Critical Reviews in Biochemistry and Molecular Biology, 41:179–208, 2006

ISSN: 1040-9238 print / 1549-7798 online
DOI: 10.1080/10409230600648751

Taylor & Francis
Taylor & Francis Group

Dynamics of Loading the *Escherichia coli* DNA Polymerase Processivity Clamp

Linda B. Bloom
Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, USA

ABSTRACT Sliding clamps and clamp loaders are processivity factors required for efficient DNA replication. Sliding clamps are ring-shaped complexes that tether DNA polymerases to DNA to increase the processivity of synthesis. Clamp loaders assemble these ring-shaped clamps onto DNA in an ATP-dependent reaction. The overall process of clamp loading is dynamic in that protein–protein and protein–DNA interactions must actively change in a coordinated fashion to complete the mechanical clamp-loading reaction cycle. The clamp loader must initially have a high affinity for both the clamp and DNA to bring these macromolecules together, but then must release the clamp on DNA for synthesis to begin. Evidence is presented for a mechanism in which the clamp-loading reaction comprises a series of binding reactions to ATP, the clamp, DNA, and ADP, each of which promotes some change in the conformation of the clamp loader that alters interactions with the next component of the pathway. These changes in interactions must be rapid enough to allow the clamp loader to keep pace with replication fork movement. This review focuses on the measurement of dynamic and transient interactions required to assemble the *Escherichia coli* sliding clamp on DNA.

KEYWORDS DNA replication, processivity clamp loader, AAA+ ATPase, protein–DNA interactions, protein–protein interactions

INTRODUCTION

The *Escherichia coli* sliding clamp and clamp loader were first identified as factors that enhanced the processivity of DNA synthesis (Bonner *et al.*, 1992; Fay *et al.*, 1981; Fay *et al.*, 1982; Hurwitz and Wickner, 1974; Maki and Kornberg, 1988; O'Donnell, 1987; Wickner, 1976; Wickner and Hurwitz, 1974). Further investigations revealed that one of these processivity factors is a ring-shaped protein complex that functions as a sliding clamp to encircle DNA and tether a DNA polymerase to the template being copied (Kong *et al.*, 1992; Stukenberg *et al.*, 1991). In the absence of clamps, DNA synthesis is relatively inefficient, because polymerases frequently dissociate from DNA templates. When bound to a sliding clamp, the processivity of a DNA polymerase typically increases from tens of nucleotides per DNA binding event to thousands of nucleotides

Address correspondence to Linda B. Bloom, Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, 32610-0245. E-mail: lbloom@ufl.edu

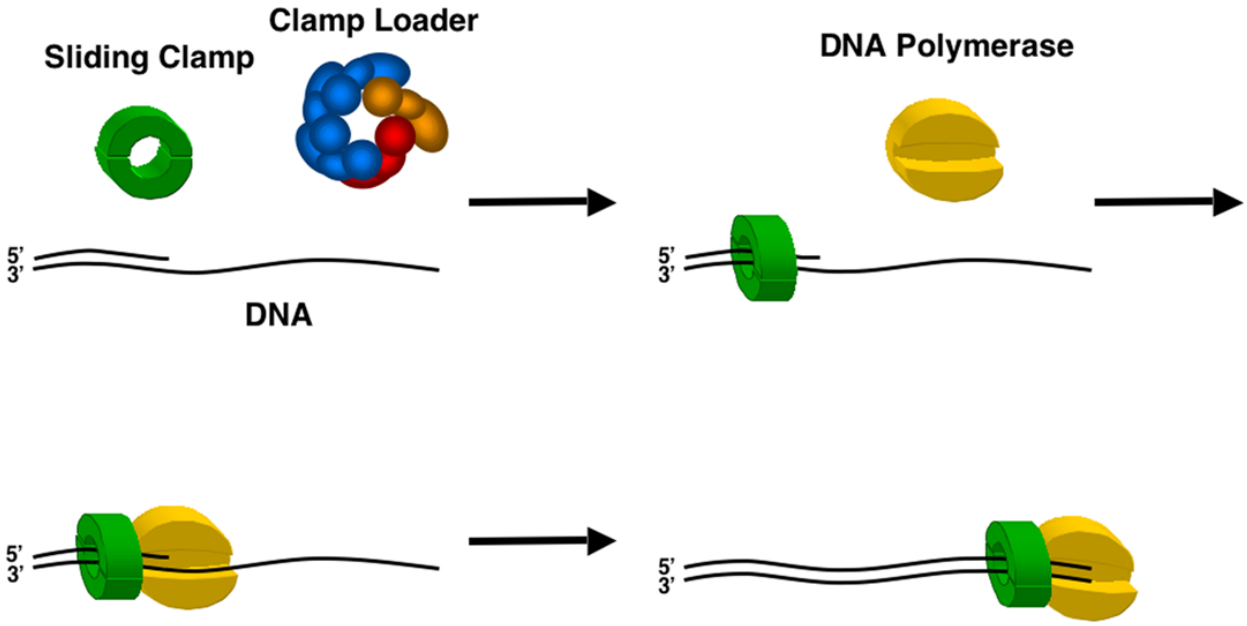

FIGURE 1 **Sliding clamps increase the processivity of synthesis by DNA polymerases. Clamps slide freely along DNA to tether polymerases to the templates being copied, yet allow DNA polymerases move at a rate limited by the rate nucleotide incorporation.**

(Figure 1). The increase in processivity provided by sliding clamps makes complete replication of entire genomes possible. On the leading strand, where DNA synthesis is continuous, one clamp is required at each replication fork. On the lagging strand, where DNA is synthesized discontinuously, a clamp is needed for each Okazaki fragment synthesized. In *E. coli*, where the replication fork progresses at rates of at least 500 nt/sec, clamp loading on the lagging strand must be rapid. Sliding clamps have also been shown to interact with a number of proteins required for different aspects of DNA metabolism and are likely to have other significant roles in the cell in addition to DNA replication (reviewed in Lopez de Saro and O'Donnell, 2001; Maga and Hubscher, 2003; Vivona and Kelman, 2003; Warbrick, 2000).

A second factor required to stimulate *E. coli* DNA polymerase III processivity is a protein complex that possesses DNA-dependent ATPase activity (Lee and Walker, 1987; Onrust *et al.*, 1991). This processivity factor functions as a clamp loader to catalyze the assembly of the ring-shaped sliding clamp onto DNA by opening the clamp and placing DNA through the center of the ring (Figure 2) (Stukenberg *et al.*, 1991). This reaction requires ATP binding and hydrolysis and, as such, clamp loaders are part of a large family of enzymes that couple NTP binding and hydrolysis to mechanical reactions (Burgers and Kornberg, 1982; Onrust *et al.*, 1991; Wickner, 1976). Structural features of clamp loaders are conserved among clamp loaders from different species (reviewed in Bowman *et al.*, 2005; Davey *et al.*, 2002; Ellison and Stillman, 2001; Jeruzalmi *et al.*, 2002). Five subunits comprise the core of clamp loaders. Individual subunits within this core have structural features in common with other proteins that are members of the AAA+ family of ATPases (Davey *et al.*, 2002; Jeruzalmi *et al.*, 2002; Ogura and Wilkinson, 2001).

The overall process of loading clamps onto DNA requires the clamp loader to perform many individual reactions or steps, which may include binding the clamp, opening the clamp, binding DNA, threading DNA through the clamp, closing the clamp, and releasing the clamp on DNA. These individual steps require different interactions between the clamp loader and the clamp and between the clamp loader and DNA. Initially, the clamp loader must have a high affinity for both the clamp and DNA to bring these macromolecules together. After placing the clamp at the appropriate site on DNA, or alternatively placing DNA through the clamp in the appropriate orientation, the affinity of the clamp loader for these macromolecules must decrease, so that loaded clamps can be "handed off" to DNA polymerases to allow DNA synthesis to begin. These "*inter*molecular" interactions are modulated

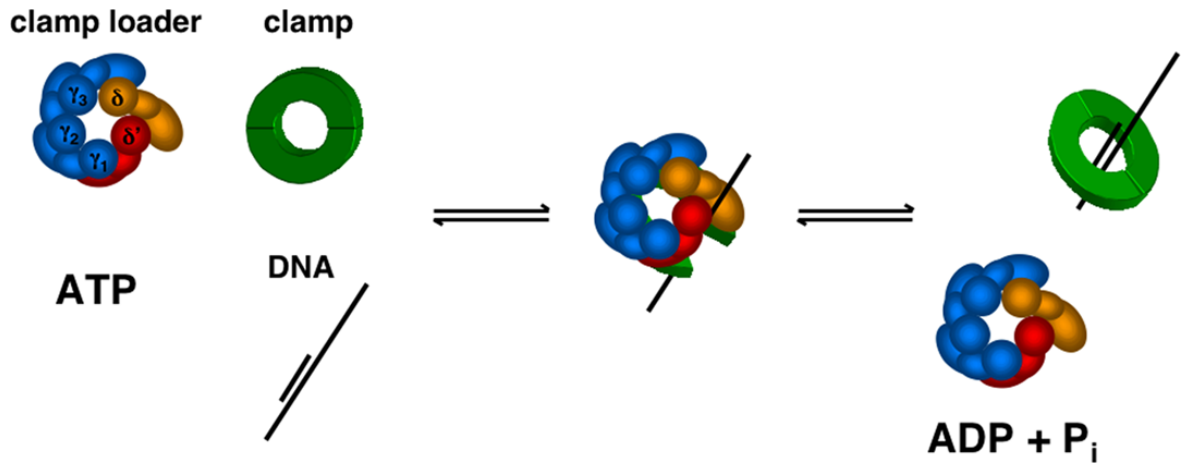

FIGURE 2 **Clamp loaders assemble ring-shaped sliding clamps onto DNA. Clamp loaders are molecular machines that couple ATP binding and hydrolysis to the process of loading clamps onto DNA. The *E. coli* β clamp is a ring-shaped dimer of identical monomers. The minimal *E. coli* clamp loader contains five subunits, three copies of γ (*blue*) that contain ATP binding sites, and one copy each of δ' (*red*) and δ (*gold*). The cartoon drawings are based on the structures of the *E. coli* β clamp and $\gamma_3\delta\delta'$ clamp loader in Figures 3 and 4. Five subunits are drawn for the clamp loader, and each subunit is divided into three components representing the three domains of the polypeptides. The clamp loader is drawn from a perspective looking down on the C-terminal domains, which are depicted as spheres.**

by changes in "*intra*molecular" interactions within the clamp loader. ATP-dependent changes in the interactions between and within clamp-loader subunits promote conformational changes in the complex that alter interactions with the clamp and DNA. Binding interactions with the clamp and DNA also promote changes in the clamp loader that alter interactions for the next step in the pathway. The main focus of this review will be on the mechanism of loading the *Escherichia coli* sliding clamp onto DNA, with special emphasis on the dynamic and transient interactions that are required to catalyze the reaction. Reviews on the complete replisomes and clamp loaders from *E. coli* (Johnson and O'Donnell, 2005; Marians, 2004; McHenry, 2003; Wijffels *et al.*, 2005), bacteriophage T4 (Benkovic *et al.*, 2001; Trakselis and Benkovic, 2001; Trakselis *et al.*, 2001b), and eukaryotes (Bell and Dutta, 2002; Garg and Burgers, 2005; Johnson and O'Donnell, 2005; Kao and Bambara, 2003; Majka and Burgers, 2004; Waga and Stillman, 1998) have been published recently.

STRUCTURAL FEATURES OF CLAMPS AND CLAMP LOADERS

Sliding Clamp Structures

The overall structures of sliding clamps are conserved from bacteria to humans. Sliding clamps required for DNA replication are composed of identical arc-shaped monomers that oligomerize to form ring-shaped structures (Figure 3). Bacteriophage RB69 (Shamoo and Steitz, 1999), bacteriophage T4 (Moarefi *et al.*, 2000), archaeal Proliferating Cell Nuclear Antigen (PCNA) (Matsumiya *et al.*, 2001), and eukaryotic PCNA (Gulbis *et al.*, 1996; Krishna *et al.*, 1994) clamps are composed of three subunits, whereas the *E. coli* (β) clamp (Kong *et al.*, 1992) contains two protomers. Although the number of subunits differs, the overall structure of the β ring is very similar to that of PCNA. Both rings contain six domains of similar fold, but the domains within an individual subunit, three in β and two in PCNA, differ in sequence.

The β ring is approximately 34Å thick, so that, when bound to DNA, the ring covers about one helical turn. The opening in the center of the β ring is about 35 Å in diameter and is lined with 12 α-helices. When DNA was modeled through the center of the ring, the axes of the α-helices were oriented perpendicular to the phosphate backbone (Kong *et al.*, 1992). This geometry, along with the inner diameter that is somewhat larger than B-DNA, suggest that β freely slides along duplex DNA and a layer or two of water rather than twisting around the grooves of DNA. In support of this model, biochemical studies have shown that the β dimer remains stably associated with circular duplex DNA, but on linearization of the DNA, rapidly dissociates, most likely by sliding off the ends (Stukenberg *et al.*, 1991).

In the crystal structures, each of the clamps forms a closed ring; however, in solution, open and closed

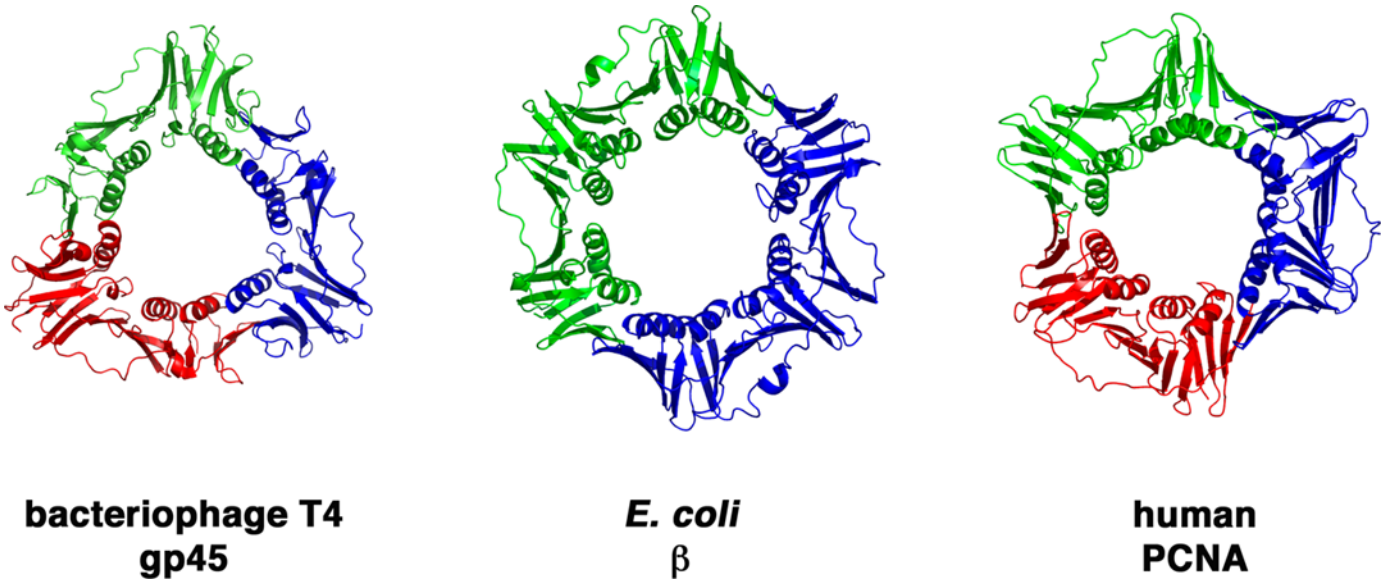

FIGURE 3 **Structures of sliding clamps. Ribbon diagrams are shown for sliding clamps from bacteriophage T4 (gp45, Moarefi *et al.*, 2000), *E. coli* (β, Kong *et al.*, 1992), and humans (PCNA, Gulbis *et al.*, 1996). Individual subunits are shown in different colors.**

forms of the clamp may exist in equilibrium (Alley *et al.*, 1999b; Griep and McHenry, 1988; Millar *et al.*, 2004). When the β clamp is loaded onto closed circular DNA, a stable complex is formed that can be isolated by gel filtration chromatography (Stukenberg *et al.*, 1991). In these complexes, β has a half-life on DNA of over an hour at 37°C (Leu *et al.*, 2000; Stewart *et al.*, 2001). This would suggest that, at least after being loaded on DNA, the monomer interfaces of the β ring are largely closed. Yeast and human PCNA also form a stable gel-filterable complex when loaded onto DNA, suggesting that a closed form predominates in solution (Burgers, 1991; Lee and Hurwitz, 1990; Yao *et al.*, 1996). In contrast, the gp45 clamp does not form such a stable interaction with DNA (Richardson *et al.*, 1990; Yao *et al.*, 1996). Solution-based studies have shown that one of the subunit interfaces in gp45 is in an open conformation (Alley *et al.*, 1999b; Millar *et al.*, 2004). In the presence of the bacteriophage T4 DNA polymerase, the gp45 clamp remains stably bound to DNA, because the C-terminal tail of the polymerase binds in the open interface to effectively close the ring (Alley *et al.*, 1999a; Alley *et al.*, 2001; Capson *et al.*, 1991).

Sliding clamps contain a rotational axis of symmetry through the center of the ring. A twofold axis is present in the β clamp and a threefold axis is present in gp45 and in PCNA. This symmetry creates two distinct faces to which proteins can bind. Thus, it is theoretically possible for different proteins to bind to different faces of a clamp simultaneously. However, in *E. coli*, DNA polymerase III core and the clamp loader bind to the same face of the β clamp, and even at the same site (Jeruzalmi *et al.*, 2001b; Lopez De Saro *et al.*, 2003a; Naktinis *et al.*, 1995; Naktinis *et al.*, 1996). This means that some mechanism must regulate the polymerase and clamp loader so that the enzymes do not compete with each other for binding the clamp, allowing each to bind at the appropriate time (Kim *et al.*, 1996c; Naktinis *et al.*, 1996). The same situation is true for bacteriophage T4 and eukaryotes in that the polymerases and clamp loaders bind to the same face of the clamp (Fotedar *et al.*, 1996; Latham *et al.*, 1997; Mossi *et al.*, 1997).

Common binding motifs have been identified in proteins that interact with the β clamp and PCNA. A key feature of both motifs is the presence of hydrophobic amino acid residues, including Phe. Based on sequence alignments and binding studies, consensus β binding sequences of QL(S/D)LF (Dalrymple *et al.*, 2001) and QxxL(x)F (Lopez De Saro *et al.*, 2003a) have been proposed. A similar consensus sequence, Qxx(I/L/M)xxF(F/Y), has been proposed for proteins that interact with PCNA (Warbrick, 1998; Warbrick, 2000). The similarity in binding motifs between β-binding and PCNA-binding proteins is remarkable because the primary sequences of β and PCNA are not conserved. The α subunit of *E. coli* DNA polymerase III core potentially contains two β binding motifs (Dohrmann and McHenry, 2005). One site located internally at residue 920, QADMF, is absolutely required for high-affinity binding to the β clamp and

processive DNA synthesis (Dohrmann and McHenry, 2005; Kim and McHenry, 1996). A second site located at the extreme C-terminus of the α subunit at residue 1154, QEVLEF, also contributes to clamp binding and processive synthesis (Lopez De Saro *et al.*, 2003a; Lopez De Saro *et al.*, 2003b). This second site may be important for maintaining the polymerase–clamp interaction during processive synthesis, but allowing the polymerase to dissociate on completing an Okazaki fragment (Kim *et al.*, 1996c; Leu *et al.*, 2003). A six-amino-acid sequence, QAMSLF, present in the in the δ subunit of the *E. coli* clamp loader is required for the δ–β interaction and contributes to the high-affinity interaction between the clamp loader and clamp (Jeruzalmi *et al.*, 2001b).

Clamp-Loader Structures

At the replication fork, the *E. coli* DNA polymerase III holoenzyme is composed of two core polymerases ($\alpha\varepsilon\theta$ subunits), a clamp loader, and two copies of the β sliding clamp (reviewed in Johnson and O'Donnell, 2005; McHenry, 2003). The clamp loader contains seven subunits with the stoichiometry of three copies of the *dnaX* gene products, τ and γ, and one copy each of the δ, δ', χ, and ψ subunits (Jeruzalmi *et al.*, 2001a; Pritchard *et al.*, 2000). The amino acid sequence of the γ subunit (47.5 kDa) is the same as the N-terminal two-thirds of the τ subunit (71.1 kDa). The γ subunit is produced by a translational frameshift that adds one unique amino acid residue before prematurely truncating the protein (Blinkowa and Walker, 1990; Flower and McHenry, 1990; Tsuchihashi and Kornberg, 1990). The form of the clamp loader in the holoenzyme most likely contains two copies of τ and one copy of γ (Glover and McHenry, 2000; Onrust *et al.*, 1995b; Pritchard *et al.*, 2000). Both the γ and τ subunits bind ATP (Tsuchihashi and Kornberg, 1989), but the C-terminal end of τ provides properties unique to τ that are important at the replication fork. A region in the C-terminus of the τ subunit interacts with the α subunit of the core polymerase, forming a dimeric DNA polymerase III complex (core–τ)$_2$ that couples leading and lagging strand synthesis (Kim *et al.*, 1996b; McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991). The activity of the DnaB helicase is stimulated by interactions with the τ subunit coupling duplex unwinding at the replication fork to DNA synthesis (Kim *et al.*, 1996a). The τ subunit protects the β clamp from being removed while the DNA polymerase is actively synthesizing DNA (Kim *et al.*, 1996c) but then mediates DNA polymerase dissociation and recycling on completing synthesis of an Okazaki fragment (Leu *et al.*, 2003). Clamp loaders containing either three copies of the τ subunit (τ complex, $\tau_3\delta\delta'\chi\psi$) or γ subunit (γ complex, $\gamma_3\delta\delta'\chi\psi$) are fully active in clamp loading (Dallmann *et al.*, 1995; Onrust *et al.*, 1995a). The minimal complex of *E. coli* proteins with clamp-loading activity has the composition $\gamma_3\delta\delta'$ (Olson *et al.*, 1995; Onrust and O'Donnell, 1993; Xiao *et al.*, 1993).

Recent structures of a minimal *E. coli* clamp loader ($\gamma_3\delta\delta'$), a *S. cerevisiae* clamp loader–clamp complex, and a *P. furiosus* clamp loader–clamp–DNA complex have made a significant contribution to our understanding of these molecular machines (Bowman *et al.*, 2004; Jeruzalmi *et al.*, 2001a; Kazmirski *et al.*, 2004; Miyata *et al.*, 2004; Miyata *et al.*, 2005). The structures of the individual subunits within the *E. coli* clamp loader, γ, δ, and δ', are similar in that each comprises three domains arranged in a "C"-shaped geometry (Figure 4, top) (Guenther *et al.*, 1997; Jeruzalmi *et al.*, 2001a; Podobnik *et al.*, 2003). Domains 1 and 2 are similar to the nucleotide binding domains of proteins in the AAA+ family of ATPases, although only the γ subunits are functional ATPases (Onrust and O'Donnell, 1993; Onrust *et al.*, 1991; Xiao *et al.*, 1993; Xiao *et al.*, 1995). The five subunits are arranged such that C-terminal domains of each protein form a more tightly packed ring-shaped collar (Figure 4, bottom). The relative orientations of the three domains within each subunit are different so that the overall structure is asymmetric with the N-terminal end being more open. The three γ subunits occupy adjacent positions within the complex, the δ' subunit contacts γ_1, and the δ subunit contacts γ_3. Although the C-terminal domains (domain 3) form a closed ring, there is a large gap in the structure between the domains 1 and 2 of the δ' and δ subunits such that these regions of the adjacent proteins do not interact.

The γ subunits form the motor of the clamp loader in that these subunits bind and hydrolyze ATP during the course of the clamp-loading reaction (Onrust and O'Donnell, 1993; Onrust *et al.*, 1991; Tsuchihashi and Kornberg, 1989; Xiao *et al.*, 1993; Xiao *et al.*, 1995). The ATP binding sites are located at the interface of two domains within a single subunit, and at an interface between two adjacent subunits within the complex (Podobnik *et al.*, 2003). Within a γ subunit, ATP is bound in a cleft between domains 1 and 2 (Figure 4, top). Conserved Walker A and Walker B

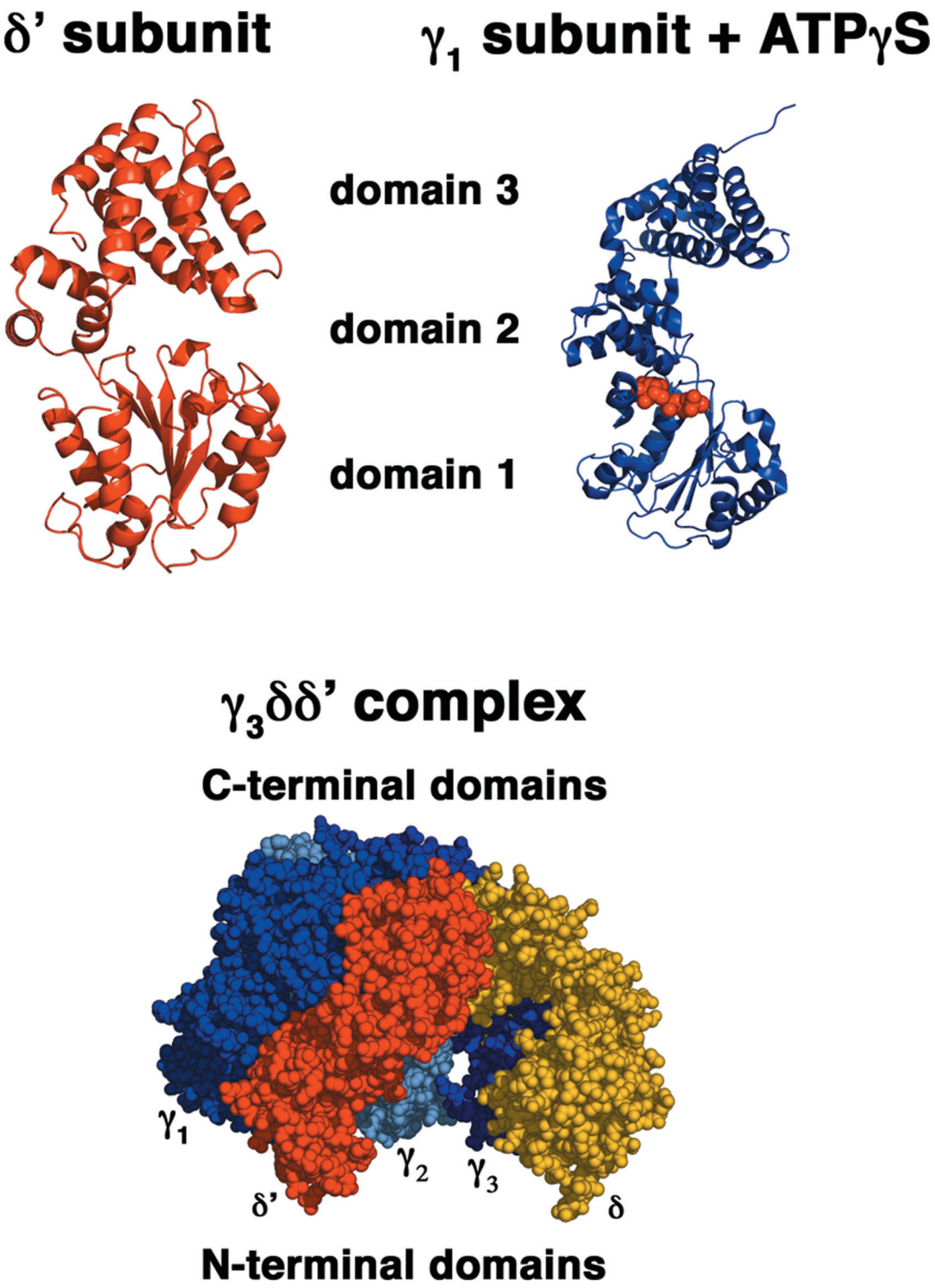

FIGURE 4 Structural features of the minimal *E. coli* clamp loader, $\gamma_3\delta\delta'$. ***Top.*** A ribbon diagram of the δ' subunit taken from the crystal structure of the free subunit is shown on the left (Guenther *et al.*, 1997). Shown on the right is a ribbon diagram of the γ_1 subunit bound to ATPγS (*red spheres*) taken from the crystal structure of the $\gamma_3\delta\delta'$ complex (Kazmirski *et al.*, 2004). Both subunits are oriented with the C-terminal domains at the top and N-terminal domains at the bottom. ***Bottom.*** A space-filled diagram of the $\gamma_3\delta\delta'$ complex with the δ' subunit in red, γ subunits in shades of *blue*, and δ subunit in *gold* (Jeruzalmi *et al.*, 2001a). The individual subunits in the complex are aligned in a parallel fashion with the C-terminal domains (*top*) forming a tightly packed ring. The complex is more open on the side containing the N-terminal domains (*bottom*) and a large gap exists between the δ' and δ subunits.

nucleotide binding motifs are located in domain 1 (Podobnik *et al.*, 2003; Walker *et al.*, 2000). A sensor 2 element, thought to sense nucleotide binding and to transmit binding into conformational changes in AAA+ proteins, is located in domain 2, and Arg-215 in this region that is conserved in clamp loaders interacts with the γ-phosphate of ATP. The location of these binding elements in different domains is likely to result in differences in the conformation between domains within a γ subunit on nucleotide binding and hydrolysis (Podobnik *et al.*, 2003). A second nucleotide sensing element, sensor 1, is located in domain 2 (residues 157 to 170). A conserved Ser-Arg-Cys (SRC) motif is present in this region that is found in all clamp loaders and is located at the interface between subunits. The "Arg finger" present in the SRC motif extends toward the ATP binding site in the adjacent subunit and may sense ATP binding and/or hydrolysis in the neighboring subunit (Johnson and O'Donnell, 2003; Ogura *et al.*, 2004; Snyder *et al.*, 2004). Although the δ' subunit does not bind ATP, an SRC motif is present that could potentially interact with nucleotide bound in the adjacent γ_1 subunit (Jeruzalmi *et al.*, 2001a; Johnson and O'Donnell, 2003; Snyder *et al.*, 2004). Given the location of ATP binding sites at the interface between domains within a γ subunit and at the interface between subunits within the complex, it is easy to envision a mechanism in which ATP binding and hydrolysis at individual sites promote conformational changes in each subunit that are translated to changes within the complex that alter interactions with other molecules.

Crystal structures of the nucleotide-free (Jeruzalmi *et al.*, 2001a) and ATPγS-bound (Kazmirski *et al.*, 2004) forms of the $\gamma_3\delta\delta'$ complex have been solved. There is very little difference in the conformations of the two complexes; however, only two of the three ATP binding sites (γ_1 and γ_3 in Figure 4, lower panel), are occupied by ATPγS. In both structures, the nucleotide binding sites of the γ_1 and γ_3 subunits are open to allow nucleotide entry, whereas the binding site in the γ_2 subunit is physically blocked by the γ_1 subunit. Nucleotide binding to the $\gamma_3\delta\delta'$ complex measured in solution by isothermal titration calorimetry also indicates that only two molecules of ATP or ATPγS bind this minimal clamp loader (Kazmirskit *et al.*, 2004). It is also interesting to note that, in these structures, the Arg fingers do not approach the nucleotide binding sites of the adjacent subunits closely enough to interact with the γ-phosphates of ATP. Based on these findings, this conformation of the $\gamma_3\delta\delta'$ complex is likely to represent a stable, but inactive, state of the clamp loader. The reason that nucleotide-dependent changes in conformation are not observed is not clear. One suggestion is that all three sites must be occupied by ATP to produce conformational changes that activate the clamp loader and interactions with the β clamp allow the third site, γ_2, to bind ATP. Another likely possibility is that the χ and ψ subunits that are missing from this minimal clamp loader stabilize a conformation in which the third ATP binding site in γ_2 is open and an active state is more stable.

The ability of a single γ complex subunit, δ, to stimulate dissociation of the β clamp from circular DNA molecules indicates that the δ subunit alone is capable of opening the β clamp (Leu *et al.*, 2000; Turner *et al.*, 1999). A crystal structure of a complex between the N-terminal domain (domain 1) of δ and a β monomer suggests the mechanism by which this might be possible (Jeruzalmi *et al.*, 2001b). Conserved residues in δ, Leu-73 and Phe-74, make a hydrophobic "finger" that binds in a hydrophobic pocket on the face of β. Binding in this way creates steric clashes between residues in the δ subunit and a loop on the surface of β that is connected to α-helix that forms part of the β-dimer interface (Jeruzalmi *et al.*, 2001b). This steric clash is likely to alter the conformation of the helix to disrupt interactions at the dimer interface and open the clamp. The conformation of the inactive state of the $\gamma_3\delta\delta'$ complex described above is such that this $\delta - \beta$ interaction required for clamp opening cannot occur due to steric clashes between the clamp and other subunits in the clamp loader (Jeruzalmi *et al.*, 2001a).

The *S. cerevisiae* RFC clamp loader has many structural features in common with the *E. coli* clamp loader (Bowman *et al.*, 2004; Jeruzalmi *et al.*, 2001a). Like the human clamp loader, the *S. cerevisiae* RFC is composed of four small subunits, RFC2-5, and one large subunit, RFC1 (Cullmann *et al.*, 1995; Fien and Stillman, 1992; Yoder and Burgers, 1991). These five subunits assemble in a five-membered ring to form a structure similar to the minimal *E. coli* $\gamma_3\delta\delta'$ complex (Bowman *et al.*, 2004; Jeruzalmi *et al.*, 2001a). The small subunits are composed of three domains similar to the γ subunit and the large subunit also contains 3 domains, but with larger N-terminal and C-terminal ends. The large RFC1 subunit is analogous to the δ subunit in its clamp binding site and position in the complex, but differs in that it is a functional ATPase (Ca *et al.*, 1997; Fotedar *et al.*, 1996;

Podust *et al.*, 1998; Schmidt *et al.*, 2001; Uhlmann *et al.*, 1997). The RFC5 subunit is analogous to δ' in its position in the complex and in that it does not hydrolyze ATP but possesses and Arg finger that can interact with the ATP binding site on the adjacent subunit (Bowman *et al.*, 2004). The RFC2-4 subunits are functional ATPases and occupy positions in the clamp loader analogous to the γ subunits (Bowman *et al.*, 2004; Cai *et al.*, 1997; Jeruzalmi *et al.*, 2001a; Schmidt *et al.*, 2001).

Structures of the *E. coli* clamp loader, *S. cerevisiae* RFC–PCNA complex, and *P. furiosus* RFC–PCNA–DNA complex show how clamps and clamp loaders may interact during the clamp loading reaction as well as how clamp-loaders bind DNA (Figure 5). The clamp loaders bind one face of the clamp via the N-terminal domains of the clamp-loader subunits (Bowman *et al.*, 2004; Jeruzalmi *et al.*, 2001a; Jeruzalmi *et al.*, 2001b; Miyata *et al.*, 2004; Miyata *et al.*, 2005). One interface between adjacent monomers in the clamp is opened possibly through movement both in the plane of the ring and out of the plane of the ring formed by clamp monomers (Kazmirski *et al.*, 2005; Miyata *et al.*, 2005). Individual clamp-loader subunits adopt a conformation such that they spiral around an axis going from the C-terminal to N-terminal domains of the proteins in the complex. Duplex DNA at the 3′ primer/template junction enters the clamp-loading complexes through the open side formed by the N-terminal domains, and the spiraling conformation of the subunits matches the spiral of the DNA helix such that the clamp loader resembles a "screw-cap" (Bowman *et al.*, 2004; Miyata *et al.*, 2005; Zhuang *et al.*, 2004). Single-stranded DNA exits through the gap between the *E. coli* δ' and δ subunits, or the analogous *S. cerevisiae* RFC5 and RFC1 subunits or *P. furiosus* large and small RFC subunits.

Although there are many similarities, there are some significant differences in the structures of the RFC–PCNA complexes from *S. cerevisiase* and *P. furiosus*. One

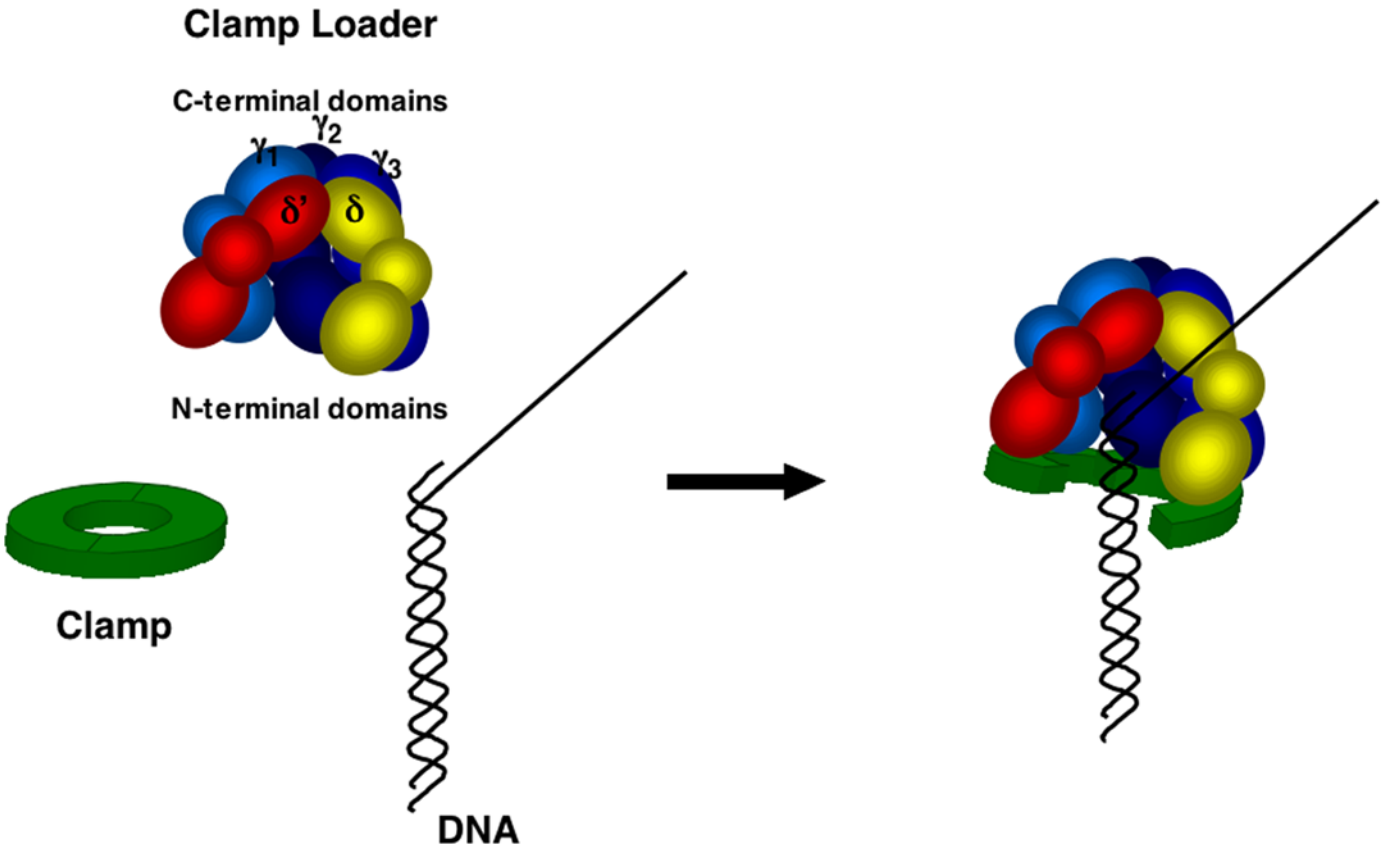

FIGURE 5 **Model for the *E. coli* clamp loader-clamp complex binding DNA. The side of the $\gamma_3\delta\delta'$ complex containing the N-terminal domains of the subunits interacts with one face of the β clamp (*green*) such that the conserved binding motif in the δ' subunit can interact with the hydrophobic pocket on β. The β clamp is open at one interface between adjacent subunits, possibly by motion both in the plane and out of the plane of the β ring. The duplex portion of p/t DNA enters the clamp loader through the open end on the N-terminal side and the single-stranded DNA overhang exits through the gap between the δ' (*red*) and δ (*gold*) subunits. This model has been proposed based on data for the *S. cerevisiae* RFC–PCNA (Bowman *et al.*, 2005; Bowman *et al.*, 2004; Kazmirski *et al.*, 2005) and *P. furiosus* RFC–PCNA–DNA complexes (Miyata *et al.*, 2005). The cartoon drawing shows five subunits of the clamp loader, each of which is divided into three components representing the three domains. The view is side-on with the N-terminal domains that contact β at the bottom.**

key difference is that the PCNA clamp is in an open state in the archaeal structure but closed in the yeast structure. There is also a difference in the contacts made between RFC and PCNA that are most likely related to the difference in the open and closed conformations of PCNA. All five subunits in the archaeal RFC complex make extensive interactions with PCNA whereas only three of the five yeast RFC subunits interact with PCNA. Both complexes were formed in the presence of the non-hydrolyzable ATP analog, ATPγS, to activate the clamp loader but to limit hydrolysis. It is possible that the different conformations observed in the complexes represent different states that are formed during the course of the clamp-loading reaction. The yeast complex could represent a state that forms just prior to clamp opening, or possibly on closing the clamp. The archaeal complex, on the other hand, is likely to represent the open state that is needed to thread DNA through the clamp. Another possibility is that mutations made to the Arg fingers (Arg to Gln) in the yeast RFC subunits to limit hydrolysis of ATPγS also inhibit ATP-induced conformational changes that allow RFC to open the clamp. Answers to these questions await further studies.

REAL-TIME TECHNIQUES USED TO INVESTIGATE CLAMP-LOADING REACTIONS

The process of loading clamps is dynamic. Protein–protein interactions within the clamp-loader complex and between the clamp loader and clamp as well as protein–DNA interactions must change in order for the mechanical reaction of loading clamps to take place. Not only do the interactions change, but interactions change rapidly over milliseconds. Although much has been learned from structural and biochemical studies that measure end-state products of clamp-loading reactions, techniques that allow the investigator to monitor these reactions directly in solution and in real time are key to uncovering the dynamic and transient interactions that are required for clamp loading. Fluorescence spectroscopy is a useful tool for monitoring these reactions, because it offers the sensitivity necessary to measure interactions between biomolecules at biologically relevant concentrations in solution and because it can be adapted to real time assays. A general overview of several fluorescence-based techniques that have been used successfully in investigations of the mechanism of clamp loading will be presented in this section. This is by no means an exhaustive list of techniques that may be used, but instead highlights some of the approaches that have been used. These techniques are generally applicable to the measurement of pre-steady-state enzyme kinetics. For a more detailed description of pre-steady-state kinetic techniques and analyses, the reader is referred to the following reviews (Fersht, 1999; Fierke and Hammes, 1995; Johnson, 1986; Johnson, 1992).

Fluorescence Anisotropy Measurements of Protein–Protein and Protein–DNA Interactions

Fluorescence anisotropy, or depolarization, is commonly used to measure protein–protein and protein–nucleic acid interactions in solution (for more detailed references on the technique, see Hill and Royer, 1997; Lakowicz, 1999; Rusinova *et al.*, 2002; Steiner, 1991). Fluorescence depolarization reports on the rate of rotational diffusion of molecules in solution, and because larger molecules tumble more slowly than smaller ones, fluorescence depolarization provides a measure of the overall size of a molecule. This sensitivity to molecular size or volume makes fluorescence anisotropy experiments ideally suited for investigations of macromolecular interactions where binding reactions create larger complexes from smaller components and where dissociation reactions create smaller species from larger ones. Typically, a biomolecule of interest is covalently labeled with a fluorescent dye, and the fluorescence depolarization of the extrinsic dye is measured. On binding another macromolecule, the effective size of the labeled species increases, which in turn decreases its rate of rotational diffusion and the rate of fluorescence depolarization of the probe (Figure 6). Similarly, dissociation events that create smaller species from a larger complex increase the rate of rotational diffusion and fluorescence depolarization of the probe. One advantage of this technique is that it is generally applicable, in that all interactions between macromolecules or between a macromolecule and a labeled ligand will change the effective size of the species to which the fluorophore is attached and thus affect the fluorescence anisotropy of the probe. It is usually advantageous to label the smallest biomolecule, because this will give the largest difference in size when a complex is formed or dissociates. A second advantage is that anisotropy measurements do not necessarily require knowledge of the structure of

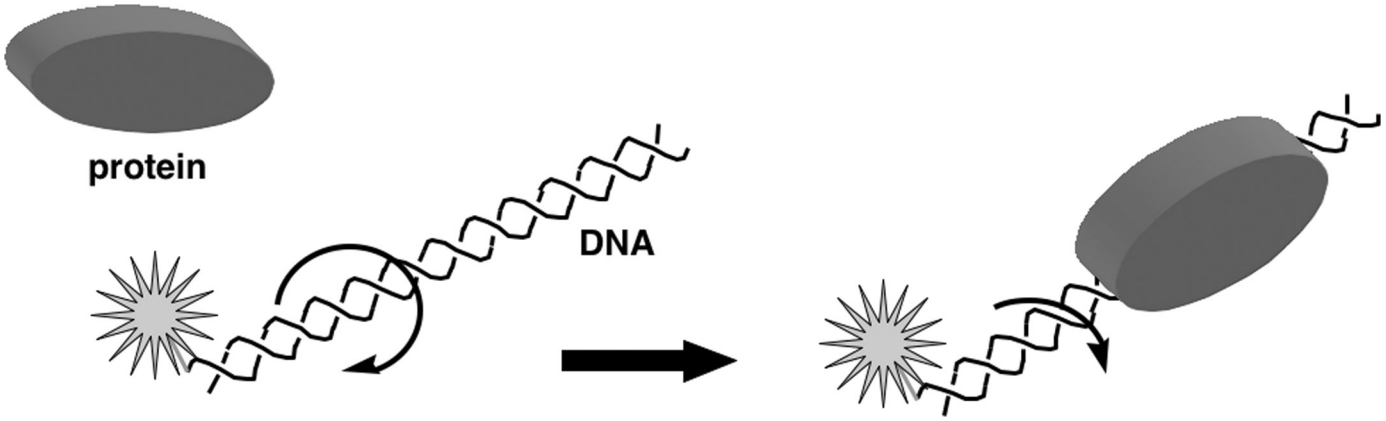

FIGURE 6 **Fluorescence anisotropy, or depolarization, reports on the rotational dynamics of macromolecules in solution. A free DNA molecule labeled with an extrinsic fluorophore (*star*) will tumble relatively rapidly in solution giving rise to a large depolarization of fluorescence or relatively small anisotropy. When a protein binds the DNA molecule, the rate of rotational diffusion decreases which decreases the fluorescence depolarization and yields a large anisotropy.**

macromolecules. As long as the fluorophore does not interfere with the activity of the biomolecule, it can be attached to any chemically reactive site, such as an amino group or sulfhydryl group. In contrast, other fluorescence techniques often require some knowledge of structure so that the fluorophore can be located at a site where its environment changes in some way to produce a signal on forming a complex.

The clamp-loading reaction is not a traditional enzyme-catalyzed reaction in the sense that the clamp loader catalyzes the making or breaking of covalent bonds to convert a substrate into a product. Instead, this reaction uses the energy from ATP binding and hydrolysis to make and break noncovalent bonds (Purich, 2001). The clamp loader catalyzes the formation of a macromolecular complex, a clamp on DNA, from two "free" macromolecules. Fluorescence anisotropy experiments report on exactly this type of reaction, formation of a complex. Not only does the clamp-loading reaction form a complex on going from "substrates" to "product," but formation and dissociation of intermediate complexes are required to assemble clamps on DNA. The clamp loader must bind both the clamp and DNA to ultimately form a ternary complex, and then, the clamp loader must dissociate to release the clamp on DNA for use by the polymerase. Fluorescence anisotropy provides a powerful tool for monitoring the formation and decomposition of intermediate complexes directly in solution in real time.

Using fluorescence anisotropy to measure molecular interactions is possible because fluorophores provide the appropriate time scale for the measurements and information on molecular orientation (Figure 7). A fluorophore acts as a molecular stopwatch to provide a defined time window in which to measure the extent of tumbling of molecules in solution. When a fluorophore is excited by light, it spends some time in an excited state before emitting a photon as fluorescence to relax to the ground state. Excited state lifetimes are typically on the order of nanoseconds, which is precisely the time scale on which macromolecules tumble in solution. Because fluorophores have specific transition dipole moments for excitation and emission, they provide information on the orientation of macromolecules in solution (Figure 7). In the simplest terms, when a population of fluorophores is excited by polarized light, those molecules with excitation transition moments aligned with the polarization of light are selectively excited. It is actually a little more complicated, in that the probability of exciting a fluorophore decreases as the angle between the polarized light and the transition dipole increases, but the general idea is the same. If those excited molecules are held absolutely motionless during the lifetime of the excited state, then the emitted fluorescence will also be polarized, because fluorophores have a specific transition dipole for emission. In solution, however, the molecules tumble during the excited state lifetime, and this leads to depolarization of fluorescence emission. Thus, the rate of fluorescence depolarization reflects the rate of rotational diffusion of the fluorophore in solution.

Because the fluorophore is acting as a molecular stopwatch to provide the time scale for anisotropy measurements, the choice of a fluorophore for binding measurements depends on the lifetime of the probe and the time scale of the rotational motions of the free

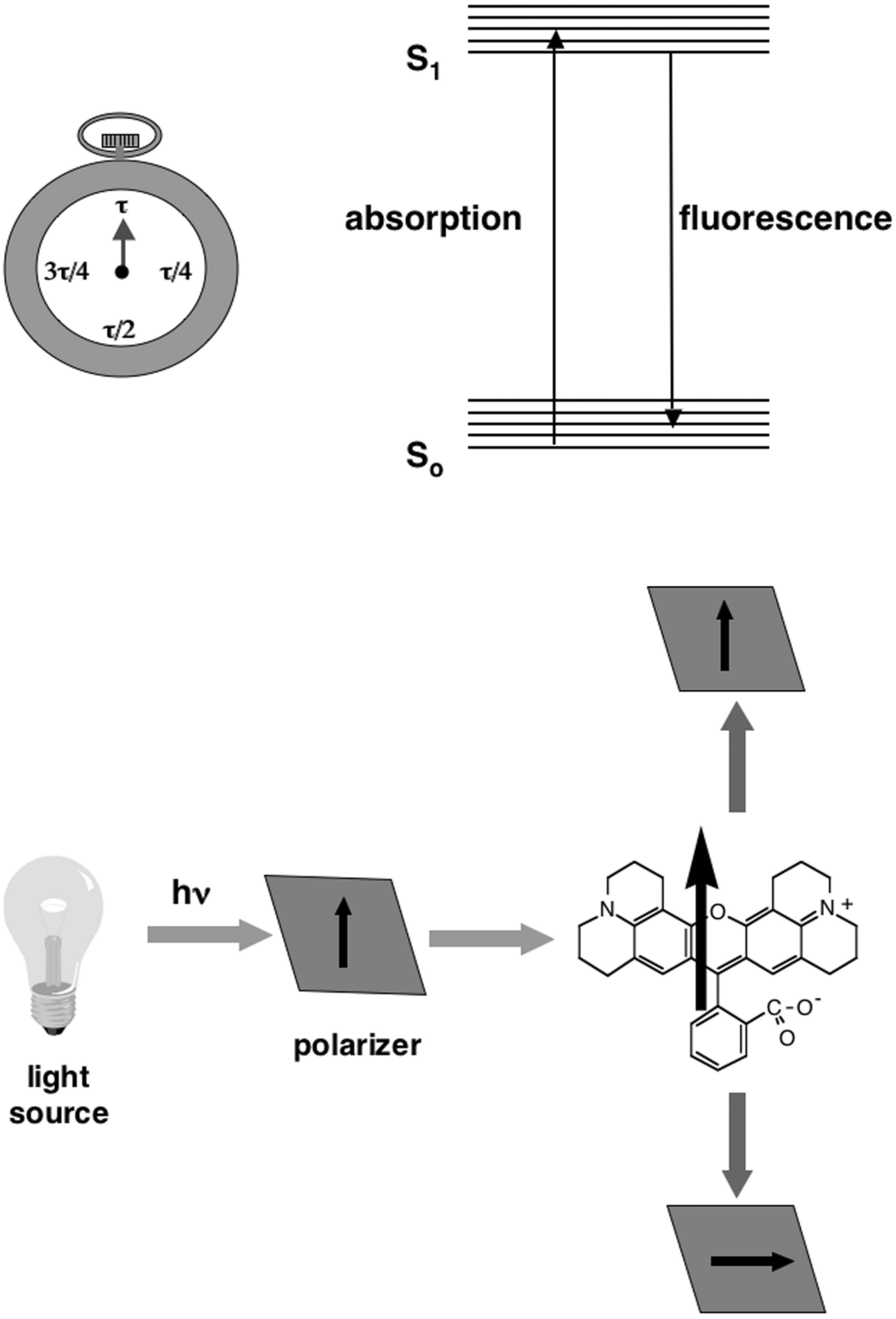

FIGURE 7 Fluorophores provide a time window in which to observe rotational dynamics of macromolecules and information about molecular orientation in solution. *Top*. Following excitation, a fluorophore spends some time in an excited state before emitting a photon as fluorescence. The lifetime of a fluorophore provides the time scale on which to measure the motion of a population of molecules in solution. *Bottom*. Fluorophores have specific transition dipoles for excitation and emission. Excitation of a solution of fluorophores with polarized light selectively excites molecules with transition dipoles aligned with the light. (Note: An arbitrary transition dipole is depicted for X-rhodamine.) In the absence of rotational diffusion, fluorescence emission is also polarized. Rotational diffusion during the lifetime of the excited state depolarizes fluorescence emission.

and bound macromolecules. The goal, as always, is to maximize the observed signal change, which in this case is the difference in the anisotropy values for the free and bound states of the labeled molecule. Ideally, the free molecule should tumble rapidly relative to the rate of fluorescence decay, and the bound molecule should tumble less, or virtually not at all, during the lifetime of the excited state. Fluorophores genenerally have characteristic fluorescence lifetimes. For example, fluorescein and rhodamine dyes typically have lifetimes on the order of 3 to 5 ns, and pyrene can have a lifetime as long as 100 ns. The lifetimes of some fluorophores are very sensitive to the environment, whereas others are less sensitive. Equation 1 describes how the lifetime of a fluorophore and tumbling rate affect the observed anisotropy.

$$r = r_o/(1 + \tau/\phi) \quad (1)$$

As shown in equation 1, the observed anisotropy (r) of a fluorophore is a function of three terms: the fundamental anisotropy value, r_o; the lifetime of the fluorophore, τ; and the rotational correlation time, ϕ, of the fluorophore. The r_o value is the anisotropy in the absence of rotational diffusion and is defined by the angle between the excitation and emission dipole moments. Theoretical values for r_o range from 0.4, when the excitation and emission dipoles are collinear, to −0.2 when the dipoles are orthogonal. The value of r_o sets a limit on the observed anisotropy, which ranges from 0 when the molecules tumble rapidly to r_o when the molecules tumble slowly relative to the lifetime of the fluorophore. The value τ, is the average time that a fluorophore stays in the excited state, and is inversely related to the rate of decay of fluorescence. The rotational correlation time, ϕ, is a parameter that defines the time scale of the rotational motion of a molecule in solution, and similar to the lifetime, is inversely related to the rate of anisotropy decay. For the purposes of choosing candidate probes, probe lifetimes should be on the order of the rotational correlation time of the macromolecule, which can be approximated based on the molecular weight (Lakowicz, 1999).

Anisotropy measurements are made by exciting a sample with polarized light and measuring the intensity of polarized emission parallel and perpendicular to the direction of the excitation light (Figure 7, lower panel). The steady-state anisotropy, r, is calculated from the polarized emission intensities using equation 2 in which I_{VV} and I_{VH} are the intensities of vertical and horizontal emission when the sample is excited with vertically polarized light and g is the g-factor.

$$r = (I_{VV} - gI_{VH})/(I_{VV} + 2gI_{VH}) \quad (2)$$

The g-factor is a measure of the polarization bias of the detection system and corrects for differences in the efficiencies with which the detection system measures vertically and horizontally polarized light. The g-factor is experimentally determined by measuring the intensity of vertical and horizontal emission when exciting with horizontally polarized light (equation 3).

$$g = I_{VV}/I_{VH} \quad (3)$$

Because the difference in vertically and horizontally polarized emission is normalized to the total intensity of fluorescence ($I_{VV} + 2gI_{VH}$), anisotropy values are independent of the concentration of a fluorophore in solution; that is, assuming that the concentration differences do not affect a binding equilibrium in such a way as to change the populations of free and bound species. This makes physical sense; the concentration of a fluorophore should not affect its rate of rotational diffusion unless some concentration-dependent binding interaction takes place.

Figure 8 illustrates how steady-state anisotropy measurements can be used to monitor clamp-loading reactions. In this experiment, primed template DNA is labeled on the 5′ end of the template with X-rhodamine (RhX) via a C6 amino linker. When attached to DNA, X-rhodamine has a mean lifetime of on the order of 4.5 ns (Bloom *et al.*, 1996) and an r_o on the order of 0.34 to 0.35 (Bloom, unpublished observations). The anisotropy value of RhX is about 0.22 on free DNA (DNA) and does not change on addition of the β clamp ($+\beta$) because the β clamp does not spontaneously assemble on DNA. Addition of the γ complex clamp loader, ($+\gamma_c$), results in an increase in anisotropy to a value of about 0.32. This increase in anisotropy reflects the establishment of a steady-state clamp-loading reaction in which the γ complex loads clamps on DNA, clamps slide off the ends of the short linear substrates and are reloaded (Bloom *et al.*, 1996). After 15 minutes, the clamp loader has consumed the ATP present and the reaction cycle stops. When additional ATP is added, (+ATP), the clamp loading cycle resumes. These changes anisotropy between free DNA and bound DNA can be measured

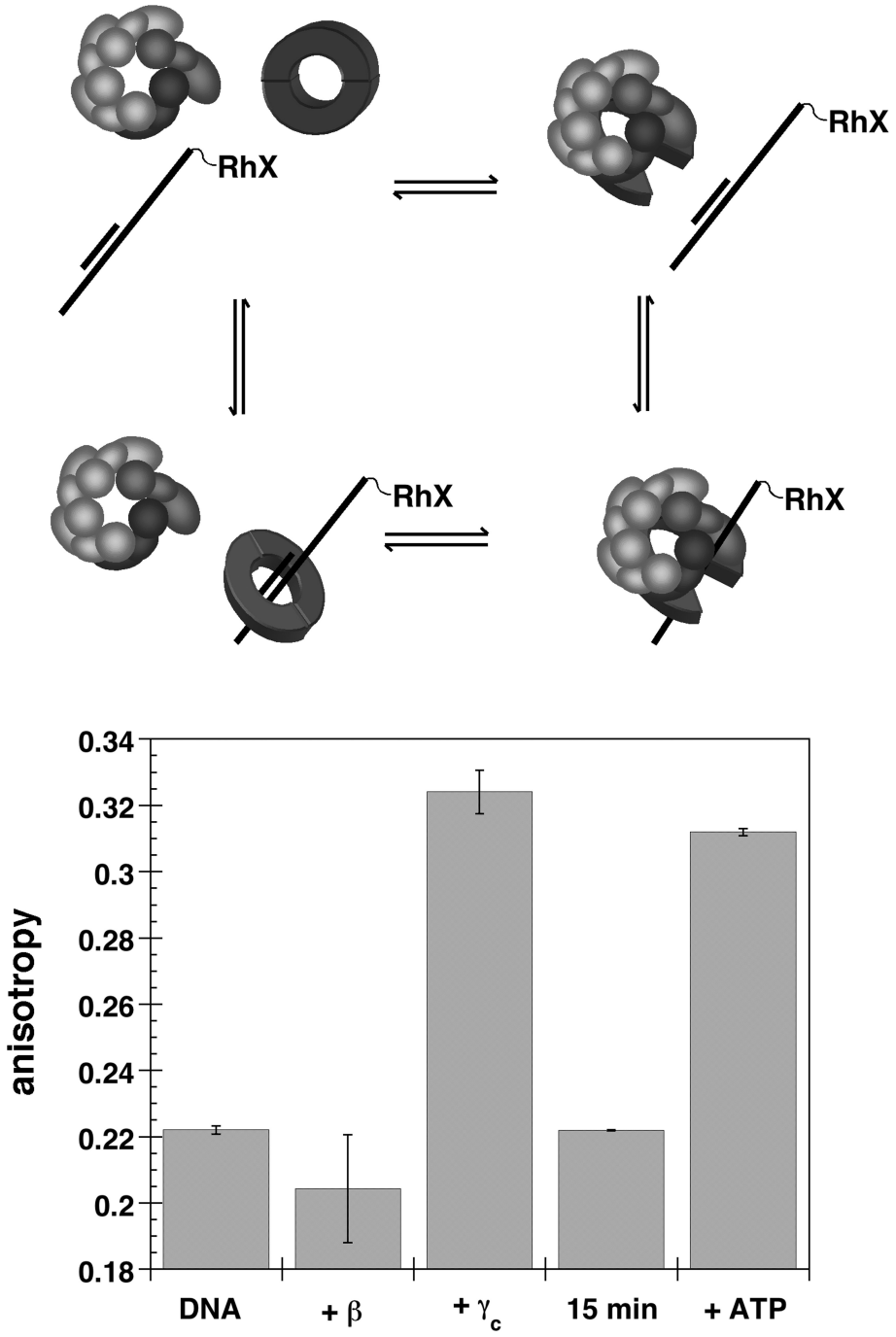

FIGURE 8 **Fluorescence anisotropy measurements of a steady-state clamp-loading reaction cycle. A DNA template, 105 nucleotides, was covalently labeled on the 5′-end with X-rhodamine and annealed to a 30-nucleotide primer. The anisotropy value for free DNA is about 0.22 (DNA) and does not change on addition of β ($+\beta$). Addition of the γ complex clamp loader ($+\gamma_c$) increases the anisotropy to a value just over 0.32 because a steady-state clamp-loading cycle is established. As illustrated in the diagram above the graph, the β clamp is loaded onto DNA, slides off the short linear template, and must be reloaded. After 15 min, the ATP initially present in the reaction is consumed and the reaction cycle stops. The observed anisotropy decreases to that of free DNA (*15 min*). When additional ATP is added to the mixture, the clamp loading reaction cycle resumes and the anisotropy increases to a value just under 0.32 ($+$ATP). Concentrations were 50 nM DNA, 500 nM γ complex, 2 μM β and 250 μM ATP. The second addition of ATP gave a final concentration of 1 mM.**

as a function of concentrations of components or as a function of reaction time to investigate the mechanism of clamp loading reactions.

Anisotropy measurements can be used to quantitate binding interactions measured under equilibrium conditions or in real time. In both cases, the observed anisotropy at any time (t) is a population weighted average of the anisotropy values for all free and bound species as in equation 4, where x_i is the mole fraction of species i at time t, and r_i is the anisotropy of species

i. For equilibrium experiments, time t is time at which the system has come to equilibrium.

$$r_{obs}(t) = \Sigma\, x_i(t) r_i \quad (4)$$

Because the observed anisotropy is a function of the fraction of free and bound species, equilibrium binding constants can be determined from experiments in which the concentrations of the binding partner is varied. For example, in measuring an equilibrium binding constant for a simple two-state system, in which there is a single free and single bound state, equation 4 can be rewritten as shown in equation 5.

$$\begin{aligned} r_{obs} &= x_{free}\, r_{free} + x_{bound}\, r_{bound} \\ &= x_{bound}\,(r_{bound} - r_{free}) + r_{free} \end{aligned} \quad (5)$$

The anisotropy values for the free and bound states can be determined experimentally. The mole fraction bound, x_{bound}, can be expressed in terms of a dissociation constant and substituted into equation 5. Equation 5 then becomes equation 6, where K_d is the dissociation constant, M_L is the total concentration of labeled molecule and M_U is total concentration of the unlabeled binding partner. Titration data can be fit by nonlinear regression to equation 6 to calculate a K_d value.

$$\begin{aligned} r_{obs} &= \frac{K_d + M_L + M_U - \sqrt{(K_d + M_L + M_U)^2 - 4M_L M_U}}{2M_L} \\ &\quad \times (r_{bound} - r_{free}) + r_{free} \end{aligned} \quad (6)$$

This approach has been used to measure the equilibrium binding of γ complex to the β clamp (Snyder *et al.*, 2004). In these experiments, the β clamp, the smaller of the two species, was covalently labeled with pyrene via a cysteine residue (β^{PY}). Pyrene was chosen as a probe over fluorescein or rhodamine because pyrene typically has a much longer mean lifetime and results in a larger signal difference between free and bound β. Figure 9 shows an equilibrium binding experiment measuring the affinity of a γ complex Arg finger mutant, Arg-158 to Ala in the δ' subunit, for β^{PY} in the presence (Figure 9, *filled circles*) and absence (Figure 9, *open circles*) of ATP. ATP binding only slightly increases the affinity, less than a factor of three, of this mutant clamp loader for the β clamp. Dissociation constants calculated assuming a simple two-state binding model for γ_c binding β as in equation 6 were 123 ± 1 nM in the absence of ATP and 51 ± 4 nM (Snyder *et al.*, 2004). In contrast, ATP binding increases the affinity of wild-type γ complex for β by about three orders of magnitude (Naktinis *et al.*, 1995; Snyder *et al.*, 2004). These results suggest that this γ complex mutant is deficient in the ability to undergo ATP-induced conformational changes that produce a high affinity binding state of the clamp loader.

Anisotropy measurements can also be made in real time during the time course of a binding reaction to determine the kinetics of binding and dissociation. As an example, the β^{PY} anisotropy-based binding assay described above can be used to measure the dissociation of γ complex from β^{PY} (Figure 10, S.G. Anderson and L.B. Bloom, unpublished results). Two types of dissociation events are possible: passive dissociation of the clamp from the clamp loader and active release of the clamp on DNA during a clamp-loading reaction. To measure passive dissociation, the γ complex, β^{PY}, and ATP are pre-mixed to form a γ–β^{PY} complex and then rapidly added to a solution containing a 20-fold excess of unlabeled β trap. To measure active dissociation, primed template DNA along with the unlabeled β trap is rapidly mixed with the solution of γ–β^{PY} complex. In both reactions, when γ complex dissociates from β^{PY}, the anisotropy of β^{PY} decreases, and free γ complex is trapped by the excess unlabeled β. Comparison of the rates of decrease in anisotropy shows that passive dissociation of β from the γ complex is significantly slower, about 80 times, than active release β on DNA (Figure 10). It takes about 80 sec ($k_{off} = 0.05\ s^{-1}$) for γ complex to dissociate from g β^{PY} in the absence of DNA and only about 1 sec ($k_{off} = 4\ s^{-1}$) in the presence of p/t DNA. The latter dissociation event is the result of a productive clamp-loading reaction. By performing anisotropy-based assays with PY-labeled β and RhX-labeled DNA, the clamp-loading reaction can be monitored from the perspectives of DNA and the β clamp. The combination of measurements provide information on steps that involve changes in interactions with DNA versus steps that involve changes in interactions with the clamp, as well as two independent measurements of steps that involve both the clamp and DNA.

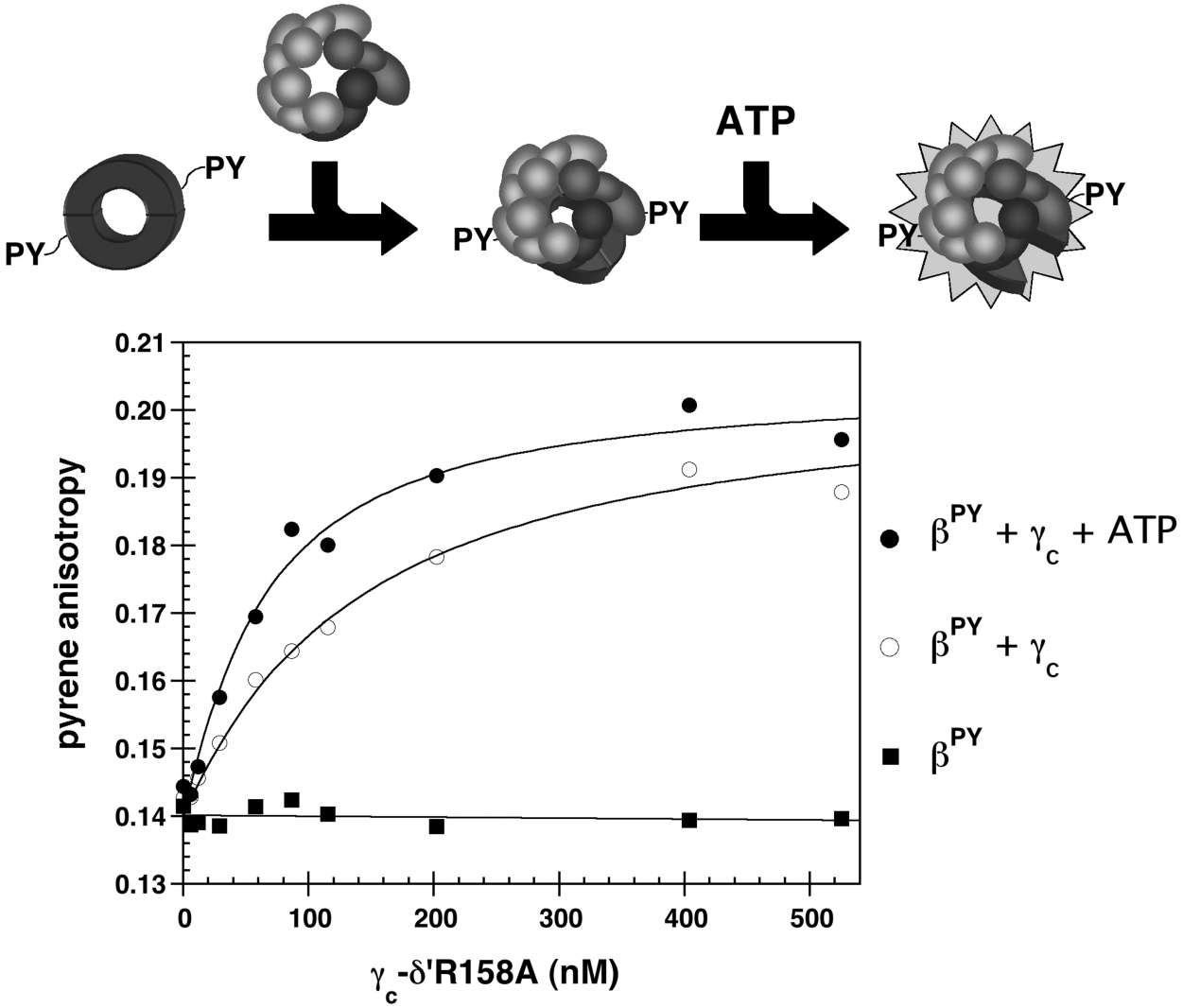

FIGURE 9 Titration of pyrene-labeled β with a γ complex mutant (Snyder *et al.*, 2004). In this titration, β^{PY}, a γ complex mutant containing an Arg-158 to Ala mutation in the δ' subunit (γ_c-δ'R158A), and ATP were sequentially added to a cuvette and the anisotropy was measured after each addition. This procedure was repeated at several concentrations of the γ complex mutant, γ_c-δ'R158A. Anisotropy values determined for free β (*filled squares*) illustrate of the reproducibility of these measurements. Anisotropy values measured following addition of $\gamma_c - \delta'$R158A provide a measure of the ATP-independent interaction between this γ complex mutant and the β clamp. Finally, anisotropy values measured after addition of ATP provide a measure of the ATP-dependent interactions between this γ complex mutant and β. The observation that the affinity of this γ complex mutant for β does not increase substantially on addition of ATP suggests that this mutant is defective in the ability to undergo ATP-induced conformational changes that give the clamp loader a high affinity for the clamp.

Fluorescence Intensity Measurements of ATP Hydrolysis

ATP binding and hydrolysis by the clamp loader modulate protein–protein and protein–DNA interactions to allow the clamp loader to assemble clamps onto DNA. Measurement of the temporal correlation between ATP binding or hydrolysis events and changes in protein–protein and protein–DNA interactions is essential for defining how ATP-dependent changes contribute to the affinity modulation of clamp-loader interactions. An ATPase assay has been developed by Martin Webb and colleagues that permits the measurement of ATP hydrolysis directly in solution and in real time (Brune *et al.*, 1994; Brune *et al.*, 1998). This assay makes use of the *E. coli* phosphate binding protein (PBP), the product of the *phoS* gene, and monitors ATP hydrolysis by measuring the inorganic phosphate product of the hydrolysis reaction.

In *E. coli*, PBP is made under low-phosphate conditions. Phosphate binding protein is present in the periplasm and functions to bind and transfer P_i to a membrane-bound transporter for transfer to the cytoplasm. The overall structure of PBP

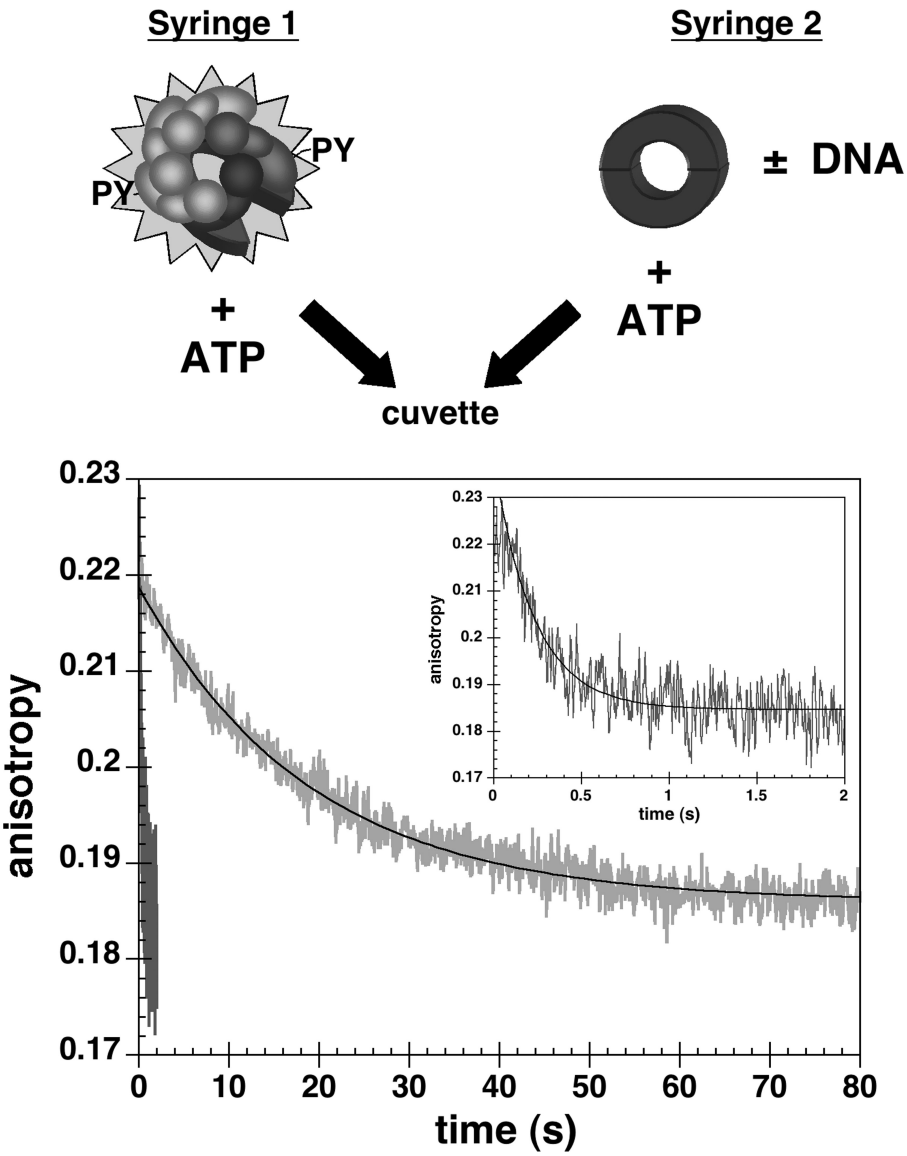

FIGURE 10 Stopped-flow anisotropy measurements of passive dissociation and active release of β^{PY} from γ complex. Passive dissociation of β^{PY} from γ complex (*light gray reaction trace* over 80 sec) was measured by rapid addition of a solution of a 20-fold excess of unlabeled β and ATP to a solution of γ complex, β^{PY}, and ATP. Active release of the β clamp (*dark gray* reaction trace over 2 sec in both the main plot and inset) was measured in an identical reaction except that primed template DNA was added along with the unlabeled β clamp. The diagram above the graph illustrates the stopped-flow mixing scheme in which preincubation of the γ complex, β, and ATP results in formation of an ATP-activated clamp loader-clamp complex indicated by the star. Solid black lines through the reaction traces are the result of a fit to a single exponential decay. Simple dissociation of β^{PY} from γ complex is significantly slower, k_{off} = 0.05 s^{-1}, than active release of β^{PY}, $k_{off} = 4\ s^{-1}$, resulting from a productive clamp loading reaction (S.G. Anderson and L.B. Bloom, unpublished results). Final concentrations were 300 nM γ complex, 300 nM β^{PY}, 500 μM ATP, 6 μM unlabeled β, and 330 nM p/t DNA when present.

contains two globular domains with a cleft between the domains. Inorganic phosphate binds in the cleft produces a conformational change that closes the cleft (Hirshberg *et al.*, 1998; Ledvina *et al.*, 1996; Luecke and Quiocho, 1990). A mutation from Ala-197 to Cys allows PBP to be site-specifically labeled with a coumarin dye, N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) near the phosphate binding cleft. When MDCC-labeled PBP binds inorganic phosphate, a large increase in MDCC fluorescence is produced presumably from the P_i-dependent conformational change which alters the environment of the probe (Brune *et al.*, 1994; Brune *et al.*, 1998; Hirshberg *et al.*, 1998). The fluorescence emission increases by about 10-fold, and there is a blue-shift in the emission maximum from 474 nm in the absence of P_i to 464 nm in the presence of P_i.

Because PBP rapidly binds P_i with high affinity ($k_{on} = 1.36 \times 10^8\ M^{-1}s^{-1}$, Brune *et al.*, 1994), MDCC-labeled PBP provides an excellent tool for measuring

inorganic phosphate produced by nucleotide hydrolysis. ATP hydrolysis is measured by including MDCC-PBP in enzyme-catalyzed reactions and monitoring the fluorescence emission of MDCC, typically at 465 nm when exciting at 425 nm. Given that there is a one-to-one binding stoichiometry, one molar equivalent of MDCC-PBP is required for each mole of P_i produced in an ATP hydrolysis reaction. Fluorescence measurements must be made over a concentration range in which the response of MDCC fluorescence is linear with increasing P_i concentration. This concentration range can be determined empirically by titrating MDCC-PBP with inorganic phosphate. As the fraction of protein bound approaches saturation, the fluorescence response becomes nonlinear. When P_i product is measured within the linear range of the fluorescence response, the increase in fluorescence is directly proportional to the amount of P_i product. The observed MDCC fluorescence is a function of the fraction of MDCC-PBP that is bound to P_i, as in equation 7, where x_{bound} is the mole fraction of MDCC–PBP bound to P_i, x_{free} is the mole fraction of free MDCC–PBP, I_{bound} and I_{free} are the intensities of MDCC-PBP–P_i and MDCC–PBP, respectively.

$$\begin{aligned} I_{obs(t)} &= x_{free(t)}\, I_{free} + x_{bound(t)}\, I_{bound} \\ &= (1 - x_{bound(t)})(I_{bound} - I_{free}) + I_{free} \end{aligned} \qquad (7)$$

Before MDCC-PBP can report on ATP hydrolysis, inorganic phosphate must be released by the enzyme hydrolyzing ATP. Thus, the MDCC-PBP-based ATPase assay truly measures the rate of release of inorganic phosphate from the enzyme active site. However, if the rate of phosphate release is rapid relative to the rate of ATP hydrolysis, then the rate of ATP hydrolysis limits the rate of the reaction, and rates measured in a MDCC-PBP-based assay reflect rates of ATP hydrolysis. Rapid-quench experiments using radio-labeled ATP measure the total concentration of ADP product produced in an enzyme-catalyzed reaction regardless of whether the product remains bound in the enzyme active site or is free in solution. A comparison of rates of ATP hydrolysis measured in rapid-quench experiments and in MDCC-PBP experiments will demonstrate whether the rates determined in the MDCC-PBP assay reflect the rate of ATP hydrolysis or P_i release. If both assays give the same reaction rates, then the rate of P_i release is rapid relative to ATP hydrolysis and the MDCC-PBP-based assay provides a measure of the rate of ATP hydrolysis. On the other hand, if the rate measured in MDCC-PBP-based assays is slower than in rapid-quench assays, then P_i release is rate-limiting.

By correlating the timing of ATP hydrolysis with binding and dissociation reactions that occur during the clamp-loading reaction cycle, steps that require ATP hydrolysis can be identified. Figure 11 shows an example of the time courses for ATP hydrolysis measured using the MDCC-PBP-based assay and clamp release measured using the β^{PY} anisotropy assay under identical conditions (S.G. Anderson and L.B. Bloom, unpublished results). ATP hydrolysis by the γ complex is triggered by DNA binding; ATP hydrolysis in the absence of DNA is significantly slower (Bertram *et al.*, 1998; Hingorani *et al.*, 1999; Onrust *et al.*, 1991; Turner *et al.*, 1999). The time course of P_i release as measured by the increase in MDCC fluorescence shows a small lag, reflecting the time it takes to bind DNA followed by a "burst" of ATP hydrolysis and a linear steady-state phase of the reaction mirroring the time course measured in rapid-quench experiments (Bertram *et al.*, 2000; Hingorani *et al.*, 1999). The time course for clamp release as measured by the anisotropy of β^{PY} shows a substantial lag in which there is very little (if any) change in the anisotropy preceding a decrease in anisotropy, reflecting clamp release. Comparison of the timing of ATP hydrolysis and β^{PY} release shows that the majority of the burst of ATP hydrolysis occurs approximately within the first 120 ms of the reaction. Over this time period, there is very little (if any) change in anisotropy showing that the clamp has not yet been released. A significant decrease in anisotropy does not begin until the burst of ATP hydrolysis is nearly complete. This correlation in timing indicates that ATP hydrolysis occurs prior to release of the clamp on DNA. Similar experiments measuring the temporal correlation clamp loading from the perspective of RhX-labeled DNA and ATP hydrolysis have yielded the same result that ATP hydrolysis occurs prior to clamp release (see Figure 13, Bertram *et al.*, 2000).

Fluorescence Resonance Energy Transfer Measurements of Protein–Protein Interactions

Fluorescence resonance energy transfer (FRET) occurs when a fluorescent donor transfers its excited state energy to an acceptor that emits fluorescence (for more detailed references on FRET, see Clegg, 1995; Lakowicz,

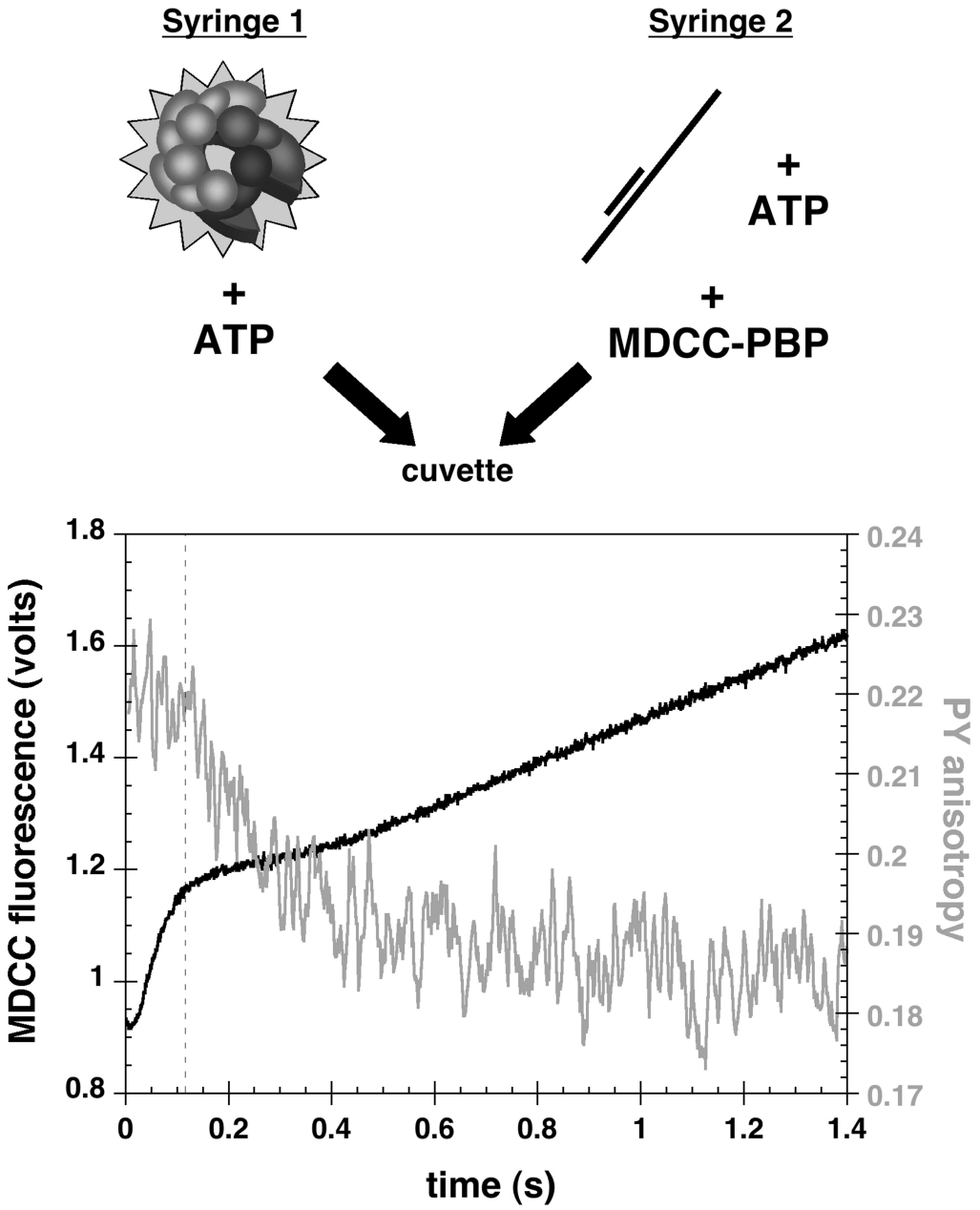

FIGURE 11 **Temporal correlation of ATP hydrolysis and β clamp release. Stopped-flow reactions were done using the MDCC-PBP-based assay to measure ATP hydrolysis (*black reaction trace*) and the β^{PY}-anisotropy-based assay to measure clamp release (*gray reaction trace*). In both reactions, a solution of primed template DNA and ATP was rapidly added to a solution of γ complex, β, and ATP as illustrated in the stopped-flow reaction scheme above the graph. Preincubation of the γ complex, β, and ATP results in formation of an ATP-activated clamp loader-clamp complex indicated by the star. In the ATPase reaction, MDCC-PBP was included in the solution with DNA and in the anisotropy experiment, β^{PY} was used along with a 20-fold excess unlabeled β as in Figure 10. A rapid increase in MDCC fluorescence due to ATP hydrolysis occurs over the first approximately 120 ms of the clamp loading reaction. During this time period, there is little if any change in the anisotropy of PY indicating that the β clamp is not released on DNA. A significant decrease in PY anisotropy resulting from clamp release occurs over the time period of approximately 120 to 800 ms. These data indicate that ATP hydrolysis by the γ complex occurs before release of the clamp on DNA during the clamp loading reaction (S.G. Anderson and L.B. Bloom, unpublished results). Final concentrations were 330 nM p/t DNA, 300 nM γ complex, 300 nM β^{PY}, 500 μM ATP, 6 μM unlabeled β in the clamp release assay and 4.5 μM MDCC-PBP in the ATPase assay.**

1999; Wu and Brand, 1994). Transfer of energy is a nonradiative process that results from dipole-dipole interactions between the donor and the acceptor. The efficiency of energy transfer (E) is a function of the photophysical properties of the donor-acceptor pair and the distance between donor and acceptor shown in equation 8 in which r is the distance between donor and acceptor and R_o is the Förster distance or the distance

at which energy transfer is 50% efficient.

$$E = \frac{1}{1 + \left(r/R_o\right)^6} \quad (8)$$

FRET typically occurs over distances of 10 to 100 Å, making it ideal for measurements of the proximity of two macromolecules or two regions within the same macromolecule (reviewed in Hillisch *et al.*, 2001; Lakowicz, 1999; Lilley and Wilson, 2000; Selvin, 1995; Stryer, 1978; Wu and Brand, 1994). When one macromolecule is labeled with a donor and a second with an acceptor, changes in interactions that bring the donor and acceptor closer together will increase the FRET efficiency, and changes that move them farther will decrease the FRET efficiency. FRET is most easily used to measure changes in distances between donor and acceptor that occur during the course of a reaction. FRET can also be used to measure absolute distances between donor and acceptor sites, but these measurements are more complicated, in that absolute FRET efficiencies must be accurately measured and R_o for the donor-acceptor pair must be determined which requires measuring a number of photophysical properties of the pair, including the lifetime of the donor and orientation factor.

The choice of a donor-acceptor pair depends on the distance range being measured and on the R_o value for a particular pair. As can be seen from equation 8, the FRET efficiency is very sensitive to distance when the distance between donor and acceptor, r, is close to R_o. Ideally, a donor-acceptor pair should be used that has an R_o value within a factor of 2 of the predicted donor-acceptor distance. A donor-acceptor distance of $0.5R_o$ gives a FRET efficiency of 98% and a distance of $2R_o$ gives a FRET efficiency of 1.5%. When the goal is to measure a change in distance, a pair should be chosen based on R_o so that the change in donor-acceptor distance has a large effect on FRET efficiency. Extrinsic donors and acceptors can be site-specifically incorporated into proteins by covalently modifying amino or sulfhydryl groups. Often, the terminal amino group can be selectively labeled (over lysine residues) by performing the labeling reaction around neutral pH. Tryptophan can also be used as a donor with several different extrinsic acceptors. Use of tryptophan as a donor is particularly useful when a donor and acceptor need to be incorporated in the same polypeptide.

FRET measurements have proved to be an extremely valuable tool for measuring dynamic and transient interactions that occur during the clamp loading reaction cycle (Alley *et al.*, 2000; Alley *et al.*, 1999b; Millar *et al.*, 2004; Soumillion *et al.*, 1998; Trakselis *et al.*, 2001a; Trakselis *et al.*, 2003) and between different components in the bacteriophage T4 replisome (Alley *et al.*, 2001; Xi *et al.*, 2005a; Xi *et al.*, 2005b; Zhang *et al.*, 2005). In the bacteriophage T4 clamp-loading reaction, FRET has been particularly useful in defining the stages at which the gp45 clamp opens and closes. By placing a donor on one monomer near the interface and placing an acceptor on the adjacent monomer across the interface, changes in FRET report on opening and closing of the interface which increase and decrease the distance between donor and acceptor. By mixing labeled and unlabeled clamps, subunit exchange can also be monitored by FRET. Subunit exchange studies done in the presence and absence of the clamp loader indicate that the gp45 clamp is loaded onto DNA by opening an interface rather than by a disassembly/reassembly mechanism (Soumillion *et al.*, 1998). One of the key findings of FRET studies of the gp45 clamp is that one interface between adjacent subunits is open in solution, whereas all three monomer interfaces are closed in the crystal structure (Alley *et al.*, 1999b; Millar *et al.*, 2004). On binding the open clamp in solution, the gp44/62 clamp loader opens the clamp further in a process that most likely requires hydrolysis of two molecules of ATP. Following DNA binding, hydrolysis of two more molecules of ATP is required to close the clamp, but the clamp is not closed completely (Alley *et al.*, 2000; Sexton *et al.*, 1998; Trakselis *et al.*, 2001a; Trakselis *et al.*, 2003). The bacteriophage T4 DNA polymerase ultimately binds the clamp in a manner in which the C-terminus is bound in the small opening left at the interface between clamp subunits to effectively close the ring (Alley *et al.*, 1999a; Alley *et al.*, 2001; Trakselis *et al.*, 2001a).

DYNAMICS OF CLAMP LOADING

Unlike other systems in which a protein must simply have a high affinity for its target to accomplish its function, the mechanics of clamp loading require that the affinity of the clamp loader for its targets, the clamp and DNA, be rapidly modulated during the course of the reaction. The clamp loader must bind the clamp and DNA to bring these macromolecules together, but

then must release both the clamp and DNA to allow the DNA polymerase to bind. Not only must the affinity be rapidly modulated, but the affinity must also be regulated in a coordinated fashion. If the clamp loader were to lose affinity for one substrate prior to binding the other, then this cycle of clamp loading would be unproductive. Changes in the intermolecular association of the clamp loader with the clamp and DNA are likely to be associated with changes in interactions within the clamp loading complex itself. ATP binding and hydrolysis are likely to alter interactions within individual subunits and between subunits of the clamp loader that alter the overall structure of the complex to modulate its interactions with the clamp and DNA. In addition to the bound nucleotide, interactions between the clamp loader and its targets, the clamp and DNA, must also affect changes. For example, if the ATP-bound form of a clamp loader were simply activated for binding the clamp and DNA, there would not necessarily be any driving force to release the clamp on DNA. However, if interactions between the clamp loader and one of its targets, say DNA, were to trigger a change that made the complex competent for ATP hydrolysis, then ATP hydrolysis could produce an ADP-bound state with lower affinity for the clamp and DNA. Alternatively, if the clamp loader were to spontaneously hydrolyze ATP and oscillate between high- and low-affinity states in the absence of its targets, then clamp loading would be inefficient, because the clamp loader would be spending much its time in the wrong binding state. The process of clamp loading is likely to comprise a series of binding reactions, each of which promotes some change in the clamp loader that alters its interaction with the next component of the pathway. This dynamic process would allow for the coordinated formation individual clamp-loader states that are required to perform each of the steps in the overall clamp loading reaction. The following section describes work that has been done to define the dynamic and transient interactions that are required for clamp loading using the *E. coli* clamp-loading reaction as a model system.

Clamp-Loader Interactions with DNA

Early studies of the DNA polymerase III holoenzyme demonstrated that the holoenzyme requires ATP to form a pre-initiation complex on DNA that is capable of rapid, on the order of 500 nt/sec, and processive DNA synthesis (Burgers and Kornberg, 1982; Fay *et al.*, 1981; Fay *et al.*, 1982; Hurwitz and Wickner, 1974; Wickner, 1976; Wickner and Hurwitz, 1974). The holoenzyme only hydrolyzes ATP in formation of the pre-initiation complexes and not during the elongation phase of synthesis (Burgers and Kornberg, 1982; O'Donnell, 1987; Wickner, 1976). In addition to contributing to the formation of a processive pre-initiation complex, the *E. coli* clamp loader has DNA-dependent ATPase activity (Lee and Walker, 1987; Onrust *et al.*, 1991). The steady-state ATPase activity of the clamp loader is stimulated to the greatest degree by primed template DNA and is greater in the presence of β than in the absence (Onrust *et al.*, 1991; Turner *et al.*, 1999). Although the DNA-dependent ATPase activity of the clamp loader shows that the clamp loader interacts with DNA in the absence of the clamp, this interaction is dynamic and requires real time techniques for characterization.

High affinity binding of the *E. coli* γ complex clamp loader ($\gamma_3\delta\delta'\chi\psi$ subunits) to DNA requires ATP binding, but the interaction with primed template DNA is transient (Ason *et al.*, 2000; Hingorani and O'Donnell, 1998; Turner *et al.*, 1999). Fluoresence-anisotropy-based experiments in which DNA substrates are covalently labeled with X-rhodamine have been used to characterize ATP-dependent γ complex–DNA interactions (Ason *et al.*, 2000; Ason *et al.*, 2003). The experiments show that γ complex binds p/t DNA rapidly, but on binding, the affinity of γ complex for DNA decreases so that DNA is rapidly released (Figure 12). In the steady-state regime of the reaction, after about 1 sec, the γ complex cycles between a high-affinity state that binds DNA rapidly and a low-affinity state that dissociates from DNA rapidly and relatively slowly converts back to the high-affinity binding state (Figure 12, diagram). This cycling reaction makes it appear that the clamp loader weakly binds p/t DNA in steady-state experiments because only a small population is transiently bound at any time. The p/t-DNA-triggered dissociation of γ complex is associated with ATP hydrolysis. The nonhydrolyzable ATP analog, ATPγS, supports binding of γ complex to p/t DNA, but the DNA-triggered dissociation of a low-affinity form is not observed in reactions with ATPγS (Ason *et al.*, 2000). In steady-state reactions, a greater population of p/t DNA is bound in reactions with ATPγS because the cycling reaction is inefficient in the absence of ATP hydrolysis (Ason *et al.*, 2000; Turner *et al.*, 1999). Consistent with these results, experiments measuring the temporal correlation

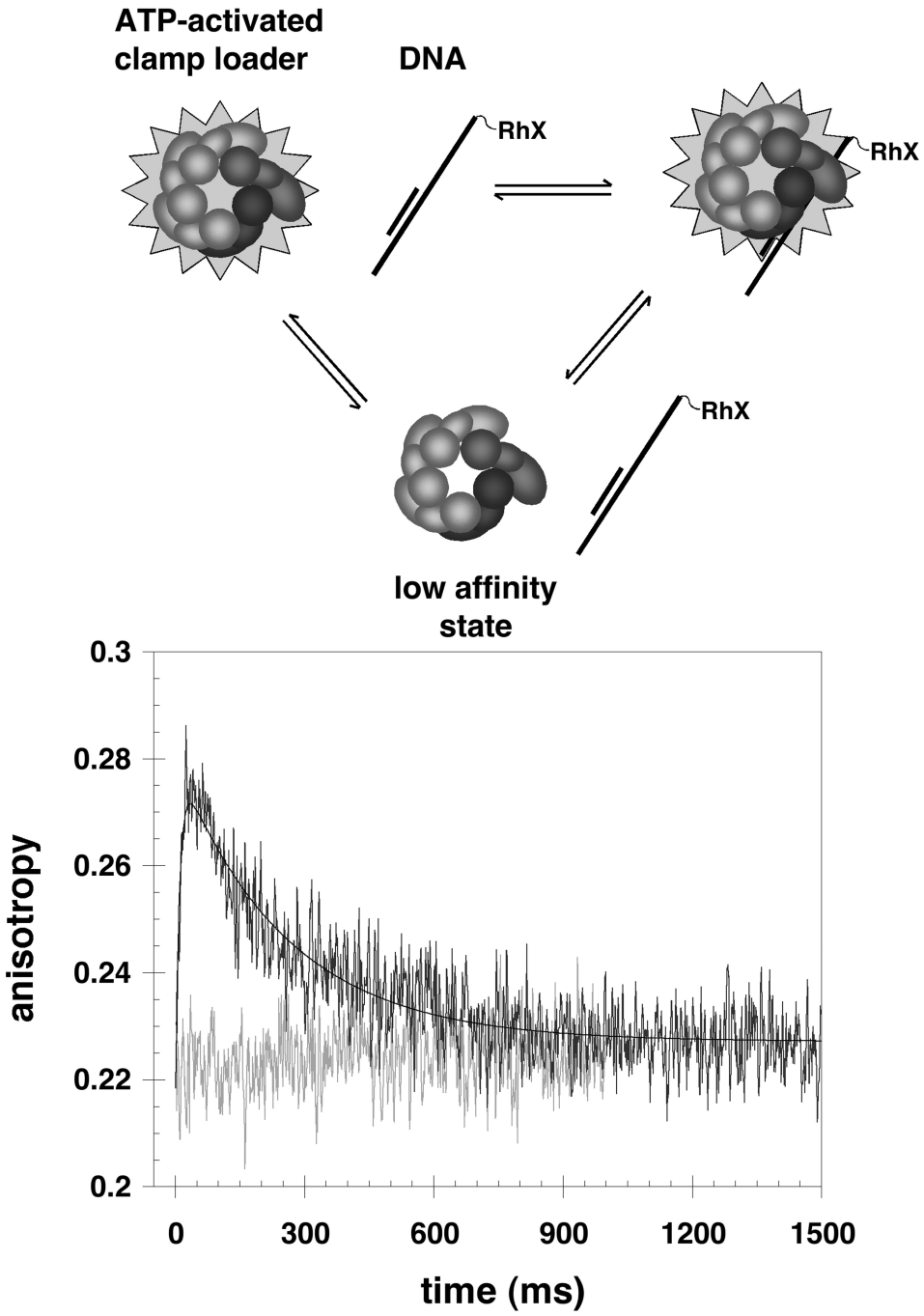

FIGURE 12 **Dynamic interaction of γ complex with primed template DNA (Ason *et al.*, 2000). A stopped-flow anisotropy assay was used to measure the kinetics of γ complex binding to a primed template covalently labeled on the 5′ template end with X-rhodamine. A solution of primed template DNA and ATP was rapidly added to a solution of γ complex and ATP. The reaction time course (*dark gray trace*) shows a rapid increase in anisotropy due to γ complex binding DNA followed by a decrease due to dissociation of γ complex from DNA. The diagram above the graph illustrates reactions that occur to give rise to the observed reaction time course. An ATP-activated form of the γ complex rapidly binds DNA with high affinity. DNA binding triggers a change in the γ complex converting it to a state with low affinity for DNA that rapidly dissociates. Conversion from the low affinity binding state of the clamp loader to the high affinity state is the slowest step in the reaction cycle so that at steady state times greater than about 1 s, a very small population of the DNA is bound at any given time. The light gray trace is the result of a mock reaction done by mixing labeled DNA with buffer and shows that there is no anisotropy change for DNA in the absence of the clamp loader. Final concentrations were 50 nM p/t DNA, 300 nM γ complex, and 500 μM ATP.**

of DNA binding and ATP hydrolysis show that γ complex binds p/t DNA rapidly prior to hydrolyzing ATP and subsequently dissociating (Ason *et al.*, 2003). DNA substrates of the correct structure for synthesis by a DNA polymerase, a single-stranded (ss) double-stranded (ds) DNA junction with a 5′ template overhang, preferentially trigger ATP hydrolysis and release of DNA from the γ complex. Single-stranded DNA

and ss/ds DNA junctions of incorrect polarity are significantly less efficient at triggering this reaction. As a result, binding interactions measured under steady-state conditions give the appearance that the clamp loader binds more tightly to DNA structures that would not be appropriate substrates for clamp loading and DNA synthesis. These results suggest a model in which ATP binding by the γ complex alters the conformation in some way to increase binding to DNA, subsequent binding to p/t DNA alters the conformation to make the complex competent for ATP hydrolysis, and hydrolysis alters the complex so that DNA is released.

The p/t-DNA-triggered ATP hydrolysis and release of DNA from the clamp loader is not an artifact of DNA binding experiments done in the absence of the clamp, but is relevant to the clamp loading reaction. Stopped-flow anisotropy experiments measuring clamp loading onto RhX-labeled DNA show that a pre-formed clamp-loader-clamp complex rapidly binds DNA and on binding, the γ complex releases the clamp onto DNA (Bertram *et al.*, 2000; Bloom *et al.*, 1996). In similar reactions with either single-stranded DNA or ATPγS, the clamp/DNA release step is inhibited showing that p/t DNA and ATP hydrolysis are required for efficient clamp release (Bertram *et al.*, 1998; Turner *et al.*, 1999). The temporal correlation between DNA binding and ATP hydrolysis in clamp-loading reactions was measured in identical stopped-flow experiments using anisotropy to measure clamp loading on RhX-labeled p/t DNA and the MDCC-PBP-based assay to measure the inorganic phosphate product of hydrolysis (Figure 13 (Bertram *et al.*, 2000)). These experiments show that a pre-formed β–γ complex rapidly binds DNA in a step that does not require ATP hydrolysis. Once bound, ATP is hydrolyzed and the γ complex dissociates from the clamp–DNA complex.

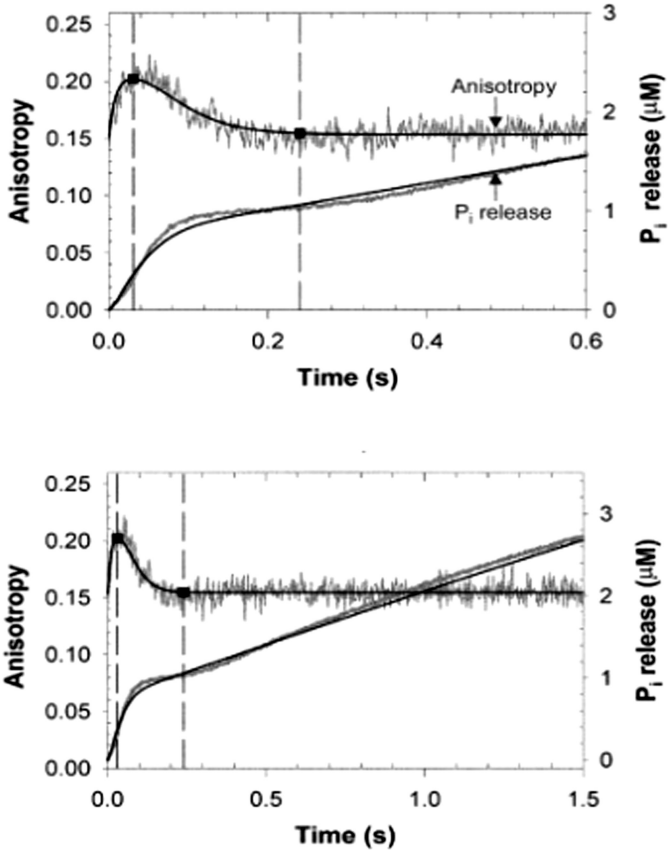

FIGURE 13 **Temporal correlation of DNA binding and ATP hydrolysis during a clamp loading reaction (Bertram *et al.*, 2000). Side-by-side experiments were done measuring DNA binding in an X-rhodamine-anisotropy-based assay and inorganic phosphate release from ATP hydrolysis in the MDCC-PBP-based ATPase assay. A pre-incubated solution of β, γ complex and ATP was rapidly mixed with a solution of primed template DNA and ATP. In the DNA binding reaction, DNA was labeled on the 5′ template end with X-rhodamine and in the ATPase reaction MDCC-PBP was included. The temporal correlation between DNA binding and P_i release shows that DNA binding occurs rapidly prior to ATP hydrolysis. P_i release is correlated with a decrease in anisotropy resulting from the clamp loader releasing the clamp and DNA. These results indicate that the DNA-triggered ATP hydrolysis is coupled to release of the clamp on DNA by the clamp loader. Final concentrations were 500 nM primed template DNA, 500 nM γ complex, 500 nM β, and 200 μM ATP.**

Physical measurement of clamps loaded onto circular DNA templates confirms the observations made in these real time kinetic assays. The γ complex exhibits specificity in clamp loading reactions and preferentially loads clamps on DNA substrates that contain an ss/ds DNA junction with a 5′ ss overhang (Yao *et al.*, 2000).

This DNA-triggered switch in the affinity of the clamp loader for the clamp and DNA makes two contributions to the clamp loading mechanism. First, it provides a dynamic mechanism by which the clamp loader recognizes the appropriates sites for loading clamps because those sites preferentially trigger the clamp loader to "fire" and release the clamp on DNA. Second, it provides a mechanism that allows the clamp loader to "hand" the loaded clamp to the polymerase. When the clamp loader fires at a p/t junction, the clamp and DNA are released, and the clamp loader transiently cycles into a state with reduced affinity for both. This gives the DNA polymerase the opportunity to bind the loaded clamp without competing with the clamp loader for binding. And, because both the clamp loader and the polymerase are part of the same holoenzyme complex, the polymerase is positioned to bind the clamp as soon as it is released.

Clamp Loader Interactions with the Clamp

Binding of ATP dramatically increases the affinity of the *E. coli* γ complex for the β clamp (Hingorani and O'Donnell, 1998; Naktinis *et al.*, 1995), such that the binding constant in the presence of ATP is about 1000-fold greater than in the absence (Naktinis *et al.*, 1995; Snyder *et al.*, 2004). In the ATP-bound state, the γ complex is also able to open the clamp (Hingorani and O'Donnell, 1998; Turner *et al.*, 1999). Studies with a clamp mutant containing a cross-link at one dimer interface to prevent opening indicate that the clamp loader only needs to open one interface to load clamps (Turner *et al.*, 1999). Binding to the β clamp does not stimulate the γ complex to hydrolyze ATP in the absence of DNA, whereas p/t DNA triggers hydrolysis by the clamp loader in the absence of β. Moreover, the presence of the β clamp inhibits the weak ATPase activity of the γ complex that occurs in the absence of DNA (Hingorani *et al.*, 1999; Onrust *et al.*, 1991; Turner *et al.*, 1999). In this respect, the interactions between the *E. coli* and bacteriophage T4 clamp loader and clamp differ. The gp44/62 clamp loader binds ATP prior to binding the gp45 clamp, but on binding gp45, gp44/62 most likely hydrolyzes two of the four molecules of ATP (Alley *et al.*, 2000; Sexton *et al.*, 1996; Sexton *et al.*, 1998; Trakselis *et al.*, 2001a; Trakselis *et al.*, 2003). However, it must be noted that another study suggests that formation of an open gp44/62–gp45 complex does not require ATP hydrolysis (Pietroni *et al.*, 2001). In solution, one interface between neighboring monomers of gp45 is in an open conformation, on hydrolysis of ATP, gp44/62 opens this interface further (Alley *et al.*, 2000; Alley *et al.*, 1999b; Millar *et al.*, 2004; Trakselis *et al.*, 2001a; Trakselis *et al.*, 2003). Although, ATP binding by the γ complex is sufficient to allow for clamp opening, the degree of opening is not known. It is possible that ATP binding partially opens the clamp, and ATP hydrolysis triggered by DNA opens the subunit-subunit interface further.

Biochemical studies of interactions between individual subunits of the γ complex and the β clamp showed that a single subunit, δ, binds the clamp with relatively high affinity (Naktinis *et al.*, 1995; Turner *et al.*, 1999). Experiments measuring the dissociation of clamps that were assembled on circular DNA substrates showed that the δ subunit dramatically increased the rate of clamp dissociation indicating that the δ subunit alone is capable of opening the clamp (Leu *et al.*, 2000; Stewart *et al.*, 2001). The clamp binding and opening activity of the δ subunit is regulated when δ is a part of the γ complex. Binding of ATP most likely induces conformational changes in the γ complex that exposes the β-binding site of the δ subunit in such a way as to allow the interaction between δ and β that opens the clamp (Hingorani and O'Donnell, 1998; Naktinis *et al.*, 1995; Turner *et al.*, 1999). Weaker interactions between other clamp loader subunits and the clamp may enhance this interaction and assist in clamp opening (Leu and O'Donnell, 2001).

ATP Binding and Hydrolysis

Clamp loaders contain multiple subunits that bind ATP. The *E. coli* complex contains three ATP binding sites, and bacteriophage T4 and eukaryotic complexes contain four ATP binding sites. Binding and hydrolysis of ATP could be an "all or none" process in which binding of ATP or hydrolysis at all of the sites is required to effect a change in affinity. Complete filling of the sites with ATP could activate the clamp loader for

binding both the clamp and DNA. And, hydrolysis of ATP at all sites could be required to release the clamp and DNA. Alternatively, ATP binding and hydrolysis could occur incrementally to effect changes that promote sequential binding and release of the clamp and DNA. In either case, interactions with the clamp and DNA are likely to promote changes in the clamp loader that affect the ATP binding and hydrolysis activity of the complex. Binding to the clamp or DNA could enhance ATP binding or promote ATP hydrolysis at some or all of the sites, or could do both. In work done to date, the major differences in the clamp-loading reactions catalyzed by the *E. coli*, bacteriophage T4, and *S. cerevisiae* clamp loaders seem to be at the steps in which ATP is bound and/or hydrolyzed.

ATP binding by the γ complex is sufficient to drive all the steps in the clamp-loading reaction up to the formation of a ternary complex between the clamp loader, clamp and DNA (Hingorani and O'Donnell, 1998; Naktinis *et al.*, 1995; Turner *et al.*, 1999). However, the number of molecules of ATP bound to the clamp loader at each step and the order in which each of the three ATP sites are filled has not yet been firmly established. Gel filtration experiments done under equilibrium conditions indicate that the γ complex ($\gamma_3\delta\delta'\chi\psi$ subunits) binds three molecules of ATP in the absence of the clamp and DNA, and that all three sites bind with similar affinity, a K_d of 1 to 2 μM (Johnson and O'Donnell, 2003). Isothermal titration calorimetry (ITC) experiments show that a minimal clamp loader complex ($\gamma_3\delta\delta'$ subunits) binds two molecules of ATP with equal affinity, K_d of 3.7 μM, in the absence of the clamp and DNA (Kazmirski *et al.*, 2004). The ITC results are consistent with the crystal structure of the minimal clamp loader, in which two molecules of ATPγS are bound and the third binding site is physically blocked (Jeruzalmi *et al.*, 2001a; Kazmirski *et al.*, 2004). The reason for the difference in the number of ATP molecules bound to the free clamp loaders is not yet clear. One possibility is that the χ and ψ subunits that are present in the γ complex but not in the minimal complex stabilize a conformation of the clamp loader in which the third ATP binding site is open allowing the third molecule of ATP to bind. Another possibility is that the free clamp loader only binds two molecules of ATP, and a stepwise series of binding events as have been seen in the yeast system occurs. Studies with yeast RFC suggest that RFC binds, in the following order, two molecules of ATP, PCNA, a third molecule of ATP, DNA, and a fourth molecule of ATP (Gomes *et al.*, 2001). Regardless of whether two or three molecules of ATP bind γ complex in the absence of the clamp and DNA, both experiments indicate that the sites that bind ATP do so with equal affinity and that there is little or no cooperativity in ATP binding. This would suggest that the ATP sites in the free clamp loader are not filled in a specific order.

On binding ATP, the γ complex undergoes conformational changes that increase the affinity of the clamp loader for the clamp and DNA (Ason *et al.*, 2000; Bertram *et al.*, 1998; Hingorani and O'Donnell, 1998; Naktinis *et al.*, 1995; Turner *et al.*, 1999). Structural data for an ATP-activated complex is not available so the physical changes that take place are not yet known. However, the kinetics of formation of a complex that is activated by ATP for DNA binding has been determined (Williams *et al.*, 2004). The ATP-induced conformational changes required for high affinity DNA binding are relatively slow. ATP-activation of the γ complex is likely to be the sum of several discrete conformational changes that produce the activated complex at an overall rate of 6.5 s^{-1}. When ATPase assays are initiated by the addition of ATP to the γ complex, hydrolysis is relatively slow and limited by the rate of these conformational changes (Williams *et al.*, 2004). It remains to be determined whether conformational changes that result in high affinity clamp binding occur at the same rate. It is possible that a different set of conformational changes within the γ complex modulates interactions with the clamp. Modeling the kinetics of ATP hydrolysis in the absence of the clamp suggests that the ATP-activated state binds and hydrolyzes three molecules of ATP (Williams *et al.*, 2004). The conformational changes that produce the ATP-activated state could occur after binding the third molecule of ATP, or alternatively, could reflect conformational changes that open a site to allow the third molecule of ATP to bind. The latter possibility is intriguing given that the minimal clamp loader ($\gamma_3\delta\delta'$) bound only two molecules of ATPγS in the crystal and the third site was physically blocked by an adjacent subunit.

Whereas ATP binding is sufficient for the formation of a ternary clamp loader–clamp–DNA complex, ATP hydrolysis is required for releasing the clamp on DNA (Bertram *et al.*, 1998; Turner *et al.*, 1999). Incubation of the γ complex ($\gamma_3\delta\delta'\chi\psi$ subunits) with ATP and the clamp produces a β–γ complex that is activated for DNA binding and clamp loading. Binding of this ATP-activated β–γ complex to p/t DNA triggers a burst of

ATP hydrolysis (Figures 11 and 13). Careful measurement of the total protein concentration and concentration of ADP product indicate three molecules of ATP per molecule of γ complex are likely to be hydrolyzed in this step (Williams *et al.*, 2004). These results suggest that all three sites in the γ complex hydrolyze ATP at the same global stage of the reaction, releasing the clamp on DNA (Bertram *et al.*, 2000). This differs from the bacteriophage T4 reaction clamp loading reaction in which the gp44/62 clamp loader most likely hydrolyzes two molecules of ATP on binding the clamp and the remaining two molecules on binding DNA (Alley *et al.*, 2000; Pietroni *et al.*, 2001; Sexton *et al.*, 1996; Sexton *et al.*, 1998; Trakselis and Benkovic, 2001; Trakselis *et al.*, 2003; Young *et al.*, 1996). There may also be some interesting differences in the stage at which ATP is hydrolyzed in the clamp-loading reaction catalyzed by the clamp loader associated with the *E. coli* DNA polymerase III holoenzyme. The composition of the clamp loader in the holoenzyme is most likely $\tau_2\gamma\delta\delta'\chi\psi$ and each τ subunit is bound to a core polymerase via contacts with the α subunit (reviewed in Johnson and O'Donnell, 2005; McHenry, 2003). Experiments with the nonhydrolyzable analog, ATPγS, show that the holoenzyme can load one clamp to form a processive DNA polymerase complex (Glover and McHenry, 2001). Further clamp loading requires ATP hydrolysis. This suggests that at the replication fork, ATP binding may be sufficient to drive all steps leading to the formation of a pre-initiation complex on the leading strand and subsequent loading of clamps on the lagging strand requires ATP hydrolysis (Glover and McHenry, 2001). It is possible that differences in protein–protein interactions are responsible for this difference in requirement for ATP hydrolysis between the γ complex and clamp loader in the holoenzyme. The τ subunits in the holoenzyme contain an additional C-terminal region and make contacts with the core polymerase that are not present in the γ complex. Alternatively, it is possible that the physical association of the polymerase with the clamp loader in the holoenzyme allows the formation of a functional replication complex by a mechanism that differs from the ATP-hydrolysis-dependent formation of a pre-initiation complex such as a passive dissociation reaction (see Figure 11).

Although the clamp does not induce the γ complex to hydrolyze ATP in the absence of DNA, β does increase the overall rate of hydrolysis in the presence of DNA (Bertram *et al.*, 1998; Hingorani *et al.*, 1999; Onrust *et al.*, 1991; Turner *et al.*, 1999). In the absence of the clamp, the ATP-activated form of the γ complex hydrolyzes two molecules of ATP more rapidly than the third (Williams *et al.*, 2004). Equilibration of the γ complex with ATP and β produces an ATP-activated β–γ complex that hydrolyzes all three molecules of ATP at the same rapid rate as the two molecules hydrolyzed in the absence of the clamp (Williams *et al.*, 2004). These results show that binding of β to the ATP-activated clamp loader alters the complex in some way to increase the efficiency of ATP hydrolysis at one of the three sites. This is consistent with the notion that each component that binds the clamp loader produces a change that affects the reaction. DNA binding alters the γ complex in some way to trigger hydrolysis, but clamp binding is required to produce a change that allows one of the three sites to hydrolyze ATP efficiently.

The ATP binding sites in the γ subunits are located at the interface between two adjacent subunits (Jeruzalmi *et al.*, 2001a; Kazmirski *et al.*, 2004; Podobnik *et al.*, 2003). This interfacial location is likely to provide a mechanism by which ATP binding and hydrolysis at individual sites could be communicated between subunits and translated to changes in the whole complex. Conserved Arg fingers are present in the δ' subunit and γ subunits that extend to the ATP binding site of the adjacent subunit and that could sense changes in the bound nucleotide or possibly trigger hydrolysis (Jeruzalmi *et al.*, 2001a; Kazmirski *et al.*, 2004; Ogura *et al.*, 2004). Although these Arg fingers are too far from the ATP binding sites in the crystal structures of the "inactive" states of the minimal clamp loader, biochemical evidence suggest that these Arg residues play a role in nucleotide-dependent changes in the clamp loader (Johnson and O'Donnell, 2003; Kazmirski *et al.*, 2004; Snyder *et al.*, 2004). Asymmetry in the ATP sites can be introduced by reconstituting γ complex with either an Arg finger mutant of δ' (δ'R158A) or Arg finger mutants of γ (γR169A) (Figure 14). The Arg finger mutant γ complexes bind ATP with the same affinity as wild-type γ complex, but the ATP hydrolysis activities of the mutant complexes are reduced differentially (Johnson and O'Donnell, 2003). The Arg mutations in the γ subunit have a much more pronounced effect on hydrolysis of ATP than the δ' mutation. Interestingly, mutations in the γ versus δ' subunits also have a differential effect on the ATP-dependent binding to the clamp and DNA (Snyder *et al.*, 2004). The ATP-dependent β binding activity of γ complex with the δ'R158A mutation

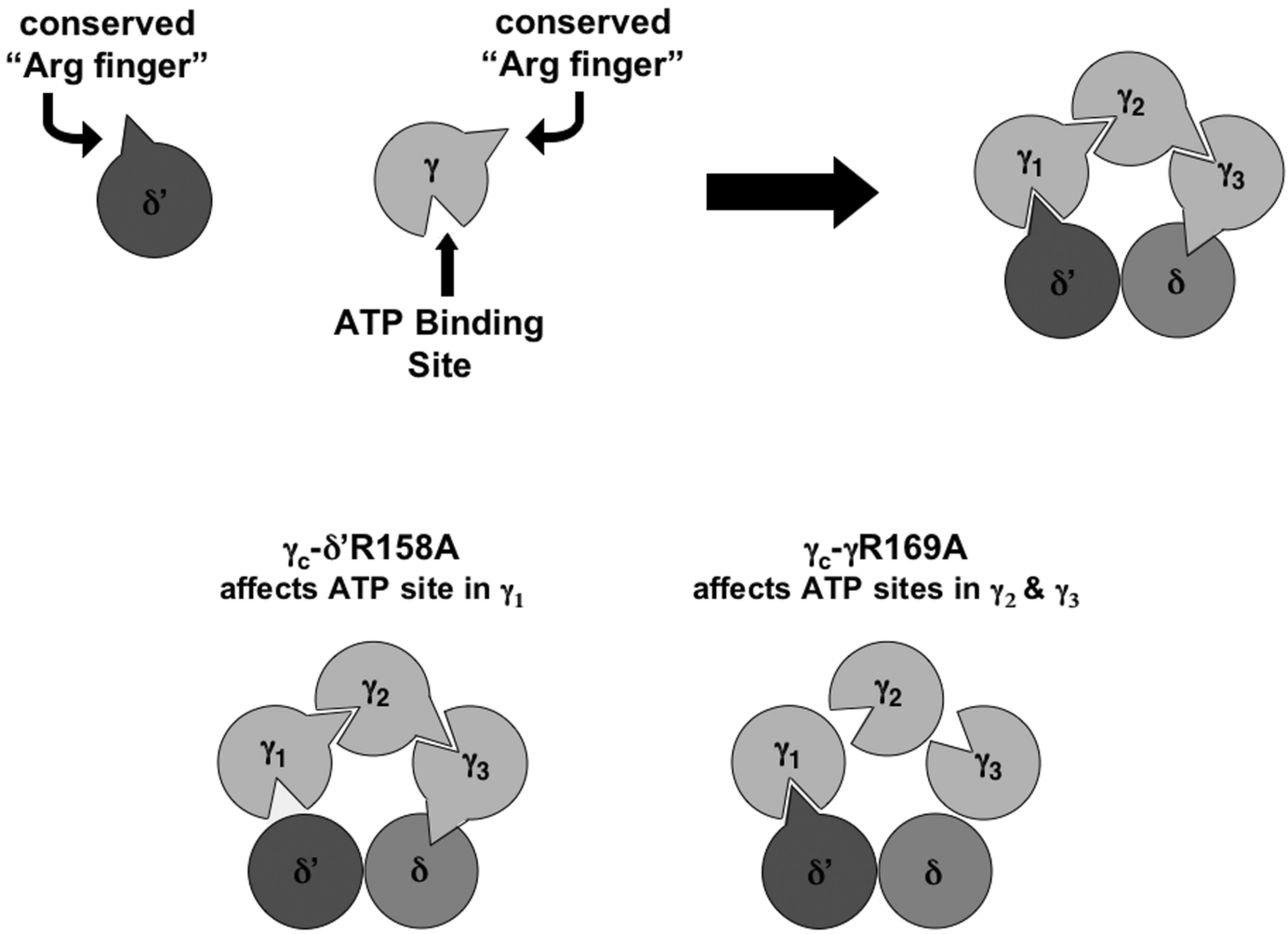

FIGURE 14 **Diagram illustrating the juxtaposition of conserved arginine fingers and ATP binding sites in the $\gamma_3\delta\delta'$ complex. Each of the three γ subunits contain an arginine (Arg) finger and ATP binding site. Arg fingers from one subunit extend towards ATP binding sites in the adjacent subunit as illustrated (Jeruzalmi *et al.*, 2001a; Kazmirski *et al.*, 2004). The δ' subunit contains an Arg finger but lacks an ATP binding site. Mutation of the Arg finger in the δ' subunit potentially affects ATP binding and hydrolysis in the γ_1 subunit and mutation of the Arg fingers in the γ subunits potentially affects ATP binding and hydrolysis in the γ_2 and γ_3 subunits.**

(see Figure 9) is reduced to a greater extent than that for the γR169A mutant. Conversely, the γR169A mutant shows very little binding to DNA, whereas the δ'R158A mutant complex binds DNA with about the same affinity as the wild-type γ complex. These studies revealed that the Arg fingers in the γ complex play an important role in sensing bound ATP and translating ATP binding into conformational changes increase the affinity of the γ complex for β and DNA. It is possible that the Arg fingers also either sense or are needed to catalyze ATP hydrolysis, but these functions may be more difficult to establish because these mutations affect binding of the γ complex to β and DNA and these binding interactions in turn affect ATP hydrolysis. Because of the differential effects of the γ and δ' mutations on the binding activities of the complexes to β and DNA, these results suggest that ATP binding at one subset of sites, γ_2 and/or γ_3, is largely responsible for increasing the affinity of the complex for DNA, and ATP binding at another, γ_1, is largely responsible for increasing the affinity of the complex for β (Snyder *et al.*, 2004). The possibility that one subset of sites is largely responsible for modulating interactions with β whereas another modulates interactions with DNA is supported by kinetics of ATP hydrolysis. In the absence of the clamp, the γ complex hydrolyzes two molecules of ATP faster than the third, suggesting that DNA binding efficiently triggers hydrolysis at two sites, but β binding is required for efficient hydrolysis at the third (Williams *et al.*, 2004).

SUMMARY

The combined efforts of many laboratories have started to unravel the ordered series of binding

interactions and subsequent changes in the *E. coli* clamp loader that are necessary to complete the mechanical clamp-loading cycle. Binding of ATP alters the clamp loader in some way as to increase the affinity of the γ complex for the clamp and DNA. It is likely that ATP binding at one subset of sites, γ_2 and/or γ_3, promotes changes that enhance the interaction with DNA and binding to another, γ_1, enhances interactions with the clamp. Binding to β alters the γ complex in some way that increases the efficiency with which one of the three sites hydrolyzes ATP. On binding primed template DNA, the DNA triggers a change in the clamp loader that induces hydrolysis of ATP. ATP hydrolysis reduces the affinity of the clamp loader for the clamp and DNA so that the γ complex releases the clamp on DNA and transiently cycles into an inactive state so as not to compete with the polymerase for binding loaded clamps. Nucleotide exchange, ADP for ATP, is presumably required to repeat the cycle. Although structural and biochemical studies have answered many questions about the clamp loading reaction cycle, many more remain. One key question is whether or not interactions between the clamp loader other proteins at the replication fork alter the clamp-loading reaction cycle. Lessons learned from the investigation of the clamp loading mechanism may also be relevant to the mechanism of other enzymes that couple NTP binding and hydrolysis to mechanical reaction cycles.

ACKNOWLEDGMENTS

Research in L. B. Bloom's laboratory is supported by National Institutes of Health grant GM055596. I thank Stephen G. Anderson for critically reading this review.

REFERENCES

Alley, S.C., Abel-Santos, E., and Benkovic, S.J. 2000. Tracking sliding clamp opening and closing during bacteriophage T4 DNA polymerase holoenzyme assembly. *Biochemistry* 39:3076–3090.

Alley, S.C., Jones, A.D., Soumillion, P., and Benkovic, S.J. 1999a. The carboxyl terminus of the bacteriophage T4 DNA polymerase contacts its sliding clamp at the subunit interface. *J Biol Chem* 274:24485–24489.

Alley, S.C., Shier, V.K., Abel-Santos, E., Sexton, D.J., Soumillion, P., and Benkovic, S.J. 1999b. Sliding clamp of the bacteriophage T4 polymerase has open and closed subunit interfaces in solution. *Biochemistry* 38:7696–7709.

Alley, S.C., Trakselis, M.A., Mayer, M.U., Ishmael, F.T., Jones, A.D., and Benkovic, S.J. 2001. Building a replisome solution structure by elucidation of protein-protein interactions in the bacteriophage T4 DNA polymerase holoenzyme. *J Biol Chem* 276:39340–39349.

Ason, B., Bertram, J.G., Hingorani, M.M., Beechem, J.M., O'Donnell, M., Goodman, M.F., and Bloom, L.B. 2000. A model for *Escherichia coli* DNA polymerase III holoenzyme assembly at primer/template ends: DNA triggers a change in binding specificity of the γ complex clamp loader. *J Biol Chem* 275:3006–3015.

Ason, B., Handayani, R., Williams, C.R., Bertram, J.G., Hingorani, M.M., O'Donnell, M., Goodman, M.F., and Bloom, L.B. 2003. Mechanism of loading the *Escherichia coli* DNA polymerase III beta sliding clamp on DNA. Bona fide primer/templates preferentially trigger the gamma complex to hydrolyze ATP and load the clamp. *J Biol Chem* 278:10033–10040.

Bell, S.P. and Dutta, A.2002. DNA replication in eukaryotic cells. *Annu Rev Biochem* 71:333–374.

Benkovic, S.J., Valentine, A.M., and Salinas, F. 2001. Replisome-mediated DNA replication. *Annu Rev Biochem* 70:181–208.

Bertram, J.G., Bloom, L.B., Hingorani, M.M., Beechem, J.M., O'Donnell, M., and Goodman, M.F. 2000. Molecular mechanism and energetics of clamp assembly in *Escherichia coli*. The role of ATP hydrolysis when γ complex loads β on DNA. *J Biol Chem* 275:28413–28420.

Bertram, J.G., Bloom, L.B., Turner, J., O'Donnell, M., Beechem, J.M., and Goodman, M.F. 1998. Pre-steady state analysis of the assembly of wild type and mutant circular clamps of *Escherichia coli* DNA polymerase III onto DNA. *J Biol Chem* 273:24564–24574.

Blinkowa, A.L., and Walker, J.R. 1990. Programmed ribosomal frameshifting generates the *Escherichia coli* DNA polymerase III gamma subunit from within the tau subunit reading frame. *Nucleic Acids Res* 18:1725–1729.

Bloom, L.B., Turner, J., Kelman, Z., Beechem, J.M., O'Donnell, M., and Goodman, M.F. 1996. Dynamics of loading the β sliding clamp of DNA polymerase III onto DNA. *J Biol Chem* 271:30699–30708.

Bonner, C.A., Stukenberg, P.T., Rajagopalan, M., Eritja, R., O'Donnell, M., McEntee, K., Echols, H., and Goodman, M.F. 1992. Processive DNA synthesis by DNA polymerase II mediated by DNA polymerase III accessory proteins. *J Biol Chem* 267:11431–11438.

Bowman, G.D., Goedken, E.R., Kazmirski, S.L., O'Donnell, M., and Kuriyan, J. 2005. DNA polymerase clamp loaders and DNA recognition. *FEBS Lett* 579:863–867.

Bowman, G.D., O'Donnell, M., and Kuriyan, J.2004. Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex. *Nature* 429:724–730.

Brune, M., Hunter, J.L., Corrie, J.E.T., and Webb, M.R. 1994. Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase. *Biochemistry* 33:8262–8271.

Brune, M., Hunter, J.L., Howell, S.A., Martin, S.R., Hazlett, T.L., Corrie, J.E., and Webb, M.R. 1998. Mechanism of inorganic phosphate interaction with phosphate binding protein from *Escherichia coli*. *Biochemistry* 37:10370–10380.

Burgers, P.M. 1991. Saccharomyces cerevisiae replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerases delta and epsilon. *J Biol Chem* 266:22698–22706.

Burgers, P.M.J. and Kornberg, A. 1982. ATP activation of DNA polymerase III holoenzyme of *Escherichia coli*. I. ATP-dependent formation of an initiation complex with a primed template. *J Biol Chem* 257:11468–11473.

Cai, J., Gibbs, E., Uhlmann, F., Phillips, B., Yao, N., O'Donnell, M., and Hurwitz, J. 1997. A complex consisting of human replication factor C p40, p37, and p36 subunits is a DNA-dependent ATPase and an intermediate in the assembly of the holoenzyme. *J Biol Chem* 272:18974–18981.

Capson, T.L., Benkovic, S.J., and Nossal, N.G. 1991. Protein-DNA crosslinking demonstrates stepwise ATP-dependent assembly of T4 DNA polymerase and its accessory proteins on the primer-template. *Cell* 65:249–258.

Clegg, R.M. 1995. Fluorescence resonance energy transfer. *Curr Opin Biotechnol* 6:103–110.

Cullmann, G., Fien, K., Kobayashi, R., and Stillman, B. 1995. Characterization of the five replication factor C genes of Saccharomyces cerevisiae. *Mol Cell Biol* 15:4661–4671.

Dallmann, H.G., Thimmig, R.L., and McHenry, C.S. 1995. DnaX complex of *Escherichia coli* DNA polymerase III holoenzyme. Central role of tau in initiation complex assembly and in determining the functional asymmetry of holoenzyme. *J Biol Chem* 270:29555–29562.

Dalrymple, B.P., Kongsuwan, K., Wijffels, G., Dixon, N.E., and Jennings, P. 2001. A universal protein-protein interaction motif in the eubacterial DNA replication and repair systems. *Proc Natl Acad Sci USA* 98:11627–11632.

Davey, M.J., Jeruzalmi, D., Kuriyan, J., and O'Donnell, M. 2002. Motors and switches: AAA+ machines within the replisome. *Nat Rev Mol Cell Biol* 3:826–835.

Dohrmann, P.R. and McHenry, C.S. 2005. A bipartite polymerase-processivity factor interaction: only the internal beta binding site of the alpha subunit is required for processive replication by the DNA polymerase III holoenzyme. *J Mol Biol* 350:228–239.

Ellison, V. and Stillman, B. 2001. Opening of the clamp: an intimate view of an ATP-driven biological machine. *Cell* 106:655–660.

Fay, P.J., Johanson, K.O., McHenry, C.S., and Bambara, R.A. 1981. Size classes of products synthesized processively by DNA polymerase III and DNA polymerse III holoenzyme of *Escherichia coli. J Biol Chem* 256:976–983.

Fay, P.J., Johanson, K.O., McHenry, C.S., and Bambara, R.A. 1982. Size classes of products synthesized processively by two subassemblies of *Escherichia coli* DNA polymerase III holoenzyme. *J Biol Chem* 257:5692–5699.

Fersht, A. 1999. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*. New York: W.H. Freeman and Company, pp. 132–158.

Fien, K. and Stillman, B. 1992. Identification of replication factor C from Saccharomyces cerevisiae: a component of the leading-strand DNA replication complex. *Mol Cell Biol* 12:155–163.

Fierke, C.A. and Hammes, G.G. 1995. Transient kinetic approaches to enzyme mechanisms. *Methods Enzymol* 249:3–37.

Flower, A.M. and McHenry, C.S. 1990. The gamma subunit of DNA polymerase III holoenzyme of *Escherichia coli* is produced by ribosomal frameshifting. *Proc Natl Acad Sci USA* 87:3713–3717.

Fotedar, R., Mossi, R., Fitzgerald, P., Rousselle, T., Maga, G., Brickner, H., Messier, H., Kasibhatla, S., Hubscher, U., and Fotedar, A. 1996. A conserved domain of the large subunit of replication factor C binds PCNA and acts like a dominant negative inhibitor of DNA replication in mammalian cells. *EMBO J* 15:4423–4433.

Garg, P. and Burgers, P. M. 2005. DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit Rev Biochem Mol Biol* 40:115–128.

Glover, B.P. and McHenry, C.S. 2000. The DnaX-binding subunits δ' and ψ are bound to γ and not τ in the DNA polymerase III holoenzyme. *J Biol Chem* 275:3017–3020.

Glover, B.P. and McHenry, C.S. 2001. The DNA polymerase III holoenzyme: an asymmetric dimeric replicative complex with leading and lagging strand polymerases. *Cell* 105:925–934.

Gomes, X.V., Schmidt, S.L., and Burgers, P.M. 2001. ATP utilization by yeast replication factor C. II. Multiple stepwise ATP binding events are required to load proliferating cell nuclear antigen onto primed DNA. *J Biol Chem* 276:34776–34783.

Griep, M.A. and McHenry, C.S. 1988. The dimer of the β subunit of *Escherichia coli* DNA polymerase III holoenzyme is dissociated into monomers upon binding magnesium (II). *Biochemistry* 27:5210–5215.

Guenther, B., Onrust, R., Sali, A., O'Donnell, M., and Kuriyan, J. 1997. Crystal structure of the delta' subunit of the clamp-loader complex of *E. coli* DNA polymerase III. *Cell* 91:335–345.

Gulbis, J.M., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. 1996. Structure of the C-terminal region of $p21^{WAF1/CIP1}$ complexed with human PCNA. *Cell* 87:297–306.

Hill, J.J. and Royer, C.A. 1997. Fluorescence approaches to the study of protein–nucleic acid complexation. *Methods Enzymol* 278:390–416.

Hillisch, A., Lorenz, M., and Diekmann, S. 2001. Recent advances in FRET: distance determination in protein-DNA complexes. *Curr Opin Struct Biol* 11:201–207.

Hingorani, M.M., Bloom, L.B., Goodman, M.F., and O'Donnell, M. 1999. Division of labor-sequential ATP hydrolysis drives assembly of a DNA polymerase sliding clamp around DNA. *EMBO J* 18:5131–5144.

Hingorani, M.M. and O'Donnell, M. 1998. ATP binding to the *Escherichia coli* clamp loader powers opening of the ring-shaped clamp of DNA polymerase III holoenzyme. *J Biol Chem* 273:24550–24563.

Hirshberg, M., Henrick, K., Haire, L.L., Vasisht, N., Brune, M., Corrie, J.E., and Webb, M.R. 1998. Crystal structure of phosphate binding protein labeled with a coumarin fluorophore, a probe for inorganic phosphate. *Biochemistry* 37:10381–10385.

Hurwitz, J. and Wickner, S. 1974. Involvement of two protein factors and ATP in in vitro DNA synthesis catalyzed by DNA polymerase 3 of *Escherichia coli. Proc Natl Acad Sci USA* 71:6–10.

Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. 2001a. Crystal structure of the processivity clamp loader gamma (γ) complex of *E. coli* DNA polymerase III. Cell 106:429–441.

Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. 2002. Clamp loaders and sliding clamps. *Curr Opin Struct Biol* 12:217–224.

Jeruzalmi, D., Yurieva, O., Zhao, Y., Young, M., Stewart, J., Hingorani, M., O'Donnell, M., and Kuriyan, J. 2001b. Mechanism of processivity clamp opening by the delta-subunit wrench of the clamp loader complex of *E. coli* DNA polymerase III. *Cell* 106:417–428.

Johnson, A. and O'Donnell, M. 2003. Ordered ATP hydrolysis in the gamma complex clamp loader AAA+ machine. *J Biol Chem* 278:14406–14413.

Johnson, A. and O'Donnell, M. 2005. Cellular DNA replicases: components and dynamics at the replication fork. *Annu Rev Biochem* 74:283–315.

Johnson, K.A. 1986. Rapid kinetic analysis of mechanochemical adenosinetriphosphotases. *Methods Enzymol* 134:677–705.

Johnson, K.A. 1992. Transient-state kinetic analysis of enzyme reaction pathways. *Enzymes* 20:1–61.

Kao, H.I. and Bambara, R.A. 2003. The protein components and mechanism of eukaryotic Okazaki fragment maturation. *Crit Rev Biochem Mol Biol* 38:433–452.

Kazmirski, S.L., Podobnik, M., Weitze, T.F., O'Donnell, M., and Kuriyan, J. 2004. Structural analysis of the inactive state of the *Escherichia coli* DNA polymerase clamp-loader complex. *Proc Natl Acad Sci USA* 101:16750–16755.

Kazmirski, S.L., Zhao, Y., Bowman, G.D., O'Donnell, M., and Kuriyan, J. 2005. Out-of-plane motions in open sliding clamps:Molecular dynamics simulations of eukaryotic and archaeal proliferating cell nuclear antigen. *Proc Natl Acad Sci USA* 102:13801–13806.

Kim, D.R. and McHenry, C.S. 1996. Identification of the beta-binding domain of the alpha subunit of *Escherichia coli* polymerase III holoenzyme. *J Biol Chem* 271:20699–20704.

Kim, S., Dallman, H.G., McHenry, C.S., and Marians, K.J. 1996a. Coupling of a replicative polymerase and helicase: A tau-DnaB interaction mediates rapid replication fork movement. *Cell* 84:643–650.

Kim, S., Dallmann, H.G., McHenry, C.S., and Marians, K.J. 1996b. tau couples the leading- and lagging-strand polymerases at the *Escherichia coli* DNA replication fork. *J Biol Chem* 271:21406–21412.

Kim, S., Dallmann, H.G., McHenry, C.S., and Marians, K.J. 1996c. Tau protects beta in the leading-strand polymerase complex at the replication fork. *J Biol Chem* 271:4315–4318.

Kong, X.-P., Onrust, R., O'Donnell, M., and Kuriyan, J. 1992. Three-dimensional structure of the β subunit of *E. coli* DNA polymerase III holoenzyme: A sliding DNA clamp. *Cell* 69:425–437.

Krishna, T.S., Kong, X.-P., Gary, S., Burgers, P.M., and Kuriyan, J. 1994. Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell* 79:1233–1243.

Lakowicz, J.R. 1999. *Principles of Fluorescence Spectroscopy.* New York: Plenum Publishers, pp. 291–319.

Latham, G.J., Bacheller, D.J., Pietroni, P., and von Hippel, P.H. 1997. Structural analyses of gp45 sliding clamp interactions during assembly of

the bacteriophage T4 DNA polymerase holoenzyme. II. The Gp44/62 clamp loader interacts with a single defined face of the sliding clamp ring. *J Biol Chem* 272:31677–31684.
Ledvina, P.S., Yao, N., Choudhary, A., and Quiocho, F.A. 1996. Negative electrostatic surface potential of protein sites specific for anionic ligands. *Proc Natl Acad Sci USA* 93:6786–6791.
Lee, S.H. and Hurwitz, J. 1990. Mechanism of elongation of primed DNA by DNA polymerase delta, proliferating cell nuclear antigen, and activator 1. *Proc Natl Acad Sci USA* 87:5672–5676.
Lee, S.H. and Walker, J.R. 1987. Escherichia coli DnaX product, the tau subunit of DNA polymerase III, is a multifunctional protein with single-stranded DNA-dependent ATPase activity. *Proc Natl Acad Sci USA* 84:2713–2717.
Leu, F.P., Georgescu, R., and O'Donnell, M. 2003. Mechanism of the *E. coli* tau processivity switch during lagging-strand synthesis. *Mol Cell* 11:315–327.
Leu, F.P., Hingorani, M.M., Turner, J., and O'Donnell, M. 2000. The δ subunit of DNA polymerase III holoenzyme serves as a sliding clamp unloader in *Escherichia coli*. *J Biol Chem* 275:34609–34618.
Leu, F.P. and O'Donnell, M. 2001. Interplay of clamp loader subunits in opening the beta sliding clamp of *Escherichia coli* DNA polymerase III holoenzyme. *J Biol Chem* 276:47185–47194.
Lilley, D.M. and Wilson, T.J. 2000. Fluorescence resonance energy transfer as a structural tool for nucleic acids. *Curr Opin Chem Biol* 4:507–517.
Lopez De Saro, F.J., Georgescu, R.E., Goodman, M.F., and O'Donnell, M. 2003a. Competitive processivity-clamp usage by DNA polymerases during DNA replication and repair. *EMBO J* 22:6408–6418.
Lopez De Saro, F.J., Georgescu, R.E., and O'Donnell, M. 2003b. A peptide switch regulates DNA polymerase processivity. *Proc Natl Acad Sci USA* 100:14689–14694.
Lopez de Saro, F.J., and O'Donnell, M. 2001. Interaction of the beta sliding clamp with MutS, ligase, and DNA polymerase I. *Proc Natl Acad Sci USA* 98:8376–8380.
Luecke, H. and Quiocho, F.A. 1990. High specificity of a phosphate transport protein determined by hydrogen bonds. *Nature* 347:402–406.
Maga, G. and Hubscher, U. 2003. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J Cell Sci* 116:3051–3060.
Majka, J. and Burgers, P.M. 2004. The PCNA-RFC families of DNA clamps and clamp loaders. *Prog Nucleic Acid Res Mol Biol* 78:227–260.
Maki, S. and Kornberg, K. 1988. DNA polymerase III holoenzyme of *Escherichia coli*. II. A novel complex including the γ subunit essential for processive synthesis. *J Biol Chem* 263:6555–6560.
Marians, K.J. 2004. Mechanisms of replication fork restart in *Escherichia coli*. *Philos Trans R Soc Lond B Biol Sci* 359:71–77.
Matsumiya, S., Ishino, Y., and Morikawa, K. 2001. Crystal structure of an archaeal DNA sliding clamp: proliferating cell nuclear antigen from Pyrococcus furiosus. *Protein Sci* 10:17–23.
McHenry, C.S. 1982. Purification and characterization of DNA polymerase III′. Identification of tau as a subunit of the DNA polymerase III holoenzyme. *J Biol Chem* 257:2657–2663.
McHenry, C.S. 2003. Chromosomal replicases as asymmetric dimers: studies of subunit arrangement and functional consequences. *Mol Microbiol* 49:1157–1165.
Millar, D., Trakselis, M.A., and Benkovic, S.J. 2004. On the solution structure of the T4 sliding clamp (gp45). *Biochemistry* 43:12723–12727.
Miyata, T., Oyama, T., Mayanagi, K., Ishino, S., Ishino, Y., and Morikawa, K. 2004. The clamp-loading complex for processive DNA replication. *Nat Struct Mol Biol* 11:632–636.
Miyata, T., Suzuki, H., Oyama, T., Mayanagi, K., Ishino, Y., and Morikawa, K. 2005. Open clamp structure in the clamp-loading complex visualized by electron microscopic image analysis. *Proc Natl Acad Sci USA* 102:13795–13800.
Moarefi, I., Jeruzalmi, D., Turner, J., O'Donnell, M., and Kuriyan, J. 2000. Crystal structure of the DNA polymerase processivity factor of T4 bacteriophage. *J Mol Biol* 296:1215–1223.
Mossi, R., Jonsson, Z.O., Allen, B.L., Hardin, S.H., and Hubscher, U. 1997. Replication factor C interacts with the C-terminal side of proliferating cell nuclear antigen. *J Biol Chem* 272:1769–1776.
Naktinis, V., Onrust, R., Fang, F., and O'Donnell, M. 1995. Assembly of a chromosomal replication machine: Two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. II. Intermediate complex between the clamp loader and its clamp. *J Biol Chem* 270:13358–13365.
Naktinis, V., Turner, J., and O'Donnell, M. 1996. A molecular switch in a replication machine defined by an internal competition for protein rings. *Cell* 84:137–145.
O'Donnell, M.E. 1987. Accessory proteins bind a primed template and mediate rapid cycling of DNA polymerase III holoenzyme from *Escherichia coli*. *J Biol Chem* 262:16558–16565.
Ogura, T., Whiteheart, S.W., and Wilkinson, A.J. 2004. Conserved arginine residues implicated in ATP hydrolysis, nucleotide-sensing, and inter-subunit interactions in AAA and AAA+ ATPases. *J Struct Biol* 146:106–112.
Ogura, T. and Wilkinson, A.J. 2001. AAA+ superfamily ATPases: common structure—diverse function. *Genes Cells* 6:575–597.
Olson, M.W., Dallmann, H.G., and McHenry, C.S. 1995. DnaX of *Escherichia coli* DNA polymerase III holoenzyme. The $\chi\psi$ complex functions by increasing the affinity of τ and γ for δ–δ' to a physiologically relevant range. *J Biol Chem* 270:29570–29577.
Onrust, R., Finkelstein, J., Naktinis, V., Turner, J., Fang, L., and O'Donnell, M. 1995a. Assembly of a chromosomal replication machine: Two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. I. Organization of the clamp loader. *J Biol Chem* 270:13348–13357.
Onrust, R., Finkelstein, J., Turner, J., Naktinis, V., and O'Donnell, M. 1995b. Assembly of a chromosomal replication machine: Two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. III. Interface between two polymerases and the clamp loader. *J Biol Chem* 270:13366–13377.
Onrust, R. and O'Donnell, M. 1993. DNA polymerase III accessory proteins. II. Characterization of δ and δ'. *J Biol Chem* 268:11766–11772.
Onrust, R., Stukenberg, P.T., and O'Donnell, M. 1991. Analysis of the ATPase subassembly which initiates processive DNA synthesis by DNA polymerase III holoenzyme. *J Biol Chem* 266:21681–21686.
Pietroni, P., Young, M.C., Latham, G.J., and von Hippel, P.H. 2001. Dissection of the ATP-driven reaction cycle of the bacteriophage T4 DNA replication processivity clamp loading system. *J Mol Biol* 309:869–891.
Podobnik, M., Weitze, T.F., O'Donnell, M., and Kuriyan, J. 2003. Nucleotide-induced conformational changes in an isolated *Escherichia coli* DNA polymerase III clamp loader subunit. *Structure (Camb)* 11:253–263.
Podust, V.N., Tiwari, N., Ott, R., and Fanning, E. 1998. Functional interactions among the subunits of replication factor C potentiate and modulate its ATPase activity. *J Biol Chem* 273:12935–12942.
Pritchard, A.E., Dallman, H.G., Glover, B.P., and McHenry, C.S. 2000. A novel assembly mechanism for the DNA polymerase III holoenzyme DnaX complex: association of $\delta\delta'$ with $DnaX_4$ forms $DnaX_3\delta\delta'$. *EMBO J* 19:6536–6545.
Purich, D.L. 2001. Enzyme catalysis: a new definition accounting for noncovalent substrate- and product-like states. *Trends Biochem Sci* 26:417–421.
Richardson, R.W., Ellis, R.L., and Nossal, N.G. 1990. Protein-protein interactions within the bacteriophage T4 DNA replication complex. Molecular mechanisms in DNA replication and recombination. *UCLA Symp Mol Cell Biol* 127:247–259.
Rusinova, E., Tretyachenko-Ladokhina, V., Vele, O.E., Senear, D.F., and Alexander Ross, J.B. 2002. Alexa and Oregon Green dyes as fluorescence anisotropy probes for measuring protein-protein and protein-nucleic acid interactions. *Anal Biochem* 308:18–25.
Schmidt, S.L., Gomes, X.V., and Burgers, P.M. 2001. ATP utilization by yeast replication factor C. III. The ATP-binding domains of Rfc2,

Rfc3, and Rfc4 are essential for DNA recognition and clamp loading. *J Biol Chem* 276:34784–34791.
Selvin, P.R. 1995. Fluorescence resonance energy transfer. *Methods Enzymol* 246:300–334.
Sexton, D.J., Carver, T.E., Berdis, A.J., and Benkovic, S.J. 1996. Protein–protein and protein–DNA interactions at the bacteriophage T4 DNA replication fork: Characterization of a fluorescently labeled DNA polymerase sliding clamp. *J Biol Chem* 271:28045–28051.
Sexton, D.J., Kaboord, B.F., Berdis, A.J., Carver, T.E., and Benkovic, S.J. 1998. Dissecting the order of bacteriophage T4 DNA polymerase holoenzyme assembly. *Biochemistry* 37:
Shamoo, Y. and Steitz, T.A. 1999. Building a replisome from interacting pieces: sliding clamp complexed to a peptide from DNA polymerase and a polymerase editing complex. *Cell* 99:155–166.
Snyder, A.K., Williams, C.R., Johnson, A., O'Donnell, M., and Bloom, L.B. 2004. Mechanism of loading the *Escherichia coli* DNA polymerase III sliding clamp: II. Uncoupling the β and DNA binding activites of the γ complex. *J Biol Chem* 279:4386–4393.
Soumillion, P., Sexton, D.J., and Benkovic, S.J. 1998. Clamp subunit dissociation dictates bacteriophage T4 DNA polymerase holoenzyme disassembly. *Biochemistry* 37:1819–1827.
Steiner, R.F. 1991. Fluorescence anisotropy: Theory and applications. In: J. R. Lakowicz, ed. *Topics in Fluorescence Spectroscopy*, New York: Plenum Press, pp. 1–52.
Stewart, J., Hingorani, M.M., Kelman, Z., and O'Donnell, M. 2001. Mechanism of β clamp opening by the δ subunit of *Escherichia coli* DNA polymerase III holoenzyme. *J Biol Chem* 276:19182–19189.
Stryer, L. 1978. Fluorescence energy transfer as a spectroscopic ruler. *Annu Rev Biochem* 47:819–846.
Studwell-Vaughan, P.S., and O'Donnell, M. 1991. Constitution of the twin polymerase of DNA polymerase III holoenzyme. *J Biol Chem* 266:19833–19841.
Stukenberg, P.T., Studwell-Vaughan, P.S., and O'Donnell, M. 1991. Mechanism of the sliding β-clamp of DNA polymerase III holoenzyme. *J Biol Chem* 266:11328–11334.
Trakselis, M.A., Alley, S.C., Abel-Santos, E., and Benkovic, S.J. 2001a. Creating a dynamic picture of the sliding clamp during T4 DNA polymerase holoenzyme assembly by using fluorescence resonance energy transfer. *Proc Natl Acad Sci USA* 98:8368–8375.
Trakselis, M.A. and Benkovic, S.J. 2001. Intricacies in ATP-dependent clamp loading: variations across replication systems. *Structure (Camb)* 9:999–1004.
Trakselis, M.A., Berdis, A.J., and Benkovic, S.J. 2003. Examination of the role of the clamp-loader and ATP hydrolysis in the formation of the bacteriophage T4 polymerase holoenzyme. *J Mol Biol* 326:435–451.
Trakselis, M.A., Mayer, M.U., Ishmael, F.T., Roccasecca, R.M., and Benkovic, S.J. 2001b. Dynamic protein interactions in the bacteriophage T4 replisome. *Trends Biochem Sci* 26:566–572.
Tsuchihashi, Z. and Kornberg, A. 1989. ATP interactions of the τ and γ subunits of DNA polymerase III holoenzyme of *Escherichia coli. J Biol Chem* 264:17790–17795.
Tsuchihashi, Z. and Kornberg, A. 1990. Translational frameshifting generates the γ subunit of DNA polymerase III holoenzyme. *Proc Natl Acad Sci USA* 87:2516–2520.
Turner, J., Hingorani, M.M., Kelman, Z., and O'Donnell, M. 1999. The internal workings of a DNA polymerase clamp-loading machine. *EMBO J* 18:771–783.
Uhlmann, F., Cai, J., Gibbs, E., O'Donnell, M., and Hurwitz, J. 1997. Deletion analysis of the large subunit p140 in human replication factor C reveals regions required for complex formation and replication activities. *J Biol Chem* 272:10058–10064.
Vivona, J.B. and Kelman, Z. 2003. The diverse spectrum of sliding clamp interacting proteins. *FEBS Lett* 546:167–172.
Waga, S. and Stillman, B. 1998. The DNA replication fork in eukaryotic cells. *Annu Rev Biochem* 67:721–751.
Walker, J.R., Hervas, C., Ross, J.D., Blinkova, A., Walbridge, M.J., Pumarega, E.J., Park, M.O., and Neely, H.R. 2000. *Escherichia coli* DNA polymerase III tau- and gamma-subunit conserved residues required for activity in vivo and in vitro. *J Bacteriol* 182:6106–6113.
Warbrick, E. 1998. PCNA binding through a conserved motif. *BioEssays* 20:195–199.
Warbrick, E. 2000. The puzzle of PCNA's many partners. *BioEssays* 22:997–1006.
Wickner, S. 1976. Mechanism of DNA elongation catalyzed by *Escherichia coli* DNA polymerase III, dnaZ protein, and DNA elongation factors I and III. *Proc Natl Acad Sci USA* 73:3511–3515.
Wickner, S. and Hurwitz, J. 1974. Conversion of ϕX174 viral DNA to double-stranded form by purified *Escherichia coli* proteins. *Proc Natl Acad Sci USA* 71:4120–4124.
Wijffels, G., Dalrymple, B., Kongsuwan, K., and Dixon, N.E. 2005. Conservation of eubacterial replicases. *IUBMB Life* 57:413–419.
Williams, C.R., Snyder, A.K., Kuzmic, P., O'Donnell, M., and Bloom, L.B. 2004. Mechanism of loading the *Escherichia coli* DNA polymerase III sliding clamp: I. Two distinct activities for individual ATP sites in the γ complex. *J Biol Chem* 279:4376–4385.
Wu, P. and Brand, L. 1994. Resonance energy transfer: methods and applications. *Anal Biochem* 218:1–13.
Xi, J., Zhang, Z., Zhuang, Z., Yang, J., Spiering, M.M., Hammes, G.G., and Benkovic, S.J. 2005a. Interaction between the T4 helicase loading protein (gp59) and the DNA polymerase (gp43): unlocking of the gp59-gp43-DNA complex to initiate assembly of a fully functional replisome. *Biochemistry* 44:7747–7756.
Xi, J., Zhuang, Z., Zhang, Z., Selzer, T., Spiering, M.M., Hammes, G.G., and Benkovic, S.J. 2005b. Interaction between the T4 helicase-loading protein (gp59) and the DNA polymerase (gp43): a locking mechanism to delay replication during replisome assembly. *Biochemistry* 44:2305–2318.
Xiao, H., Dong, Z., and O'Donnell, M. 1993. DNA polymerase III accessory proteins. IV. Characterization of chi and psi. *J Biol* Chem 268:11779–11784.
Xiao, H., Naktinis, V., and O'Donnell, M. 1995. Assembly of a chromosomal replication machine: Two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. IV. ATP-binding site mutants identify the clamp loader. *J Biol Chem* 270:13378–13383.
Yao, N., Leu, F.P., Anjelkovic, J., Turner, J., and O'Donnell, M. 2000. DNA structure requirements for the *Escherichia coli* gamma complex clamp loader and DNA polymerase III holoenzyme. *J Biol Chem* 275:11440–11450.
Yao, N., Turner, J., Kelman, Z., Stukenberg, P.T., Dean, F., Shechter, D., Pan, Z.-Q., Hurwitz, J., and O'Donnell, M. 1996. Clamp loading, unloading, and intrinsic stability of the PCNA, β and gp45 sliding clamps of human, *E. coli* and T4 replicases. *Genes Cells* 1:101–113.
Yoder, B.L. and Burgers, P.M. 1991. Saccharomyces cerevisiae replication factor C. I. Purification and characterization of its ATPase activity. *J Biol Chem* 266:22689–22697.
Young, M.C., Weitzel, S.E., and von Hippel, P.H. 1996. The kinetic mechanism of formation of the bacteriophage T4 DNA polymerase sliding clamp. *J Mol Biol* 264:440–452.
Zhang, Z., Spiering, M.M., Trakselis, M.A., Ishmael, F.T., Xi, J., Benkovic, S.J., and Hammes, G.G. 2005. Assembly of the bacteriophage T4 primosome: single-molecule and ensemble studies. *Proc Natl Acad Sci USA* 102:3254–3259.
Zhuang, Z., Spiering, M.M., Berdis, A.J., Trakselis, M.A., and Benkovic, S.J. 2004. 'Screw-cap' clamp loader proteins that thread. *Nat Struct Mol Biol* 11:580–581.

Editor: Michael M. Cox