta$ in a publication by Bloom et al. (17) but were repeated in this work to compare the results of wt $\beta$ with the two $\beta$ mutants.

When wt $\beta$ or $\beta$ mutants were added to p/t DNA, the anisotropy did not increase significantly over the initial value of approximately 0.16 (Figs. 1A and 2, A and C). A substantial transition occurred, however, when $\gamma$ complex was added to the reaction, increasing the anisotropy values from 0.16 to 0.25 for wt $\beta$, 0.26 for L273A mutant $\beta$ and to 0.28 for L108A mutant $\beta$ (Figs. 1A and 2, A and C). To determine whether the increases in anisotropy were caused by the association of $\gamma$ complex with p/t DNA, the order in which the $\beta$ dimer and $\gamma$ complex were added was reversed. Addition of the $\gamma$ complex to p/t DNA resulted in a small increase in anisotropy from 0.16 to 0.17 (Figs. 1B and 2, B and D), but the large anisotropy increases did not occur until either wt $\beta$ or the $\beta$ mutants were added (Figs. 1B and 2, B and D). These data demonstrate that an increase in anisotropy for rhodamine-x-labeled p/t DNA requires the presence of both the $\gamma$ complex and the $\beta$ dimer, but they do not answer the question of which proteins are bound to DNA, $\beta$ by itself or the $\gamma$ complex and $\beta$. It has been shown, however, that after the $\gamma$ complex loads $\beta$ onto DNA, $\beta$ alone remains bound to the DNA molecule (9). These initial results indicate that the $\beta$ mutants are capable of interacting with the $\gamma$ complex and p/t DNA in the same manner as wt $\beta$. One noticeable observation is that anisotropy increases for rhodamine-x-labeled p/t DNA in the presence of the $\gamma$ complex and $\beta$ mutants were slightly larger (0.26 for L273A and 0.28 for L108A) than when in the presence of $\gamma$ complex and wt $\beta$ (0.25).

Following the addition of $\gamma$ complex and wt or mutant $\beta$ to p/t DNA, the reactions were incubated for 15 min. During this 15 min incubation, the anisotropy of wt $\beta$ dropped to a value of 0.17, a value close to that of free p/t DNA (Fig. 1, A and B, 15 min). In contrast, when reactions with $\beta$ mutants were incubated for 15 min essentially no decreases in anisotropy were observed (Fig. 2, A–D, 15 min). For the L273A mutant, the anisotropy remained at 0.26 for approximately 60 min and decreased to a value of only 0.24 after 105 min. This value is still well above the level of 0.16 for free p/t DNA (Fig. 2, A and B). For L108A mutant $\beta$, it took approximately 1 h for the anisotropy to decrease to a value of 0.21 (Fig. 2, C and D). In reactions with both the wt and mutant $\beta$s, when additional ATP was added, the anisotropy values once again increased (Figs. 1 and 2, ATP). The fact that addition of fresh ATP restored high anisotropy values indicates that the fraction of p/t DNA bound by protein is due to the utilization of ATP and
can be restored if ATP is replenished to a depleted reaction. In the absence of ATP, there was no association of \( \beta \) or \( \gamma \) complex with p/t DNA (data not shown).

For reactions with wt \( \beta \), the increased anisotropy values following the addition of \( \beta \) and \( \gamma \) complex to p/t DNA actually represent a steady state population of bound protein that is cycling on and off the DNA (see kinetic experiments described below). In each cycle, \( \beta \) is loaded onto DNA in a reaction that requires hydrolysis of ATP; it then dissociates from DNA or is removed by the \( \gamma \) complex to once again be loaded at the expense of more ATP. For reactions with the \( \beta \) mutants, a much longer period of time is required before depletion of ATP, suggesting that this cycling reaction is significantly slower than that for wt \( \beta \).

**Steady State Rates of ATP Hydrolysis**—To investigate whether ATP was being hydrolyzed by the \( \gamma \) complex at slower rates in the presence of the \( \beta \) mutants than with wt \( \beta \), ATPase assays were performed. Rates of ATP hydrolysis were measured using \([\alpha-^{32}\text{P}]\text{ATP} \), the product of which, after hydrolysis, was analyzed by separating hydrolyzed ADP from ATP using thin layer chromatography and quantitating the separated fractions on a PhosphorImager. The \( \gamma \) complex (400 nM), p/t DNA (50 nM), \([\alpha-^{32}\text{P}]\text{ATP} \) (500 \( \mu \text{M} \)), and buffer with Mg\textsuperscript{2+} were combined. The reaction was initiated by adding the \( \gamma \) complex (200 nM). Aliquots were removed at 1, 5, 10, 20, 40, and 80 min and then quenched using a solution of SDS and EDTA. Previous studies have shown that \( \gamma \) complex ATPase activity is stimulated by DNA and is stimulated further in the presence of both DNA and \( \beta \) (23–25). Here we have performed ATPase assays under conditions identical to the DNA binding anisotropy experiments. Rates of ATP hydrolysis were measured in reactions with \( \gamma \) complex alone; \( \gamma \) complex and p/t DNA; and \( \gamma \) complex, p/t DNA, and wt \( \beta \). The rate of ATP hydrolysis was very slow for \( \gamma \) complex alone, 0.4 \( \mu \text{mol-min}^{-1} \) (Fig. 3).
Loading of β Clamp on DNA by γ Complex

Fig. 3. ATP hydrolysis by γ complex alone, with p/t DNA, and with p/t DNA and wild type β dimer. A, separation of ATP and ADP by thin layer chromatography after steady state ATP hydrolysis of γ complex by itself (γ complex) in the presence of DNA (γ complex + DNA) and in the presence of wild type β + DNA (γ complex + wild type β + DNA). Reactions contained 200 nM γ complex, 50 nM p/t DNA (if present), 400 nM β dimer (if present), and 500 μM ATP in reaction buffer. Aliquots of reaction mixtures were quenched after 1, 5, 10, 20, 40, and 80 min. The arrows indicate where ADP and ATP were found. B, the data were quantitated using a PhosphorImager, and these values were plotted against time to determine the steady state rate of ATP hydrolysis from the slope of the linear plot. The steady state rate of ATP hydrolysis for γ complex alone (●) was 0.4 μmol-min⁻¹. When γ complex was in the presence of DNA (■), the steady state rate of ATP hydrolysis increased to 4.3 μmol-min⁻¹, and when γ complex was in the presence of DNA and β the steady state rate increased to 12 μmol-min⁻¹ (○). ATP hydrolysis in the presence of γ complex, wt β dimer, and DNA was linear for reaction times of 20 min or less; a decrease in ATP hydrolysis rate occurred for t > 20 min, caused by the consumption of ATP substrate (data not shown).

Fig. 4. ATP hydrolysis of γ complex when present with wt β, L273A mutant β, and L108A mutant β. A, the separation of ATP and ADP using thin layer chromatography after steady state ATP hydrolysis of γ complex in the presence of DNA and wt β (γ complex + wild type β + DNA). L273A mutant β (γ complex + L273A mutant β + DNA), and L108A mutant β (γ complex + L108A mutant β + DNA). An aliquot of 50 nM p/t DNA, 400 nM β, 200 nM γ complex, and 500 μM ATP was taken after 1, 5, 10, 20, 40, and 80 min. ATP and ADP are indicated by the arrows. B, the data were quantitated using a PhosphorImager, and these values were plotted against time to determine the steady state rate of ATP hydrolysis from the slope of the linear plot. The steady state rate of ATP hydrolysis for γ complex in the presence of DNA and wt β (●) was 12 μmol-min⁻¹. When γ complex was in the presence of DNA and L273A mutant β (■), the rate of steady state ATP hydrolysis decreased from that observed by wt β to 6.2 μmol-min⁻¹. ATP hydrolysis in the presence of γ complex, wt β dimer and DNA (○) was linear for reaction times of 20 min or less; a decrease in ATP hydrolysis rate occurred for t > 20 min, caused by the consumption of ATP substrate (data not shown).

Pre-steady State Kinetics of the γ Complex-catalyzed Loading of wt β and β Mutants on p/t DNA—Real time measurements of γ complex-catalyzed loading of wt β and β mutants onto p/t DNA were obtained using stopped-flow fluorescence anisotropy. In these experiments, real time changes in the steady state anisotropy of rhodamine-x-labeled p/t DNA were measured during the rapid mixing of DNA and proteins. For the reaction shown in Scheme 1 (Fig. 5), one stopped-flow syringe was loaded with wt or mutant β (800 nM), p/t DNA (100 nM), ATP (500 μM), and buffer containing Mg²⁺. A second stopped-flow syringe was loaded with γ complex (600 nM), ATP (500 μM), and the same buffer with Mg²⁺. Reactions were initiated by delivering equal volumes of the contents of the two syringes to
The catalyzed reaction time courses for γ complex loading wt β, L273A mutant β and L108A mutant β on p/t DNA using different orders of the addition of γ complex and β to rhodamine-x-labeled DNA. Rates of loading wt and mutant β onto DNA were measured using two different mixing schemes. For the reaction shown in Scheme 1, one stopped-flow syringe contained rhodamine-x-labeled p/t DNA, wt, or mutant β dimer, and ATP. A second syringe contained a solution of γ complex and ATP. For mixing the reaction shown in Scheme 2, one stopped-flow syringe contained a solution of rhodamine-x-labeled p/t DNA and ATP. A second contained wt or mutant β, γ complex, and ATP. The reactions shown in Schemes 1 and 2 were initiated by mixing equal volumes of solutions from syringes 1 and 2. A, loading reactions for wt β under the conditions for both Scheme 1 and Scheme 2. B, loading reactions for the L273A mutant β under the conditions for both Scheme 1 and Scheme 2. C, loading reactions for the L108A mutant β under the conditions for both Scheme 1 and Scheme 2. For all six experiments and in both reactions, final concentrations were 50 nM p/t DNA, 400 nM β, 300 nM γ complex, and 500 μM ATP. The lower curve of each graph, showing a constant measurement of anisotropy, is a control experiment eliminating γ complex from the reaction as described under “Experimental Procedures.” In A, B, and C, Scheme 2 shows the results when β and γ complex were first preincubated and then combined with p/t DNA; the increase in anisotropy was much more rapid than in Scheme 1 (A–C), when β and γ complex were added separately. Schemes of both reactions are shown at the top.
anisotropy at early times (~50 ms) “overshoot” the anisotropy values measured later in the reaction (>300 ms). This small overshoot was also observed in our original study of this system (17); however, at that time, we had several possible explanations for this effect. The lack of overshoot in the mutant data, combined with the ATP hydrolysis rate data, allows for a determination of the mechanism associated with this overshoot. In the wt data, it is important to understand that the final measured anisotropy values (>500 ms in Figs. 1A and 5A) are not equilibrium values, but rather steady state values reflecting the average proportion of time that p/t DNA is “occupied” versus free in solution as the entire reaction cycles the β and γ complex on and off the DNA. Hence, the transition from the anisotropy overshoot at 50 ms to a constant value at approximately 500 ms is a direct reflection of the transition from pre-steady state to steady state kinetics in this system. This description of the anisotropy overshoot is essentially model-independent and does not directly address the nature of the bound state formed within the first 50 ms (i.e., is it a binary or ternary complex). For instance, does the anisotropy value observed at 50 ms (or at all times for the mutant complexes) represent a β-DNA binary complex, β-γ complex-DNA ternary complex, or some proportion of both binary and ternary complexes? To answer this question, off-rate kinetics of both wt cycling reactions and mutant complexes were examined. If ATP hydrolysis was required for γ complex to release a binary β-DNA complex, then the off-rates for the mutant complexes would be expected to be much slower than the off-rate of the cycling wt complex.

Pre-steady State Dissociation of β from p/t DNA—Rates of dissociation of wt β and β mutants from p/t DNA were measured in stopped-flow anisotropy experiments. These reactions were performed by combining β (800 nM), p/t DNA (100 nM), γ complex (600 nM), ATP (2 mM), and buffer containing Mg²⁺ in one syringe. A second syringe contained M13 DNA (500 nM) annealed to two primers and buffer containing Mg²⁺. Reactions were initiated by delivering equal volumes of solutions from each syringe to the stopped-flow cuvette. In these reactions, the primed M13 DNA, which was in excess, acted as a trap to bind any free β that dissociated from p/t DNA. Fig. 6 shows anisotropy measurements for the dissociation of wt β, L273A mutant β, and L108A mutant β from p/t DNA.

The L273A and L108A β mutants have greatly reduced off-rates relative to wt β. The average off-rate for wt β was 6 s⁻¹, whereas those for the mutants L273A and L108A were 0.2 and 0.5 s⁻¹, respectively. The wt β dissociates from p/t DNA somewhere between 12 and 30 times faster than the mutants. This drastic reduction in the dissociation rates for the β mutants results in a reduction in the rates of both steady state cycling and ATP hydrolysis. These results, along with results from steady state ATPase assays showing that ATP was hydrolyzed more slowly in reactions with β mutants and the results from the accompanying manuscript (26), strongly suggest that the release of γ complex, following the loading of β, is associated with the hydrolysis of ATP.

Pre-steady State Analysis in Real Time of β and γ Complex Interacting with p/t DNA Using ATPγS—To determine whether ATP binding is sufficient for γ complex to bind β and p/t DNA, whereas ATP hydrolysis is required for γ complex to release β on the DNA completing the loading reaction, pre-steady state kinetics of γ complex-catalyzed loading of β onto p/t DNA was measured in reactions with ATPγS. ATPγS, an analog of ATP, is a useful tool that can bind to the active site of a protein, but hydrolysis and release of the phosphorothioate group is significantly slower than that of the terminal phosphate in ATP. Hydrolysis of ATPγS by γ complex is slower than pre-steady state rates of loading β onto p/t DNA (26). If, as suggested above, the β mutant and γ complex union get locked, due to diminished hydrolysis of ATP, pre-steady state anisotropy experiments previously described in Fig. 5 (Scheme 1 and Scheme 2) should transform the β curves (with overshoot) into those observed in the L273A and L108A β mutants (no overshoot).

Experiments using wt β were performed in the same manner as those in Fig. 5, except that ATP was replaced with ATPγS. The anisotropy of p/t DNA for wt β when the components were mixed as outlined in Scheme 1 started at 0.15 and increased to 0.26 over time, with an estimated rate of loading of 4 s⁻¹ (Fig. 7). If the components were mixed as outlined in Scheme 2, the anisotropy started at 0.21 and increased to 0.27 over time, with an estimated rate of loading of 31 s⁻¹ (Fig. 7). ATPγS, when used in place of ATP, decreased the rate of association of β and γ complex with DNA in both cases.

What is apparent in these experiments are the qualitative differences in the curves generated in reactions with ATP and ATPγS. Reaction time courses for wt β in the presence of ATPγS no longer appeared to be similar to those shown for wt β when ATP was used, but instead resembled those observed
The catalyzed reaction time courses for γ complex loading wt β on p/t DNA using ATPγS, using different orders of the addition of γ complex and β to rhodamine-x-labeled DNA. In Scheme 1, a solution of rhodamine-x-labeled p/t DNA, wt β dimer, and ATPγS was combined with a solution of γ complex and ATPγS. In Scheme 2, a solution of rhodamine-x-labeled p/t DNA and ATPγS was combined with wt β, γ complex, and ATPγS. In Scheme 2, if β and γ complex were first preincubated and then combined with p/t DNA, the increase in anisotropy was much more rapid than in Scheme 1, in which β and γ complex were added separately. Schemes of both reactions are shown at the top.

for either of the mutants. The overshoot in anisotropy (Fig. 5A, Scheme 2) disappeared with the anisotropy now increasing to approximately 0.26 with no detectable decrease over time (Fig. 7, Scheme 2). This result was similar to what was seen when the mutants were used (Fig. 5, B and C, Scheme 2). The reaction course appears to be following the same path as that taken with β mutants, in which β and γ complex remain bound until ATP hydrolysis occurs. As a control, experiments using ATPγS were performed using L273A mutant β, and similar results were obtained. The loading rate decreased significantly when ATPγS was used in place of ATP, but the shape of the curve remained the same (data not shown).

Pre-steady State Chase Experiments Using ATP and ATPγS—To determine whether a ternary complex is actually locked on p/t DNA in the absence of ATP hydrolysis, two different chase experiments were performed. First, the reaction was allowed to proceed in the presence of ATPγS then chased with excess ATP. One syringe was filled with β (400 nM), γ complex (300 nM), p/t DNA (50 nM), ATPγS (500 μM), and buffer containing Mg2++, and another syringe was filled with ATP (5 mM) with the same buffer containing Mg2++. The contents of the two syringes were released in the mixing chamber, and anisotropy data were collected over time. If ATPγS was used in the reaction, a locked complex formed with an anisotropy of approximately 0.27 and remained locked for 60–90 min after chasing with ATP (Fig. 8A). The exchange was quite slow, but once the active site of γ complex was able to bind ATP, hydrolysis took place, and the anisotropy began to decrease. It is important to note that the anisotropy did not drop to that seen for free p/t DNA (0.18) but did drop to the level seen in the pre-steady state wt β experiments of 0.24 (Fig. 5A), at which cycling of proteins on and off DNA occurred.

The second chase experiment was the reverse of that just described. The reaction was allowed to proceed in the presence of ATP and then chased using excess ATPγS. The anisotropy began at a level (0.23) consistent with wt β cycling on and off p/t DNA and then increased to 0.27 when ATPγS was added to the reaction (Fig. 8B). The increase in anisotropy following the ATPγS chase was much more rapid than the decrease in anisotropy following the ATP chase. Thus, the intrinsic rate of ATP exchange for γ complex is rapid when γ complex is able to hydrolyze ATP but slow when γ complex is unable to hydrolyze ATP. This suggests that once hydrolysis occurred, ADP and P_i were rapidly released, and the γ complex was free to bind ATPγS. On binding ATPγS, an increase in anisotropy attributable to the locked state of the β and γ complex occurred. If γ complex was hydrolyzing ATP prior to the chase with ATPγS, and the anisotropy seen at 0.23 was that of β cycling on and off p/t DNA, then a minimum rate of ATP hydrolysis can be determined from the rate of the exponential rise of the anisotropy when chased with ATPγS. Assuming that the γ complex remains bound to the β dimer in the presence of ATPγS, the ATP hydrolysis rate is calculated to be 3 s⁻¹ and is similar to the rate observed during the fall of the pre-steady state rise fall experiments (Fig. 5A, Scheme 2). This represents a minimum value for the rate of ATP hydrolysis because it is not clear whether this rate is actually limited by the hydrolysis of ATP, the release of ADP and P_i, dissociation of γ complex from a productively loaded β-DNA complex, or the loading of another β dimer in the presence of ATPγS.

**DISCUSSION**

The β clamp is a head-to-tail ring-shaped dimer composed of two identical monomers (10) that is assembled on DNA by action of the γ complex (11, 26). Although it is known that the overall loading reaction requires ATP hydrolysis, the individual steps and the ATP dependence of each step are not known. This work and the accompanying manuscript (26) focus on defining individual steps of the clamp loading reaction and on identifying steps that require ATP binding or ATP hydrolysis.

The first question addressed in this study considers what effects mutations in the interface of the β dimer has on the kinetics of loading β onto DNA. One idea of how the clamp loading reaction works is that the γ complex opens the dimer in a hinge-type motion at one interface of the head-to-tail dimer; it is then slipped over and closed around the double-stranded DNA molecule. Another idea is that the γ clamp is separated at both interfaces of the dimer and is held apart by action of the γ complex prior to it being loaded onto DNA. In either case, the β dimer must be opened at a minimum of one interface for loading to take place, and mutations at the interface influencing the ease of opening the dimer would be expected to affect the kinetics of loading β onto DNA. The second question addressed the idea of which step in the loading reaction requires ATP binding and which step requires ATP hydrolysis. Previous work has already shown that ATP binding is required for γ complex to bind β and to bind DNA (14).²

² L. B. Bloom and M. F. Goodman, unpublished results.
DNA. The β mutants are capable of forming dimers in solution\(^3\) and support processive synthesis by the core polymerase,\(^4\) so we set out to determine how these mutations would affect the kinetics of assembling β onto DNA. Steady state anisotropy measurements showed that both the L273A and L108A mutants associate with DNA in the presence of γ complex, increasing anisotropy values from 0.16 for free DNA to 0.26 and 0.28 for bound DNA, respectively (Fig. 2). DNA bound by wt β had an anisotropy value of 0.25 (Fig. 1). The DNA in reactions with β mutants showed an overall higher anisotropy value than wt β but could apparently be loaded with a similar efficiency. When the reaction with wt β was continued for 15 min, the steady state anisotropy decreased from the level of protein-bound DNA to the level of free DNA due to the rapid consumption of ATP during the 15-min time period (Fig. 1). This indicates that ATP was being used during each cycle of the loading reaction, and when ATP was consumed, the γ complex could no longer load β onto DNA. For the mutants, the rate of ATP consumption was much slower, which is indicated by the longer times taken to decrease the protein-bound DNA anisotropy to the level of free DNA (Fig. 2).

Steady state ATP consumption was measured using ATPase assays to verify that the β mutants decrease the rate of ATP hydrolysis by the γ complex. The γ complex hydrolyzed ATP 8-fold faster in the presence of wt β than in the presence of L273A mutant β and 2-fold faster in the presence of wt β than in the presence of L108A mutant β. In addition, ATP hydrolysis by the γ complex when present with p/t DNA is greater than that of the γ complex in the presence of p/t DNA and the L273A mutant β (Fig. 3B). The reduced rates of ATP hydrolysis in reactions with β mutants suggest that the release of γ complex from β-bound DNA may be coupled to the hydrolysis of ATP.

In steady state reactions with wt β, a steady state population of p/t DNA, bound by β, is most likely formed as a result of a cycling reaction. The γ complex assembles β on DNA in a reaction requiring but not hydrolyzing ATP. ATP hydrolysis is required, however, to complete the loading reaction allowing the dissociation of γ complex. This leaves β free to slide off the end of the short linear template and establish a sequence of events that is repeated over and over again to form the cycling reaction. This cycling reaction is made possible by loading β onto linear DNA duplexes where it can dissociate by sliding off the ends of the DNA. A cycling reaction of this nature is consistent with other studies showing that the γ complex acts catalytically to assemble β onto DNA (9), and after β is loaded, the γ complex dissociates, allowing the core polymerase to bind β (27). In reactions with β mutants, anisotropy values most likely reflect a population of p/t DNA bound by both β and γ complex. Because a mutant β-γ complex-DNA combined structure is larger than a wt β-DNA structure, it will tumble more.

\(^3\) M. O’Donnell, unpublished results.
\(^4\) J. G. Bertram and M. F. Goodman, unpublished results.

FIG. 8. Reaction time course for γ complex loading wt β on p/t DNA then chased with ATP and ATPγS. A, the reaction was initiated by combining 50 nM p/t DNA, 400 nM wt β, 300 nM γ complex, and 500 μM ATPγS. This reaction was then chased with 5 mM ATP as described under “Experimental Procedures.” Before the reaction was chased with ATP, γ complex and β remained coupled to p/t DNA (β and γ complex locked using ATPγS) at the higher anisotropy of 0.27. Two h after the reaction was chased with ATP, the anisotropy dropped to 0.24, the value at which β is thought to be cycling on p/t DNA (β cycling using ATP). B. The reaction was initiated by combining 50 nM p/t DNA, 400 nM wt β, 300 nM γ complex, and 500 μM ATP. This reaction was then chased with 5 mM ATPγS. Before the reaction was chased with ATPγS, β was cycling on p/t DNA (β cycling using ATP) at an anisotropy value of 0.23. Following the chase with ATPγS, the anisotropy increased to 0.27, the level at which γ complex and β are thought to be coupled to p/t DNA (β and γ complex locked using ATPγS). A sketch of the reaction is shown at the top.
slowly and give rise to a higher anisotropy value. The fact that the β mutants reduce rates of DNA-dependent ATP hydrolysis by the γ complex is consistent with the idea that the γ complex remains bound to the β mutants much longer than when bound to wt β. Because the γ complex is not free to bind another β dimer until it dissociates from the β-DNA complex, the rate of the steady state cycling reaction and ATP hydrolysis is reduced.

In pre-steady state anisotropy experiments, the rates of protein association with DNA and dissociation from DNA were measured in real time. Because the γ complex hydrolyzed ATP much more slowly in steady state cycling reactions when the mutants were used, a decrease in either pre-steady state association or pre-steady state dissociation rates of β with p/t DNA would also be expected. Again, like wt β, the mutant β clamps were able to associate with p/t DNA as fast as or even faster than wt β. In reactions in which β and p/t DNA were added to γ complex (Scheme 1), wt β assembled on DNA at a rate of 14 s⁻¹, L273A mutant β assembled on DNA at 18 s⁻¹, and L108A mutant β assembled on DNA at 26 s⁻¹. In the second experiment, in which γ complex was preincubated with β and then added to p/t DNA (Scheme 2), wt β assembled on DNA at 85 s⁻¹, L273A mutant β assembled on DNA at 114 s⁻¹, and L108A mutant β assembled on DNA at 79 s⁻¹ (Fig. 5, A–C). The slight difference in assembly rates may result from the difference in dimer interfaces of the mutant β proteins compared with wt β. Presumably, a weaker interface between the mutant monomers aids rapid assembly of the clamps on DNA.

A striking difference between wt β and the mutant β clamps was in the anisotropy values obtained under pre-steady state association conditions. In experiments using wt β in which a preincubated solution of β and γ complex was added to p/t DNA (Scheme 2), the higher final anisotropy values for mutants were of a magnitude similar to the transient overshoot in anisotropy observed in reactions with wt β. This transient overshoot is likely the result of an initial rapid binding of wt β and γ complex to p/t DNA, followed by dissociation of γ complex from the β-DNA complex. The disappearance of the overshoot for the mutants (Fig. 5) indicates that the γ complex is slower at releasing the β mutants onto p/t DNA than at releasing wt β onto p/t DNA.

The reason for slower ATP hydrolysis rates when the mutants were used became apparent when measurements of pre-steady state anisotropy experiments using M13 trap DNA were performed. The dissociation of wt β is 6 s⁻¹. The mean dissociation rates for the L273A and L108A mutant β are 0.2 and 0.5 s⁻¹, respectively (Fig. 6). Although the mean dissociation rates do not correlate well with ATP hydrolysis data, the fast steady state dissociation rate terms of 0.5 s⁻¹ (L273A mutant β) and 3 s⁻¹ (L108A mutant β) do (Fig. 6). The origin of the biexponential dissociation phase in the mutants, however, is not currently known. The fast phase dissociation rate for wt β is 12-fold faster than that for L273A mutant β, and accordingly, the γ complex-catalyzed ATP hydrolysis rate for wt β is 8-fold faster. Similarly, the fast phase dissociation rate for wt β is 2-fold higher than that for L108A mutant β, and the wt β ATP hydrolysis rate is 2-fold faster. The amplitudes associated with the fast dissociation phases are approximately 40–50%.

Further evidence coupling ATP hydrolysis with the dissociation of γ complex from the β-DNA aggregate was seen when ATPγS was used in place of ATP. It is difficult to hydrolyze the phosphorothioate bond of ATPγS, so when γ complex, β, and ATPγS were preincubated and then mixed with p/t DNA (Scheme 2), the overshoot previously seen when ATP was used disappeared. There is no decrease in anisotropy due to the complete block of ATP hydrolysis (Fig. 7A). In addition, when γ complex, β, and DNA were mixed with ATP and then chased with excess ATPγS, the overall anisotropy increased from 0.24 (β cycling) to 0.26 (β and γ complex locked) (Fig. 8A). These anisotropy results are consistent with results of gel shift assays from the accompanying report (26), which show that ATP binding is sufficient to form a γ complex-β-DNA structure but that ATP hydrolysis is required for γ complex to release β onto DNA completing the loading reaction (26).

A comparison of wt β and β interface mutants provides interesting insights into the γ complex-catalyzed loading reaction. Earlier work suggests that opening of the β ring is rate-limiting and occurs prior to the association of β and γ complex with DNA. Our work has shown that mutations to the dimer interface had little effect on the rates and efficiency of association of β and γ complex with DNA. In contrast, the interface mutations had large effects on the rates of dissociation of β from DNA and on steady state rates of ATP hydrolysis by γ complex. Decreases in these rates were most likely due to decreases in the rates in which γ complex released the β mutants onto DNA. Perhaps mutations to the dimer interface influence the efficiency with which the γ complex is able to close the β dimer and clamp it on DNA. Thus, the dimer interface mutations may hinder the completion of the loading reaction rather than having an influence on the initial steps of the loading reaction, which require opening of the dimer interface.

These results suggest a model for the γ complex-catalyzed assembly of β on p/t DNA. First, γ complex binds ATP and goes through a conformational change. The reaction then proceeds by either of two pathways. The γ complex binds β and then binds p/t DNA, or it can bind p/t DNA and then β. We favor the former pathway, based on the observation that load-
ing \( \beta \) onto DNA is 6-fold more rapid when \( \beta \) and \( \gamma \) complex are preincubated in the absence of DNA than when \( \gamma \) complex is added to a solution containing \( \beta \) and DNA (Fig. 5A). In either case, ATP binding leads to the formation of a stable composite that contains \( \gamma \) complex, \( \beta \), and DNA. When the three components are assembled, the \( \gamma \) complex hydrolyzes ATP and dissociates from the \( \beta \)-DNA complex, leaving \( \beta \) free to translocate along the DNA until it slides off the end of the short linear DNA molecule. The \( \gamma \) complex releases the ATP hydrolysis products, \( \text{ADP} + \text{Pi} \), and cycles back to assemble another \( \beta \) clamp on p/t DNA. A "minimal" model depicting this sequence of events is shown in Fig. 9.

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