Crystal structures of an *Escherichia coli* clamp loader have provided insight into the mechanism by which this molecular machine assembles ring-shaped sliding clamps onto DNA. The contributions made to the clamp loading reaction by two subunits, χ and ψ, which are not present in the crystal structures, were determined by measuring the activities of three forms of the clamp loader, γδδ′, γδδ′ψ, and γδδ′ψχ. The ψ subunit is important for stabilizing an ATP-induced conformational state with high affinity for DNA, whereas the χ subunit does not contribute directly to clamp loading in our assays lacking single-stranded DNA-binding protein. The ψ subunit also increases the affinity of the clamp loader for the clamp in assays in which ATPγS is substituted for ATP. Interestingly, the affinity of the γδδ′ complex for β is no greater in the presence than in the absence of ATPγS. A role for ψ in stabilizing or promoting ATP- and ATPγS-induced conformational changes may explain why large conformational differences were not seen in γδδ′ structures with and without bound ATPγS. The β clamp partially compensates for the activity of ψ when this subunit is not present and possibly serves as a scaffold on which the clamp loader adopts the appropriate conformation for DNA binding and clamp loading. Results from our work and others suggest that the ψ subunit may introduce a temporal order to the clamp loading reaction in which clamp binding precedes DNA binding.

The efficiency of DNA replication is enhanced by processivity factors that enable a DNA polymerase to incorporate thousands of nucleotides in a single DNA-binding event. These processivity factors, a sliding clamp and a clamp loader, are conserved from bacteria to humans (recently reviewed in Refs. 4, 15, and 16). Clamp loaders catalyze the assembly of sliding clamps on DNA at rates limited by the rate of nucleotide incorporation (6, 7). Clamps slide freely along duplex DNA so that a DNA polymerase bound to a clamp is tethered to the template being copied yet able to move along DNA at rates limited by the rate of nucleotide incorporation (6, 7). Clamp loaders catalyze the assembly of sliding clamps on DNA. The complete clamp loader at the replication fork (reviewed in Refs. 4, 15, and 16).

The functional core of clamp loaders is composed of five subunits that are members of the AAA+ family of ATPases (ATPases associated with diverse cellular activities; for recent reviews see Refs. 17 and 18). The χ subunit, and one copy each of the δ and δ′ subunits make up the core of the *E. coli* clamp loader. This 5-polypeptide complex (γmin) is the minimal assembly of subunits with clamp loading activity (19–21). A common feature of AAA+ ATPases is that subunits are arranged in a ring in which ATP binding sites located at the interface of adjacent subunits allow dynamic coupling of ATP binding and hydrolysis to conformational changes in the complex. Each of the γmin subunits is composed of three domains, and a ring is formed by a tight interaction of the C-terminal domains (22, 23). Although the two N-terminal domains of each subunit share homology with AAA+ proteins (22, 24, 25), only the γ subunits bind and hydrolyze ATP. A conserved SRC sequence motif, containing an “Arg finger,” is present in the δ′ and γ subunits of the *E. coli* clamp loader. Subunit-subunit communication of ATP binding and hydrolysis is mediated by these Arg fingers that extend from one subunit toward the ATP site in the neighboring subunit (see Fig. 1). Interaction of an Arg finger with the γ-phosphate of ATP is believed to serve both sensing and catalytic functions, and biochemical studies have shown that ATP-dependent clamp and DNA binding, in addition to ATP hydrolysis, are reduced in Arg finger mutants (26–28).

The physical process of clamp loading requires that interactions between the clamp loader and the clamp, and between the clamp loader and DNA, be modulated in a defined temporal order. This affinity modulation is accomplished by ATP binding and hydrolysis sensed by Arg fingers and coupled to confor-

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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2 The abbreviations used are: AAA+, ATPases associated with diverse cellular activities; β-PY, pyrene-labeled β; DCC, 7-diethylaminocoumarin-3-carboxylic acid succinimidyl ester; γmin, γmin subunits; γcore, γ subunits; γanom, γ subunits; γactive, γ active subunits; γinactive, γ inactive subunits; γhetero, γ subunits of the opposite species; γcom, clamp loader containing γδδ′, γδδ′ψ, and γδδ′ψχ subunits. The γ subunits were obtained from 10 different donor DNA polymerases: DNA, N-2(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide; pT-DNA, primed template DNA; RHP, Rho; PBP, phosphate-binding protein; BSA, bovine serum albumin; ATPγS, adenosine 5′-O-(3-thiotriophosphate).
A Role for ψ in Clamp Loading

FIGURE 1. Schematic diagrams of three forms of the E. coli clamp loader. Left, five subunits, three copies of δ and one copy each of δ′ and ψ, make up the core of the E. coli clamp loader (γmin) and are arranged in a ring via tight interaction of their C-terminal domains. Each γ subunit contains an ATP binding site located at the interface of two subunits; the ATP site of γi is located at the δ′-γi interface, γ2 at the γ1-γ2 interface, and γ3 at the γ2-γ3 interface. Subunits adjacent to an ATP site, δ′ and γi, contain a conserved Arg finger (indicated by the curved arrow) that extends from one subunit to the ATP site of the next. The δ subunit does not contain an ATP binding site, and the Arg finger of γi interacts with specific amino acid residues on δ′. Two additional subunits, χ and ψ, are present in the native clamp loader. Center, the ψ subunit joins the minimal complex (γmin) via interactions with a γ subunit to form a six-subunit complex (γψψmin). Right, the χ subunit binds the ψ subunit to form a seven-subunit clamp loader (γψψψψψmin).

Experimental Procedures

Nucleotides and Oligonucleotides—Concentrations of ATP (Amersham Biosciences/GE Healthcare) and ATPγS (Roche Applied Science) were determined by measuring the absorbance at 259 nm and using an extinction coefficient of 15,400 M⁻¹ cm⁻¹. [α-³²P]ATP (10 mCi/ml) was purchased from Amersham Biosciences/GE Healthcare. Synthetic oligonucleotides were purchased from Integrated DNA Technologies (Corvalle, IA) and purified by either 12% or 8% denaturing PAGE for the 30-mer or 105-mer, respectively (32). The sequence of the 105-nucleotide template (t1) is 5′-CTG TGC CAC GTA TTC TTA CGC TTT CAG GTC AGA AGG GTT CTA TCT CTG TGG GCC AGA ATC TCC CTT TTA TTA CGT GTC GTG TGA CTG GTG AAT CTG CCA ATG TAA-3′. For anisotropy experiments, an amino linker (5′ Amino Modifier C6) was incorporated at the 5′-end of the 105-mer. This amino linker was covalently labeled with X-rhodamine isothiocyanate (Molecular Probes, Eugene, OR) (42). The sequence of the 30-nucleotide primer p1 is 5′-ACG ACC AGT AAT AAA AGG GAC ATT CTG GCC-3′, and primer p2 is 5′-ACA CGA CCA GTA ATA AAA GGG ACA TTC (C6dT)GG-3′ in which C6dT is a T with a C₆ amino linker at the 5 position. Both primers are complementary to template t1 and anneal to overlapping sites that differ in position by two nucleotides. Annealing of p1 to t1 creates a 50-nt 5′ template overhang, and annealing of p2 to t1 creates a 52-nt 5′ template overhang. Primer p2 was covalently labeled with 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester (DCC, Molecular Probes) as described for X-rhodamine (42). Primed-template DNA was annealed by mixing primer and template in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, heating to 80 °C, and slowly cooling to room temperature. Annealing reactions contained the following molar ratios of primer/template: 1.0/1.1 for p2-DCC/t1, and 1.2/1.0 for p1/t1-RhX and unlabeled p1/t1. Duplex DNA was used without further purification.

Buffers—Assay buffer consisted of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 8 mM MgCl₂. Protein storage buffer consisted of 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and 10% glycerol.

DNA Polymerase III Proteins—DNA polymerase III proteins were purified, and the clamp loader complexes were reconstituted as described previously (5, 9). Protein concentrations were determined by measuring the absorbance at 280 nm in 6 M guanidine hydrochloride and using calculated extinction coefficients (γcom = 17,900 M⁻¹ cm⁻¹, γpsi = 14,890 M⁻¹ cm⁻¹, γmin = 166,850 M⁻¹ cm⁻¹, β = 14,890 M⁻¹ cm⁻¹). Covalent modification of β on a Cys residue with N-(1-pyrene)maleimide (Molecular Probes) was performed as described (28, 43). The protein concentration of β-PY was determined from the absorbance at 280 nm under nondenaturing conditions after subtracting the contribution due to pyrene absorbance. The pyrene contribution was calculated from the absorbance at 344 nm multiplied by a factor of 0.806. The native extinction coefficient (44) for β is 17,900 M⁻¹ cm⁻¹.

Equilibrium β Binding Assays—β-PY anisotropy measurements were made using a QuantaMasterQM-1 fluorometer (Photon Technology International) as described (28). Samples were excited at 345 nm, and emission was measured at 375 nm with excitation and emission slits set at a 5 nm bandpass. Equilibrium DNA Binding Assays—Anisotropy of RhX in p1/t1-DNA-RhX was measured as described previously using a T-format (32). RhX was excited at 580 nm, and emission was measured at 610 nm using a 6 nm bandpass on both excitation and emission channels. Fluorescence emission spectra of DCC (p2-DCC/t1) were measured using a QuantaMasterQM-1 fluorometer (Photon Technology International) by exciting the sample at 435 nm and measuring the emission from 450 to 550 nm.
Pre-steady-state DNA Binding Assays—Pre-steady-state DCC fluorescence quenching assays were performed at \( \approx 20^\circ C \) using an Applied Photophysics SX18MV stopped-flow apparatus configured for sequential mixing and equipped with an excitation monochromator set at 435 nm and a 475 nm cut-on filter in the path of the emission PMT. A solution of clamp loader from one syringe was mixed with a solution of ATP in the presence or absence of \( \beta \) from a second syringe, aged for 1 s, and added to a solution of p2-DCC/t1 from a third syringe. Data were collected for a total of 2 s in intervals of 2 ms.

E. coli Phosphate-binding Protein and Pre-steady-state ATPase Assays—PBP was purified and covalently labeled with \( N-(2-(1\text{-maleimidyl})\text{ethyl})-7-(\text{diethylamino})\text{coumarin-3-carboxamide} \) (MDCC, Molecular Probes) as described (45). PBP-MDCC-based ATPase assays were performed as described previously (45) using an Applied Photophysics SX18MV stopped-flow apparatus configured for sequential mixing. MDCC was excited at 425 nm, and emission was measured using a 455 nm cut-on filter. In standard sequential mixing experiments, clamp loaders were preincubated with ATP (\( \geq \beta \)) for 1 s prior to adding p1/t1 and PBP-MDCC. In Fig. 8, the time that clamp loaders were preincubated with ATP was varied as indicated.

Single-turnover ATPase Assays—The \( ^{32}\text{P}\)-based ATPase assays were performed at room temperature (\( \approx 24^\circ C \)) by preincubating clamp loader with radiolabeled ATP and \( \beta \) for 5 s prior to initiating hydrolysis by addition of p1/t1 and ATPyS-chase. Final reactions were incubated for 5 s prior to quenching in 95% formamide, 20 mM EDTA. ADP product was then separated from ATP by TLC using polyethyleneimine cellulose F TLC plates (Merck) developed in 0.5 M LiCl and quantitated using a Storm PhosphorImager and ImageQuaNT software (Amersham Biosciences).

RESULTS

Binding of the Three Clamp Loaders to the \( \beta \)-Clamp—The goals of this experiment were 1) to assess the contributions of the \( \chi \) and/or \( \psi \) subunits to clamp loader-clamp binding and 2) to provide a measure of the specific activities of the three clamp loader preparations (i.e. \( \beta \)-binding units per mole of complex). A solution-based fluorescence anisotropy assay in which \( \beta \) was covalently labeled with pyrene (PY) was used to measure the formation of clamp loader-\( \beta \)-PY complexes (28). Thiol-reactive probes, including pyrenylmaleimide selectively label Cys-333 (43), which is located on the face of \( \beta \) opposite to that which the clamp loader binds (5, 46). Labeled \( \beta \) is active in replication assays done in our laboratory3 and by others (43, 47). Therefore, the assumption is that PY does not affect interactions with the clamp loader, and this assumption is supported by agreement between \( K_d \) values measured in this experiment and in other work (29). Binding assays were done by titrating a fixed concentration of \( \beta \)-PY with increasing concentrations of clamp loader. Because the anisotropy of \( \beta \) is greater for the larger clamp loader-clamp complexes than for free pyrene-labeled \( \beta \), the observed anisotropy increases as the fraction of \( \beta \)-PY bound increases. Assays contained ATP and were done under stoichiometric binding conditions in which the concentration of \( \beta \)-PY was large (100 nM) relative to the \( K_d \) for Py binding (3 nM (29)).

Anisotropy values are plotted as a function of clamp loader concentration in Fig. 2 (a–c). Given that one molecule of clamp loader binds each clamp, this 1:1 stoichiometry should be reflected in the binding isotherms if the clamp loader preparations were 100% active. Binding data from three independent titrations were globally fit to a quadratic equation (Equation 1),

\[
\frac{K_d + a \gamma + \beta - \sqrt{(K_d + a \gamma + \beta)^2 - 4a \gamma \beta}}{2\beta} (r_b - r_f) + r_f
\]

(Eq. 1)

to calculate dissociation constants (\( K_d \)) and the fractions of active clamp loader (\( a \)) based on total concentrations of clamp loader (\( \gamma \)) and \( \beta \)-PY (\( \beta \)). Anisotropies for free (\( r_f \)) and bound (\( r_b \)) \( \beta \)-PY were also treated as adjustable parameters and globally fit. Calculated dissociation constants were fit to the low nanomolar range (Fig. 2) for all three complexes demonstrating that the \( \chi \) and \( \psi \) subunits are not required for high affinity binding to \( \beta \) in assays containing ATP. For each clamp loader preparation, saturation in \( \beta \)-PY binding was observed at a ratio greater than one clamp loader per clamp. This result was interpreted as reflecting a population of inactive clamp loader that contributes to the total protein concentration. To quantitatively compare the activities of the three clamp loaders, the concentration of active clamp loader, as determined in this \( \beta \)-binding assay, was used in all subsequent experiments. We recognize that the \( \beta \)-binding activity is not necessarily equivalent to other activities such as ATP hydrolysis, however, this standardization of concentrations gave consistent results from preparation to preparation, and our major conclusions do not depend on this normalization.

Structural data for \( \gamma_{\text{min}} \), containing bound ATPyS suggested that productive binding to the \( \beta \)-clamp would not be possible given the conformation of the clamp loader (23). To determine whether this observation would be reflected in solution, \( \beta \)-PY was titrated with the three clamp loaders in assays containing ATPyS (Fig. 2d). Binding of \( \gamma_{\text{min}} \) to \( \beta \)-PY was reduced by \( \approx 100\) fold in assays containing ATPyS compared with ATP. Notably, the affinity of \( \gamma_{\text{min}} \) for \( \beta \) in assays with ATPyS (\( K_d = 103 \pm 24 \text{ nM} \)) was not significantly greater than those for \( \gamma_{\text{com}} \) (45) and \( \gamma_{\text{min}} \) (48) binding \( \beta \)-PY in the absence of nucleotide (\( K_d \approx 150 – 200 \text{ nM} \)). In other words, an ATP-bound conformational state with high affinity for the clamp was not formed by \( \gamma_{\text{min}} \) to a large extent in assays with ATPyS. The affinities of \( \gamma_{\psi} \) and \( \gamma_{\text{com}} \) for \( \beta \) (\( K_d \) values of \( 13 \pm 4 \text{ nM} \) and \( 22 \pm 6 \text{ nM} \), respectively) were decreased by a factor of \( \approx 10 \) (Fig. 2d) showing that the presence of the \( \psi \) subunit reduces the effect of substituting ATPyS for ATP on \( \beta \) binding activity.

Equilibrium DNA-binding Activities of the Three Clamp Loaders—A second fluorescence anisotropy-based assay was used to determine whether the \( \chi \) and/or \( \psi \) subunits contribute to the DNA-binding activity of the E. coli clamp loader. Primed template DNA was covalently labeled with X-rhodamine (RhX) on the 5'-template end. The anisotropy of RhX increases when

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A Role for $\psi$ in Clamp Loading

$\gamma_{com}$ binds p/t-DNA-RhX (42). Solutions of p/t-DNA-RhX, clamp loader, and the $\beta$-clamp were sequentially added to a cuvette containing a solution of ATP$\gamma$S, and polarized intensities were measured after each addition (Fig. 3). ATP$\gamma$S was added instead of ATP to measure DNA binding in the absence of appreciable DNA-dependent ATP hydrolysis. Anisotropy values were calculated for free p/t-DNA-RhX and for clamp loaders binding p/t-DNA-RhX in the absence and presence of $\beta$. Addition of $\gamma_{min}$ to p/t-DNA-RhX (Fig. 3a, black versus striped bars) had very little affect on the anisotropy of the probe, whereas addition of $\gamma_{pal}$ (Fig. 3b, black versus striped bars) or $\gamma_{com}$ (Fig. 3c, black versus striped bars) increased the anisotropy in a concentration-dependent manner. Weaker p/t-DNA binding by $\gamma_{min}$ compared with $\gamma_{pal}$ and $\gamma_{com}$ suggests a role for the $\psi$ subunit in DNA binding. The $\psi$ subunit may bind DNA directly, or alternatively, act indirectly by stabilizing a conformational state of the clamp loader with high affinity for DNA. DNA binding was not observed in assays with a $\chi$-$\psi$ heterodimer (supplemental Fig. S1) suggesting that $\psi$ may act indirectly to stimulate DNA binding of the clamp loader.

Addition of $\beta$ to clamp loader and p/t-DNA-RhX solutions (Fig. 3, striped versus gray bars) increased the anisotropy relative to solutions containing the clamp loader and DNA alone. Given that the clamp does not independently bind p/t-DNA (42), these anisotropies increases most likely result from a greater affinity of the clamp loader-clamp complexes for DNA. These results show that $\beta$ restores DNA-binding activity to $\gamma_{min}$ suggesting that the clamp can compensate for an indirect role of $\psi$ in stabilizing a conformational state of the clamp loader with high affinity for DNA.

A New Assay to Measure Pre-steady-state DNA Binding and Clamp Loading—One limitation of the anisotropy-based DNA binding assay is that the probe reports on binding at any site. Therefore, a new assay was developed that reports on clamp loaders binding specifically at primer/template junctions where clamps are loaded. A T located three nucleotides from the primer 3'-end

\begin{align*}
\frac{\text{anisotropy}}{\text{clamp loader concentration}} & = \frac{K_{d}}{Y_{\text{min}}}, \\
Y_{\text{com}} & = 0.7 \text{ nM}, \\
K_{d} & = 0.4 \text{ nM}, \\
Y_{\text{pal}} & = 0.02, \\
Y_{\text{com}} & = 0.59.
\end{align*}

\begin{align*}
K_{d} & = 0.9 \text{ nM,} \\
\gamma_{\text{com}} & = 0.58, \\
\gamma_{\text{pal}} & = 0.58, \\
\gamma_{\text{com}} & = 0.48.
\end{align*}

\begin{align*}
K_{d} & = 0.7 \text{ nM,} \\
\gamma_{\text{com}} & = 0.59, \\
\gamma_{\text{pal}} & = 0.59, \\
\gamma_{\text{com}} & = 0.59.
\end{align*}

\begin{align*}
K_{d} & = 0.4 \text{ nM,} \\
\gamma_{\text{com}} & = 0.58, \\
\gamma_{\text{pal}} & = 0.58, \\
\gamma_{\text{com}} & = 0.48.
\end{align*}

\begin{align*}
K_{d} & = 0.9 \text{ nM,} \\
\gamma_{\text{com}} & = 0.58, \\
\gamma_{\text{pal}} & = 0.58, \\
\gamma_{\text{com}} & = 0.48.
\end{align*}

\begin{align*}
K_{d} & = 0.7 \text{ nM,} \\
\gamma_{\text{com}} & = 0.59, \\
\gamma_{\text{pal}} & = 0.59, \\
\gamma_{\text{com}} & = 0.59.
\end{align*}
fluorescence differently. We favor the first interpretation, because anisotropy experiments in Fig. 3 that report on a different physical property of the protein-DNA complex also indicated DNA binding by $\gamma_{\text{min}}$ was weaker. Emission intensities at 475 nm were 86%, 62%, and 58% of that for free p/t-DNA-DCC in assays with $\gamma_{\text{min}}$, $\gamma_{\text{psi}}$, and $\gamma_{\text{com}}$, respectively.

Binding of a clamp loader-ATP$\gamma$S-clamp complex to p/t-DNA-DCC had a different effect on fluorescence than the clamp loader alone; the quench in DCC fluorescence was not as large, but the blue shift in the emission maximum increased (from 472 to 469 nm). In the presence of $\gamma_{\text{com}}$, the quench in DCC fluorescence was similar for each clamp loader, 69%, 71%, and 70% for $\gamma_{\text{min}}$, $\gamma_{\text{psi}}$, and $\gamma_{\text{com}}$, respectively, of the free p/t-DNA-DCC intensity. This result is consistent with that of the p/t-DNA-RhX binding assay indicating that $\beta$ increased binding of $\gamma_{\text{min}}$ to p/t-DNA.

In assays with ATP, the clamp loader binds p/t-DNA transiently due to ATP hydrolysis (32, 33), and therefore, binding cannot be measured in steady-state experiments (supplemental Fig. S2). Instead, binding of clamp loaders to p/t-DNA-DCC was measured in real time in assays with ATP (Fig. 5) to determine if weaker binding of $\gamma_{\text{min}}$ to DNA was a function of the nucleotide, ATP$\gamma$S. Clamp loaders were preincubated with ATP for 1 s to charge the clamp loader with ATP before reactions were initiated by addition of p/t-DNA-DCC. Addition of $\gamma_{\text{com}}$ to p/t-DNA-DCC (Fig. 5a, no $\beta$) produced a transient decrease in fluorescence due to DNA binding that was considerably smaller than reactions containing $\gamma_{\text{psi}}$ (Fig. 5b, no $\beta$) and $\gamma_{\text{com}}$ (Fig. 5c, no $\beta$). These results are interpreted as reflecting a defect in the ATP-dependent DNA-binding activity of $\gamma_{\text{min}}$ that is corrected by the presence of the $\psi$ subunit in $\gamma_{\text{psi}}$ and $\gamma_{\text{com}}$ reactions.
Analogous assays were done in the presence of the β clamp. In these reactions, clamp loader-clamp complexes bind DNA, and clamps are released on DNA as the clamp loader dissociates. A much larger decrease in fluorescence occurred in γ\textsubscript{min} reactions with β (Fig. 5a, + β) than without β indicating that β increases DNA-binding activity. The transient decrease in fluorescence and the total quench in fluorescence at steady-state (after ~1 s) was smaller for γ\textsubscript{min} than either γ\textsubscript{psl} (Fig. 5b, + β) or γ\textsubscript{com} (Fig. 5c, + β) indicating that the overall clamp loading reaction cycle was less efficient for γ\textsubscript{min}.

**Kinetics of ATP Hydrolysis**—Given that p/t-DNA binding triggers ATP hydrolysis, a lower affinity of γ\textsubscript{min} for DNA should be reflected in the kinetics of ATP hydrolysis. Fluorescence-labeled *E. coli* phosphate-binding protein (PBP-MDCC) was used to measure inorganic phosphate (P\textsubscript{i}) released from the clamp loaders on hydrolysis of ATP (49, 50). The fluorescence of MDCC increases when PBP-MDCC binds P\textsubscript{i}. Clamp loaders were preincubated with ATP for 1 s prior to rapid addition of p/t-DNA and PBP-MDCC (Fig. 6a). As seen previously for γ\textsubscript{com} (45), both γ\textsubscript{psl} (black trace) and γ\textsubscript{com} (light gray trace) produced a rapid increase in MDCC fluorescence resulting from a rapid burst of ATP hydrolysis followed by a slower increase due to slower hydrolysis. In contrast, little or no burst of P\textsubscript{i} product was seen in the reaction with γ\textsubscript{min} (dark gray trace); only a slow increase in MDCC fluorescence occurred. The time course for ATP hydrolysis by γ\textsubscript{min} was the same at concentrations of 200, 500, and 1000 μM ATP demonstrating that γ\textsubscript{min} was saturated with ATP in these reactions (supplemental Fig. S3a). In principle, the lack of a burst could be due to slow release of P\textsubscript{i} by γ\textsubscript{min} (P\textsubscript{i} must physically dissociate from the clamp loader before binding to PBP-MDCC). However, this defect in DNA-triggered ATP hydrolysis is consistent with a reduced affinity of γ\textsubscript{min} for DNA and instead suggests that the lack of a burst is due to poor DNA binding.

DNA binding assays showed that formation of a γ\textsubscript{min}-β complex increased the affinity of γ\textsubscript{min} for DNA, and this compensatory effect should also be reflected in the kinetics of ATP hydrolysis. Clamp loaders were preincubated with ATP and β for 1 s to form a clamp loader-β complex prior to the addition of p/t-DNA and PBP-MDCC (Fig. 6b). The burst amplitudes of P\textsubscript{i} release by γ\textsubscript{psl} (black trace) and γ\textsubscript{com} (light gray trace) increased in reactions with β relative to those without β, but burst rates were unchanged, as shown previously for γ\textsubscript{com} (45). Significantly, the γ\textsubscript{min} reaction (dark gray trace) produced a burst of P\textsubscript{i} release that was not present in the absence of β, and the burst rates for all three clamp loaders were the same. This result is consistent with the idea that β can compensate for the missing ψ subunit to increase the affinity of γ\textsubscript{min} for DNA. One notable difference in the ATPase reactions with β is that the amplitude of the γ\textsubscript{min} burst is ~70% of those for the γ\textsubscript{com} and γ\textsubscript{psl} reactions. Higher DNA concentrations did not significantly increase the burst amplitude (supplemental Fig. S3b), and a smaller amplitude for γ\textsubscript{min} compared with γ\textsubscript{com} and γ\textsubscript{psl} reac-
solutions is not an artifact of standardizing protein concentrations to β-binding activities (supplemental Fig. S4).

Number of Molecules of ATP Hydrolyzed by γ<sub>com</sub>—The burst amplitude of the γ<sub>com</sub> reaction indicates that 2.5–3 molecules of P<sub>i</sub> were produced per clamp loader. To more firmly establish this number, the ADP product was measured in single turnover hydrolysis assays as a function of γ<sub>com</sub> concentration. The clamp loader was preincubated with [α<sup>−32</sup>]<sub>P</sub>ATP and β for 5 s before adding a solution of p/t-DNA and ATP<sub>S</sub> as a chase. Excess ATPγS limits hydrolysis of ATP to a single turnover, and ATP is hydrolyzed faster than exchanged for ATPγS (45). The occurrence with preincubation up to 494 ms (Fig. 8a). Rates of ATP hydrolysis by γ<sub>psl</sub> (Fig. 8b) and γ<sub>com</sub> (Fig. 8c) increased with preincubation times from 13 to 247 ms in a similar fashion and approached a maximum by 497 ms. Thus, for all three clamp loaders the 1-s preincubation period used in ATPase assays was sufficient to allow conformational transitions to occur.

**DISCUSSION**

ψ Subunit Stabilizes an ATP-activated Conformation of the Clamp Loader—A comparison of β-binding, DNA-binding, clamp loading, and ATPase activities of three forms of the
E. coli clamp loader, $\gamma_{\text{min}}$, $\gamma_{\text{psi}}$, and $\gamma_{\text{com}}$, revealed defects in the activities of $\gamma_{\text{min}}$. The most significant was a deficiency in DNA binding, which was also reflected in the DNA-dependent ATPase activity. Addition of the $\psi$ subunit alone, to form $\gamma_{\text{psi}}$, restored DNA binding and ATPase activities to levels that were comparable to those of $\gamma_{\text{com}}$. This study is the first to uncover a direct function for the $\psi$ subunit in clamp loading and, based on the activities measured, suggests that the $\chi$ subunit does not play a direct role in loading clamps on DNA in the absence of single-stranded DNA-binding protein (38, 39). The mechanism by which $\psi$ increases the affinity of $\gamma_{\text{min}}$ for DNA is most likely by stabilizing an ATP-induced conformational state with high affinity for DNA rather than by providing a direct $\psi$-DNA binding interaction. Interestingly, the clamp increased the DNA-binding and DNA-triggered ATPase activities of $\gamma_{\text{min}}$, suggesting that $\psi$ aids in stabilization of a conformation with high affinity for DNA. Perhaps the clamp serves as a scaffold that stabilizes the appropriate conformation (Fig. 9).

A general feature of AAA+ ATPases, including clamp loaders, is that ATP binding and hydrolysis promote conformational changes that alter the affinity of these complexes for their targets. Surprisingly, no large differences in the conformation of $\gamma_{\text{min}}$ were observed in crystal structures with (23) and without (22) bound ATP$\gamma$S, and modeling studies suggested that $\gamma_{\text{min}}$ could not productively bind $\beta$ in this conformation due to steric clashes. Our solution-based measurements (Fig. 2d) confirmed that ATP$\gamma$S did not promote high affinity binding of $\gamma_{\text{min}}$ to $\beta$. If either ATP or the $\psi$ subunit is present, the affinity of $\gamma_{\text{min}}$ for $\beta$ increases. Results from these experiments support two conclusions: 1) ATP promotes a conformational change in $\gamma_{\text{min}}$ that does not occur to a great extent with ATP$\gamma$S or no nucleotide, and 2) the $\psi$ subunit aids in either the formation or stabilization of a conformational state with increased affinity for $\beta$ in the presence of the nucleotide analog, ATP$\gamma$S.

The $\psi$ Subunit May Introduce a Temporal Order to ATP Binding—The differences in the activities of $\gamma_{\text{min}}$ and $\gamma_{\text{com}}$ may arise from differences in ATP binding. In the absence of the clamp, $\gamma_{\text{com}}$ binds three molecules of ATP (26) whereas $\gamma_{\text{min}}$ binds two (23). Structural studies (23) showed that the N-terminal domain of $\gamma_1$ physically blocks nucleotide entry into the binding site of $\gamma_2$, so that the two molecules of ATP$\gamma$S were bound by the $\gamma_1$ and $\gamma_2$ subunits (see Fig. 1). The $\psi$ subunit may increase the DNA-binding activity of the clamp loader by facilitating binding of a third molecule of ATP to the $\gamma_2$ site. One possible mechanism would be for $\psi$ to stabilize a conformation in which the ATP site in $\gamma_{\text{min}}$ is “constitutively” open, as are the other sites prior to binding ATP, and allow ATP to fill all three sites. If this were the case, then a conformational state with a “closed” $\gamma_2$ site would be unique to $\gamma_{\text{min}}$, and not necessarily occur during the normal cycle of ATP binding by $\gamma_{\text{com}}$. An alternate possibility is that $\psi$ facilitates a conformational change that occurs after ATP binds the $\gamma_1$ and $\gamma_2$ sites to open the third $\gamma_2$ site to ATP. If this latter possibility were true, then the conformational state with a closed ATP binding site in the $\gamma_2$ subunit represents an intermediate state that occurs in filling the ATP sites in all three forms of the clamp loader. This latter possibility is interesting in that it could provide a mechanism for binding the clamp and DNA in a defined temporal order.

The clamp loader could adopt a conformation with high affinity for the clamp after binding ATP in one or both of the first two sites and then subsequently adopt a conformation with high affinity for DNA after filling the third site.

In the case of $\gamma_{\text{min}}$, the $\beta$ clamp could compensate for the missing $\psi$ subunit by facilitating the opening of the third ATP site allowing ATP to bind as suggested by Kazmirska et al. (23). Although this is possible, our ATP hydrolysis assays did not provide evidence to support this model. Quantitative measurements showed that $\gamma_{\text{com}}$ hydrolyzed three molecules of ATP per molecule of complex in the presence of the clamp. Relative to $\gamma_{\text{com}}$, the burst amplitude indicated that $\gamma_{\text{min}}$ hydrolyzed ~70% as much ATP in the presence of $\beta$. One possible interpretation of this result is that the clamp does not promote opening of the third site and $\gamma_{\text{min}}$ binds and hydrolyzes only two molecules of ATP. However, in the case of an enzyme complex with multiple active sites, it is difficult to distinguish between a fraction of sites that is inactive and a fraction of complex that is inactive. Thus, it is also possible that $\beta$ facilitates hydrolysis of three molecules of ATP in a fraction of $\gamma_{\text{min}}$ present, but some $\gamma_{\text{min}}$ complexes are inactive. Because our assay measures $P_i$.
released from the complex, it is also formally possible that three molecules of ATP may be hydrolyzed per $\gamma_{\text{min}}$, but a fraction of sites release Pi slowly.

**ATP Site-specific Regulation of $\beta$ and DNA Binding**—Another interesting outcome of the characterization of the activities of $\gamma_{\text{min}}$ was that the ATP-dependent $\gamma$- and DNA-binding activities were uncoupled. In assays with ATP, $\gamma_{\text{min}}$ had the same affinity as $\gamma_{\text{com}}$ and $\gamma_{\text{psi}}$ for $\gamma$ but had a significantly reduced affinity for p/t-DNA. A similar uncoupling of ATP-dependent $\gamma$- and DNA-binding activities occurs in Arg finger mutants of $\gamma_{\text{com}}$ (28) and replication factor C (27). A model is proposed in Fig. 10 that illustrates how removing the subunit or Arg fingers affects conformational changes to differentially affect $\gamma$ and DNA binding. Key to this model are local conformational changes between pairs of subunits that occur when ATP binding at one site is “sensed” by an Arg finger of the adjacent subunit. Mutations that alter ATP binding and/or sensing bound ATP have different effects depending on the subunit pair; mutation of one pair largely affects DNA binding, and another affects $\beta$ binding.

In $\gamma_{\text{com}}$, ATP bound to each $\gamma$ subunit is sensed by an Arg finger, and each interaction promotes a local conformational change between subunit pairs. The global change in the entire complex creates a conformational state with a high affinity for both $\beta$ and DNA. The exact nature of this state is not known.

**FIGURE 8.** Kinetics of an ATP-induced conformational change in the three clamp loaders. The PBP-MDCC-based ATPase assay was performed as in Fig. 6 except that the time that the clamp loaders were preincubated with ATP was varied. For each reaction trace, the length of the preincubation time is indicated by the shade of gray: darker gray indicates longer times. Preincubation times were 12, 44, 94, 245, and 494 ms for $\gamma_{\text{min}}$; 13, 47, 97, 247, and 497 ms for $\gamma_{\text{psi}}$, and 13, 46, 96, 246, and 495 ms for $\gamma_{\text{com}}$. The increase in MDCC fluorescence as a function of time after adding p/t-DNA is shown for $\gamma_{\text{min}}$ (a), $\gamma_{\text{psi}}$ (b), and $\gamma_{\text{com}}$ (c) in reactions containing 200 nM clamp loader, 500 nM DNA, 500 $\mu$M ATP, and 3 $\mu$M PBP-MDCC.

**FIGURE 9.** Model for clamp loader-clamp complexes binding DNA (51, 52). Each clamp loader subunit is composed of three domains indicated by three ovals. The five clamp loader subunits, three copies of $\gamma$, and one copy of $\delta$ and $\delta'$, are arranged in a ring formed by tight interaction of the C-terminal domains. The N-terminal domains are less tightly packed than the C termini and an opening exists on the N-terminal face of the complex, which most likely allows entry of duplex DNA at a primer 3’-end. The single-stranded template overhang then exits through a gap that exists between the N-terminal domains of $\delta$ and $\delta'$. The first domain of each clamp loader subunit interacts with the same face of the $\beta$-clamp. A spiraling conformation of clamp loader subunits relative to the DNA helical axis opens the clamp in an out-of-plane conformation so that the clamp spirals around the DNA helix.
but structural and modeling studies with other clamp loaders indicate that the N-terminal domains of the clamp loader subunits may be offset so that the clamp is opened in an out-of-plane conformation that spirals in the same manner as duplex DNA as illustrated in Fig. 9 (51, 52). Local conformational changes between $\gamma_1$ and $\gamma_2$ that occur when ATP binds $\gamma_2$ and is sensed by the Arg finger of $\gamma_1$ may be largely responsible for regulating DNA binding. In the case of $\gamma_{min}$, this local conformational change may not occur, because ATP does not bind $\gamma_2$, and thus DNA binding is weaker. Similarly, weaker DNA binding resulting from mutation of the Arg fingers in the $\gamma$ subunits ($\gamma_{com}$-$\gamma R169A$) may also be due to a defect in this local conformational change because ATP binding to $\gamma_2$ cannot be sensed by $\gamma_1$ (28). ATP binding and sensing between a different subunit pair, $\delta'$-$\gamma_1$, are also likely to regulate clamp binding. Although each clamp loader subunit likely contacts the $\beta$-clamp, high affinity binding is primarily mediated by a strong interaction between the $\delta$ subunit and a $\beta$ monomer (29). A change in the local $\delta'$-$\gamma_1$ conformation may affect the conformation of $\delta'$ relative to $\delta$ and facilitate $\beta$ binding. A mutation in the Arg finger of $\delta'$ ($\gamma_{com}$-$\delta' R158A$) that senses ATP at $\gamma_1$ reduces $\beta$-binding activity but not DNA binding (28). Substitution of ATP-$\gamma S$ for ATP in the $\gamma_1$ subunit of $\gamma_{min}$ may also affect the $\delta'$-$\gamma_1$ interaction by replacing the $\gamma$-phosphate, which is sensed by the Arg finger, with a $\gamma$-thiophosphate.

ATP-site-specific regulation of target binding may be a common feature of clamp loaders. Arg finger mutations in analogous subunit pairs in replication factor C have similar effects on clamp and DNA binding as for $\gamma_{com}$ (27). Although replication factor C only contains five subunits, an additional C-terminal extension present on the large subunit may function as $\psi$ does in $\gamma_{com}$ (35). This ATP-site-specific regulation of $\beta$ and DNA binding combined with ordered filling of ATP sites, as discussed above, would make temporal regulation of binding during the clamp loading reaction possible. If the $\gamma_2$ site is initially in a closed conformation, this would allow the $\gamma_1$ site to bind ATP prior to the $\gamma_2$ site and allow $\gamma_{com}$ to bind $\beta$ prior to DNA. After filling the $\gamma_1$ and $\gamma_2$ site(s), the $\psi$ subunit could act as a switch to open the $\gamma_2$ site, and ATP binding would then promote DNA binding. These results raise the possibility that the $\psi$ subunit, or $\psi$-like component in the eukaryotic clamp loader, may aid in defining the temporal order of events in the clamp loading reaction by facilitating the ordered binding of ATP to promote clamp binding prior to DNA binding.

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DNA: Replication, Repair, and Recombination: A Function for the ψ Subunit in Loading the *Escherichia coli* DNA Polymerase Sliding Clamp

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