Fluorescence Measurements on the E. coli DNA Polymerase Clamp Loader: Implications for Conformational Changes During ATP and Clamp Binding

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Sliding clamps are ring-shaped proteins that tether DNA polymerases to their templates during processive DNA replication. The action of ATP-dependent clamp loader complexes is required to open the circular clamps and to load them onto DNA. The crystal structure of the penta-meric clamp loader complex from Escherichia coli (the γ complex), determined in the absence of nucleotides, revealed a highly asymmetric and extended form of the clamp loader. Consideration of this structure suggested that a compact and more symmetrical inactive form may predominate in solution in the absence of crystal packing forces. This model has the N-terminal domains of the δ and δ0 subunits of the clamp loader close to each other in the inactive state, with the clamp loader opening in a crab-claw-like fashion upon ATP-binding. We have used fluorescence resonance energy transfer (FRET) to investigate the structural changes in the E. coli clamp loader complex that result from ATP-binding and interactions between the clamp loader and the β clamp. FRET measurements using fluorophores placed in the N-terminal domains of the δ and δ0 subunits indicate that the distances between these subunits in solution are consistent with the previously crystallized extended form of the clamp loader. Furthermore, the addition of nucleotide and clamp to the labeled clamp loader does not appreciably alter these FRET distances. Our results suggest that the changes that occur in the relative positioning of the δ and δ0 subunits when ATP binds to and activates the complex are subtle, and that crab-claw-like movements are not a significant component of the clamp loader mechanism.

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Keywords: DNA polymerase; DNA replication; clamp loader; sliding clamp; ATPase

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Introduction

Sliding clamps are essential, conserved processivity factors for high-speed DNA replication in prokaryotes, eukaryotes, archaeabacteria and certain bacteriophage.1-3 Once properly positioned around nucleic acid at primer/template junctions, sliding clamps can move freely along double-stranded DNA.4 The polymerase subunit is held in close proximity to the DNA by a flexible linkage to the clamps, allowing it to bind and release the template without losing its place at the replication fork.5 This interaction with the sliding clamp converts Escherichia coli DNA polymerase III from an enzyme capable of synthesizing only a small number of new base-pairs per substrate-binding event to one that is capable of synthesizing several thousand nucleotides without dissociation.6-8 The action of another conserved protein complex, the clamp loader, is required to open the circular clamp protein and load it onto DNA.

In the well-studied E. coli system, the β subunit of DNA polymerase III is the sliding clamp.6 The
β clamp is a homodimeric protein that forms a circular head-to-tail dimer. The clamp loader complex in E. coli is called the γ/τ complex, and it consists minimally of five polypeptide chains: three γ/τ subunits (48 kDa or 71 kDa), a δ subunit (39 kDa) and a δ′ subunit (37 kDa). The γ and τ subunits are isoforms that are identical in sequence except that τ contains an additional C-terminal segment that is not essential for clamp loading.\(^\text{10–12}\) The experiments described here utilize a truncated form of the γ subunit (41 kDa) that was used in the previously described crystallographic studies.\(^\text{13}\)

The δ subunit is known to bind to the clamp\(^\text{4,15}\) and open one of its dimer interfaces, and it has been termed a “wrench” subunit in the complex. It is controlled by the coordinated actions of three γ “motor” subunits, each of which can bind and hydrolyze ATP, and the catalytically inert δ′ subunit.

Biochemical studies have shown that the isolated δ subunit is capable of binding to and opening the β clamp on its own, and that this ability is masked by the addition of the δ′ subunit.\(^\text{16}\) This result suggested that the function of the other subunits in the γ complex may be to hide and release the δ subunit in an ATP-dependent manner, consistent with the fact that the γ complex associates strongly with β only in the presence of ATP. Upon interaction with DNA, the γ complex hydrolyzes bound ATP and releases the β clamp on DNA, where it is ready to engage the DNA polymerase. Hence, ATP-binding and hydrolysis by the γ subunits somehow control the intrinsic ability of the δ subunit to bind to and open the β clamp.

Each of the subunits of the E. coli clamp loader complex has a similar structure with three distinct domains.\(^\text{17}\) The first two domains together comprise a module with similarity to the AAA + ATPases.\(^\text{13,18–20}\) A flexible linker segment tethers the nucleotide-binding N-terminal AAA + domains to a helical C-terminal domain.\(^\text{21}\) The C-terminal domains of the five subunits in each clamp loader assembly form a circular collar that is the structurally stable core element of the heteropentamer\(^\text{13}\) (outlined in Figure 1). The structure of the subunits of the archaeobacterial RFC complex is similar\(^\text{22}\) and sequence similarity suggests a conserved architecture for the clamp loader subunits in eukaryotes and bacteriophage T4.\(^\text{1,2,23}\) Experiments in these organisms have been informative regarding the similarities and differences in these clamp loaders’ properties\(^\text{24–35}\) in comparison to the E. coli complex.

The crystal structure of the E. coli γ complex pentamer has been determined in the absence of nucleotides.\(^\text{13}\) While the five C-terminal domains are circularly arranged, the arrangement of the N-terminal domains is highly asymmetric (Figure 1; this figure introduces a notation in which the five subunit positions are labeled A–E). In particular, one γ–γ interface (between the subunits in positions D and C (γ1 and γ2)) is sealed shut, while the other γ–γ interface (between the subunits in positions C and B (γ2 and γ3)) is wide open in the crystal structure. The δ and δ′ subunits (positions A and E) are splayed apart from each other, and only interact via their C-terminal domains. Notably, the N-terminal β-interacting domain of δ juts out from the structure and is distant from the δ′ subunit (see Figures 1 and 2). We considered the possibility that crystal packing forces may have influenced the conformation of the complex sufficiently so as to trap it in a partially “open” state.\(^\text{13}\)

Both the δ and δ′ subunits interact extensively with other molecules in the crystal lattice, suggesting that they might be “held” open. A compact model for the solution state of the nucleotide-free clamp loader was generated by closing the γ2–γ3 interface (positions C and B) so that it resembled the closed γ1–γ2 interface (positions D and C), and maintaining the relative arrangement of the N-terminal domains of the δ and γ3 subunits. This resulted in the close juxtapositioning of the N-terminal domains of the δ and δ′ subunits, such that the β-interaction surface of δ was hidden at the δ–δ′ interface (shown schematically in Figure 1). We hypothesized that ATP-binding would trigger a large rearrangement in the γ complex that would result in the movement of the δ subunit away from the δ′ subunit, thereby allowing δ to interact with the clamp (Figure 1; crab-claw model). An alternate model for the activation of the clamp loader would regard the extended nucleotide-free crystal structure as a reasonable model for the inactive state (irrespective of the influence of the crystal lattice) because the orientation of the N-terminal domain of δ is such that the γ complex cannot engage β without large clashes between the clamp and the γ and δ′ subunits of the clamp loader (Figure 1; limited change model).

Here, we measure fluorescence resonance energy transfer (FRET) between fluorophores placed in the δ and δ′ subunits in order to evaluate the flexibility of the clamp loader complex during the process of nucleotide and clamp-binding. We find that the FRET signals observed for the clamp loader are largely in agreement with the δ–δ′ distances seen in the crystal structure of the nucleotide-free clamp loader. Furthermore, the binding of ATP and the β clamp to the clamp loader complex causes very little change in the overall distance between the N-terminal domains of the δ and δ′ subunits as reported by the FRET probes, suggesting that the crab-claw model may not be appropriate.

**Results**

**Design of cysteine-light proteins and labeling with thiol-linked fluorescent probes**

Fluorescence resonance energy transfer (FRET) between excited donor labels and acceptor molecules depends inversely on the sixth power of the distance between the two fluorophores, and this dependence can be used to estimate the distance between two positions on a suitably labeled
protein. In order to examine the relative positioning of the δ and δ' subunits under the influence of nucleotide and clamp-binding, we introduced site-specific fluorescent probes into their N-terminal domains for FRET studies.

Cysteine residues provide a convenient means for targeting thiol-linked fluorescent tags to the surface of proteins and have been employed in many other systems including cAMP-dependent protein kinase, myosin motors and the GroEL/ES chaperone. Each purified subunit of the clamp loader complex can be maintained stably in the absence of the others. This enables labels to be directed at specific subunits and subsequently combined with other unlabeled subunits to reconstitute an active clamp loader. The three γ subunits of the clamp loader are identical in sequence, making it difficult to uniquely label the complex on an individual γ subunit. We therefore restricted our studies to complexes in which the δ and δ' subunits are labeled. The crab-claw model of clamp loader activation predicts that a large increase in the distance between the N-terminal domains of the δ and δ' subunits would occur upon ATP-binding (Figure 1). We chose sites for labeling on δ and δ' that are well-suited for monitoring such potential changes.

The E. coli δ and δ' proteins each contain several native cysteine residues, and we created variant forms of these subunits in which surface-exposed (and presumably reactive) cysteine residues were replaced by serine. Examination of the crystal structure revealed that four of the seven endogenous cysteine residues of δ' (C50, C59, C62, and C65) are part of a conserved zinc-binding module and are buried inside the N-terminal domain of the protein. Another buried cysteine (C159) is part of the conserved “SRC” sequence motif that contains an “arginine finger”, which protrudes into the ATP-binding pocket of the neighboring γ subunit (designated γ1). These five cysteine residues are considered unlikely to be reactive to thiol reagents. Two non-conserved cysteine residues (C217 and C294) in δ' are solvent-exposed, and these were mutated to serine to create the “cysteine-light” variant of the δ' subunit. The wild-type δ subunit has five cysteine residues. One cysteine (C139) is buried in the structure and is unlikely to be reactive. The remaining four cysteine residues (C68, C171, C173 and C332) were mutated to serine to eliminate solvent-exposed thiols.

We next introduced a number of single cysteine mutations into these cysteine-light variants of δ and δ' at solvent-exposed positions (Q40C, D106C, N132C in δ and R2C, P33C and K83C in δ') (Figure 2(a)). We selected positions on the N-terminal domains of δ and δ' that were expected to move significantly relative to the other subunit in the crab-claw mechanism as the hypothetical compact state of the clamp loader complex was converted to an extended state (top of Figure 1). Another criterion used for the selection of cysteine placement included choosing locations having high sequence variability in other bacterial clamp loader subunits. Such sites are presumably tolerant of substitution while retaining function, and the subsequent introduction of the fluorescent label would be expected to minimally impair activity. Paramount to our choice of probe locations was the selection of sites that, based upon the available crystal structures, would not be expected to interfere with the binding of the δ subunit to the β clamp or restrict any other inferred conformational changes in the clamp loader.

Proteins were overexpressed in E. coli and purified as described in Materials and Methods. These proteins were labeled with maleimide-linked...
Figure 2. Introduction of fluorescent labels into the δ and δ′ clamp loader subunits. (a) Position in the structure of the clamp loader of solvent-exposed residues mutated to cysteine for the attachment of maleimide-linked fluorophores. A ribbon diagram of subunit A (δ) is shown in lavender with the β-interaction helix colored yellow. Subunit E (δ′) is shown in orange ribbons with space-filling diagrams of the D (γ1), C (γ2), and B (γ3) subunits shown in green, red, and blue, respectively. The image was rendered by Swiss-PdbViewer. (b) SDS/polyacrylamide gel electrophoresis of labeled proteins. Labeling conditions were 100 mM Tris (pH 6.8), 300 mM NaCl, 10% glycerol for one hour at room temperature with fivefold excess label for δ subunits and 15-fold excess label for δ′ subunits. In the upper panel, cysteine-light δ proteins were imaged for fluorescein (green) and for general protein (red) after staining with Sypro Orange (Molecular Probes). In the lower panel, cysteine-light δ′ proteins were imaged for tetramethylrhodamine (TMR, in red) and for general protein (green) after staining with Sypro Orange (Molecular Probes). Both gels were imaged with a Molecular Dynamics Typhoon scanner using ImageQuant software. (c) DNA replication assays using fluorescently labeled clamp loaders. The following estimates for the relative replication activity to wild-type, unlabeled (WT; set as 100%) were made by fitting the activity data to a line: 86% for N132C δ labeled with fluorescein (N132C-f),
DNA Polymerase Clamp Loader Fluorescence

fluorescent probes, typically with a 5–15-fold excess of labels in the reaction mixture. The upper panel of Figure 2(b) shows polyacrylamide gels of labeled δ and imaged by fluorescence scanning. Although the cysteine-light δ variant shows very little labeling, the single cysteine δ mutations (Q40C, D106C, N132C) introduced into these backgrounds afford rapid association with fluorescein-5-maleimide. Note that some engineered cysteine variants are labeled more readily than others. (For example, D106C δ is not labeled as rapidly as the other δ variants.) In the δ' variants, only K83C showed substantial labeling relative to the cysteine-light control (Figure 2(b), lower panel). It appears that the other mutants (R2C and P33C) could not be labeled specifically and are not discussed further. Unincorporated fluorophore was removed from the labeled protein by a desalting column, and the percentage of labeling on the proteins was calculated using UV spectroscopy. Typically, labeling equivalent to 50–80% of the potential reactive cysteine residues was observed. Labeled clamp loader complexes were assembled as described in Materials and Methods.

DNA replication assays using singly or doubly labeled complexes demonstrate clamp loading activity that is essentially the same as that observed with unlabeled wild-type proteins (Figure 2(c)). The data in Figure 2(c) were obtained using 20 nM clamp loader. At lower concentrations (~4 nM and below) the labeled complexes showed deficiencies in the ability to provide processivity to the polymerase (10–50% wild-type activity; data not shown). We interpret this to mean that the cysteine-light labeled forms of the clamp loader are slightly less stable than the wild-type form. All the subsequent experiments described were done at protein concentrations well above this level (500 nM or higher).

**Labeled clamp loader proteins bind the β clamp in an ATP-dependent manner**

In order to assess whether clamp loader complexes incorporating labeled δ and δ' subunits retained their nucleotide-dependent association with the β clamp, we labeled β with the conjugate fluorophore at position 253 (Figure 3). We then looked for fluorescence energy transfer (FRET) between the various singly labeled clamp loader complexes and the labeled β clamp (Figure 4). Because β is a dimeric protein, each clamp has two potential locations (one on each subunit) for a fluorescent label to attach to the protein (Figure 3). The clamp loader can bind to the symmetric β clamp in either of two ways, and this makes it difficult to extract accurate distance estimates from these data. Nevertheless, these experiments do provide a useful way to assess if our labeled clamp loader complexes continue to function in binding to the β clamp.

The results of an experiment in which δ was labeled at N132C with tetramethylrhodamine (TMR), incorporated into the clamp loader and mixed with D253C β clamp labeled with a fluorescein donor are shown in Figure 4(a). We observed a small reduction in the donor fluorescence on β in the presence of clamp loader even without the addition of nucleotide (red curve in Figure 4(a)), suggesting that there is some association between the clamp loader and the β clamp without ATP. This is not surprising, because a high concentration of protein is known to drive association of the clamp loader and clamp even without the addition of nucleotides (A.J. and M.O.D., unpublished data). Upon the addition of ATP, however, the donor fluorescence is reduced significantly and acceptor fluorescence increases (see the blue curve in Figure 4(a)), indicating enhanced energy transfer. Note that addition of ATP to the labeled β clamp alone causes very little change in the donor fluorescence (green curve). Therefore, when the clamp loader is included,
FRET is observed and an ATP-dependent association occurs between the labeled γ complex and labeled β clamp.

A similar ATP-dependent FRET signal is obtained if the positions of the donor and acceptor fluorescent probes are reversed (Figure 4(b) and (c)). Association of β clamp and labeled clamp loader complex requires the presence of both Mg²⁺ and nucleotide (Figure 4(b)), further indicating that this is a specific ATP-driven clamp/clamp loader complex. Addition of ADP does not induce this change (Figure 4(b)). Also, we note that after addition of one species of nucleotide (either ATP or ADP), a threefold excess of the other nucleotide can reverse the FRET signal to that corresponding to the bound (if ATP is in excess) or unbound state (if ADP is in excess). For example in Figure 4(c), after forming the clamp loader–β clamp complex by the addition of ATP, a threefold excess of ADP reverses the interaction. This indicates that the affinity for ATP (or the clamp loader/ clamp complex) or ADP (in the free clamp loader) appears to be similar. This experiment also demonstrates that the labeled proteins are able to undergo reversible changes and that they are not trapped in one state or another by the mutations or the addition of fluorescent labels. Clamp loaders with fluorescein-labeled Q40C and D106C δ also show ATP-dependent associations with the β clamp (data not shown).

Figure 4(d) shows the results of a similar experiment in which donor-labeled D253C β clamp and clamp loader complex containing acceptor-labeled K83C δ are mixed together. As with the δ-labeled clamp loader, introduction of ATP (but not ADP) converts the fluorescence spectrum (in a Mg²⁺-dependent fashion) to one having lower donor fluorescence and increased acceptor fluorescence, indicating that these labeled proteins are capable of interacting with each other. The magnitude of the change in signal is smaller when compared to changes seen with δ-labeled clamp loader, and may reflect an increased distance between the β clamp and the δ subunit in the complex.

The addition of primer/template DNA causes a reduction in FRET signal between the clamp loader and the clamp

Figure 5 shows the results of an experiment where N132C δ fluorescein-labeled clamp loader and TMR-labeled D253C β clamp are presented
with DNA. Following the addition of ATP (blue trace in Figure 5(a)), fluorescence intensity at 515 nm is reduced. Because the signal at this wavelength comes exclusively from the donor, this change reflects an increase in energy transfer as the clamp loader binds labeled β (as expected from the results in Figure 4). After DNA (consisting of a 30-nucleotide primer annealed to a 40-nucleotide template having a ten base 5′ overhang) is added (red trace), there is a large increase in donor fluorescence and, therefore, a reduction in energy transfer. When ADP is used instead of ATP, very little change is observed in the donor fluorescence upon addition of DNA oligonucleotide (Figure 5(b)). These results, like the DNA replication assays (Figure 2(c)), indicate that the labeled clamp loaders retain the ability to load the clamp on DNA substrates and that this occurs in an ATP-dependent manner. In these experiments, the intensity of donor signal after the addition of DNA does not return completely to the level observed prior to the addition of ATP. We speculate that the loaded clamps quickly come off the short primer/template and, because ATP is in great excess, are rebound by the ATP-primed clamp loader. Similar DNA-dependent reductions in clamp loader–D253C β clamp energy transfer were obtained with clamp loaders labeled at positions 40 and 106 in δ or at position 83 in δ′ in the presence of ATP but not ADP (data not shown).

**FRET signals between δ and δ′ are not altered significantly by addition of nucleotide or β clamp**

Figure 6 shows the results of a FRET experiment that monitors the distance between probes on N132C of δ and on K83C of δ′ within the clamp loader complex as it binds to nucleotide and unlabeled β clamp. In Figure 6(a), the fluorescence intensities of clamp loader samples containing donor and acceptor pairs (black curve) and donor only (blue curve) and acceptor only (red curve) are shown. Resonance energy transfer is indicated both by the decrease in donor fluorescence (500–550 nm) and the increase in acceptor fluorescence (550–600 nm) relative to samples in which only one subunit of the clamp loader is labeled. Using the decreased fluorescence intensity of the donor to calculate the extent of energy transfer and correcting for 75% labeling of the acceptor gives an energy transfer efficiency of 43% and an apparent distance of ~52 Å between the fluorophores (Table 1, see Materials and Methods for details). While the addition of ATP causes slight changes in the fluorescence intensity (Figure 6(b)), these correspond to changes in calculated FRET distance that are small (Figure 6(c)). Even upon the subsequent addition of wild-type β clamp, the changes in the calculated FRET distance (from 52 Å to 50 Å) are insignificant and within the 2–5 Å error that we
have observed in repeated measurements with these samples. In order to calculate the apparent distance between these two probes, a value for $R_0$ of 50 Å for fluorescein–TMR pair was assumed as measured in a previous study.\textsuperscript{38} Note that these values of $R_0$ assume that the so-called orientation factor ($k^2$) of the two dye molecules is 2/3. Although there is no way to directly measure $k^2$, it is possible to estimate limits from the anisotropies of the donor and acceptor. The following are the steady-state fluorescence anisotropy values of singly labeled clamp loaders used in this study: 0.11 for fluorescein in N132C δ, 0.16 for fluorescein in D106C δ, 0.20 for fluorescein in Q40C δ, and 0.21 for TMR in K83C δ. Considering these anisotropy values, errors introduced by the assumption that $k^2$ is 2/3 are small relative to the 2–5 Å experimental error that we have found in repeated measurements.\textsuperscript{41}

Our FRET data demonstrate that the conformational changes that occur within the δ and δ′ subunits during ATP and β clamp-binding are small, or that when conformational changes do occur, they consist of a small transiently populated subset. Given that we see relatively large fluorescence changes in the FRET experiments involving δ-labeled clamp loader and labeled β clamp, and that gel-filtration experiments with the wild-type clamp and clamp loader show a stable association between the two in the presence of nucleotide,\textsuperscript{14,42} it is unlikely that the active clamp-bound state of the clamp loader is only a transiently populated state in the presence of ATP. We conclude that the resulting nucleotide-dependent rearrangements in the clamp loader do not involve significant changes in the overall distance between the δ and δ′ subunits. This is in contrast to the ~35 Å increase in δ–δ′ distance that is expected if the complex switched from the modeled compact form (Figure 1) to an extended form upon binding ATP and β clamp.

The extent of energy transfer that is observed is dependent on the protein concentrations used (Figure 6(d)). At lower concentrations of protein (1 μM and below), the calculated FRET distances increase as the concentration decreases. This reduction in energy transfer efficiency may indicate

\begin{figure}
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\caption{FRET between N132C δ labeled with fluorescein and K83C δ′ labeled with TMR within the clamp loader. (a) Steady-state fluorescence emission spectra (in arbitrary units) obtained with excitation at 460 nm with 2.5 μM clamp loader. Samples containing donor have N132C δ 82% labeled with fluorescein. Samples containing acceptor have K83C δ′ 75% labeled with TMR. Cysteine-light δ′ subunits were added in place of acceptor-labeled δ′ subunits in donor-only samples, and cysteine-light δ subunits were added in place of donor-labeled subunits in the acceptor-only samples. Buffer conditions are 50 mM Tris (pH 7.5), 400 mM NaCl, 5 mM MgCl₂, 10% glycerol, 2 mM DTT. (b) Effect of adding ATP and β clamp to donor and donor + acceptor samples in Figure 2(a). (c) FRET distances calculated between fluorescein on N132C δ and TMR on K83C δ′. (d) Concentration dependence of the FRET distance obtained between donor on N132C δ and acceptor on K83C δ′ in the absence of nucleotide or β.
\end{figure}
that the donor–acceptor pairs are coming apart at lower concentration as suggested by the reduction in DNA replication activity at lower concentrations (data not shown). (In the assembled DNA polymerase holoenzyme there are additional interactions between the C-terminal extensions of clamp loader subunits that are likely to stabilize the complex.) Because of this observation and the other assumptions used in the calculation of FRET distances (such as the acceptor labeling percentage) the distance measurements reported here should be considered an approximate upper limit.

Table 1 shows the results of similar FRET experiments using two additional donor probes on the δ subunit. The FRET distances obtained from fluorescein at positions 40 or 106 on the δ subunit to TMR linked at position 83 on δ₀ are also similar to δ–δ₀ intersubunit distances seen in the crystal structure of the clamp loader complex. Furthermore, the addition of nucleotide and β has only minor influence on these distances (all within 2–5 Å of the values obtained in their absence). Note that the addition of ADP (which does not promote loader–clamp interactions) also does not substantially influence the FRET measurements between these pairs. In summary, the observed changes in distances between probes in the N-terminal domains of the δ and δ₀ subunits in the clamp loader are small and do not reflect a significant increase in these distances with the addition of ATP and subsequent clamp-binding.

Discussion

The clamp loader is an ATP-dependent biological machine that is able to select and open polymerase clamps and target them to nucleic acid primer template sites during DNA replication. Like other members of the extensive AAA⁺ ATPase family, the clamp loader complex requires the binding and hydrolysis of ATP to carry out this sophisticated function. Like the other AAA⁺ ATPases, the details of how nucleotide interactions drive the functional cycle of this protein machine remain poorly understood. Unlike most other members of the AAA⁺ family that form hexameric complexes (such as the Clp ATPases, P97/vasolin-containing protein (VCP) and N-ethylmaleimide-sensitive factor (NSF)), the clamp loader has a heteropentameric structure. While each of the five subunits of the clamp loader has the AAA⁺ fold, in E. coli only the three γ subunits have ATPase activity. While each of the five subunits of the clamp loader has the AAA⁺ fold, in E. coli only the three γ subunits have ATPase activity. This difference in subunit composition and heterogeneity between more canonical AAA⁺ proteins and the clamp loader likely reflects important aspects of the function of the protein but these are also poorly understood.

Structural and biochemical studies suggest that the N-terminal domain of the δ subunit plays a critical role in opening one interface between the two monomers in the dimeric β clamp. Our group previously suggested a model (see crab-claw model in Figure 1) in which nucleotide-binding leads to a large conformational rearrangement that
disrupts close interactions between δ and δ' that were inferred but not observed in the crystal structure. ATP was suggested to trigger the presentation of the N-terminal domain of δ so that it is free to bind to the β clamp and to enable the entire complex to progress to clamp loading. The FRET measurements reported here suggest that the change in distance between probes on the δ and δ' subunits upon nucleotide and clamp-binding is not large. In particular, the experiments presented here provide no evidence that the N-terminal domain of the δ subunit packs against that of the δ' subunit (Figure 1). Furthermore, gel-filtration studies of an isolated subunit comprising only the N-terminal domain of δ (δ-1140) show that without its second and third domains the molecule does not associate with isolated δ' (data not shown) although a stable, gel-filterable complex is formed with the two full-length proteins. Hence, it appears that the N-terminal domains of δ and δ' do not strongly interact either in the context of the active clamp loader or as isolated subunits. We therefore conclude that the clamp loader does not require dramatic rearrangements in the relative positioning of δ and δ' to bind to the β clamp, and that the previously proposed crab-claw model of the clamp loader pentamer may not be a relevant state of the clamp-loading cycle.

Our results suggest that the clamp loader complex is likely to maintain the asymmetric and open arrangement of the N-terminal domains, even when nucleotide is absent (the limited change model in Figure 1). It is important to note that while our fluorescence data do not provide evidence for large relative movements in the δ and δ' subunits upon ATP and clamp-binding, we do see small changes in energy transfer. Though these do not correspond to a significant change in the distance between the δ and δ' subunits, these small changes may reflect less dramatic transitions in the δ and δ' subunits that enable clamp-binding. In this view, the function of nucleotide-binding would not be to open up the β-interaction element of δ from a closed conformation of the clamp loader, but rather to remodel the entire organization of the γ complex so as to promote a productive interface between the clamp loader and the entire surface of the clamp. Indeed, recent studies show that a modified E. coli clamp loader complex that entirely lacks the N-terminal domains of δ' cannot stably bind the β clamp, indicating that the γ subunits, like δ', block the access of β to δ within the clamp loader. Therefore, these most important alterations in the active clamp loader structure that promote β-binding may occur in the γ subunits that bind ATP.

X-ray crystallography has shown that the δ subunit binds the β subunit between two of the six repeated domains present in the dimeric clamp (Figure 3). Because the other clamp loader subunits share a similar architecture, it is reasonable to suggest that each subunit may interact with the clamp in a similar fashion and bind at the other inter-domain crevices. During clamp loading, one of the clamp inter-subunit interfaces is broken to allow DNA to enter the ring, and there will remain five intact inter-domain interfaces on the β clamp that are potentially available for loader binding. We suggest that the pentameric nature of the γ complex assembly may reflect the fundamental geometry of the opened clamp, and the extended structure of the clamp loader may be necessary to allow it to maintain steric complementarity with the open clamp.

The structure of the eukaryotic clamp loader complex (replication factor C) from yeast bound to ATPγS and the eukaryotic clamp (PCNA) has been determined recently (G. D. Bowman, M.O.D. and J.K., unpublished results). In this structure the N-terminal domains of the homologs of the δ and δ' subunits (RFC1 and RFC5) are separated by ~45 Å, which is similar to the separation between the N-terminal domains of δ and δ' in the clamp-free E. coli clamp loader structure. The structure of the RFC–PCNA complex also shows the presence of an additional domain in the area between the N-terminal domains of RFC1 and RFC5, contributed by the C-terminal regions of RFC1. Along with the FRET experiments reported here, this suggests that neither the bacterial clamp loader nor the RFC complex is likely to undergo a dramatic movement of the δ and δ'-like subunits upon nucleotide and clamp-binding during the process of clamp loading.

Materials and Methods
Creation of cysteine-light δ, δ' and β mutant proteins
Surface-exposed cysteine residues (C68, C171, C173 and C332 in δ, C217 and C294 in δ' and C333 in β) were replaced by serine in previously reported expression constructs using standard site-directed mutagenesis to create cysteine-light variants of δ, δ' and β. DNA sequencing confirmed the incorporation of the desired mutations. Additional single-site mutations (Q40C, D106C or N132C) were introduced into the resulting C68S, C171S, C173S, C323S δ variant (Figure 2). Likewise, additional mutations (R2C, P33C, or K83C) were introduced individually into the δ' cysteine-light background (C217S, C294S) (Figure 2). A D253C mutation was introduced into the cysteine-light β background (C333S) (Figure 3).

Protein expression and purification
All proteins were overexpressed in BL21 E. coli cells in rich medium (LB or TB) in the presence of 100 µg/ml ampicillin. After growth to an OD_{600} of ~0.8 at 37 °C, cells were induced to express protein by the addition of IPTG to 0.2–1 mM concentrations and incubated overnight at 18 °C. Cells were frozen to ~80 °C for storage and lyzed with a French press after thawing. Insoluble cell lysate was removed by centrifugation.

Wild-type and variant forms of the δ' subunit were purified essentially as described. Briefly, the cell lysate supernatant was loaded onto a 5 ml HiTrap heparin
column and eluted with a linear gradient of NaCl (0–500 mM) at pH 7.1. Peak fractions were diluted and loaded onto a 5 ml HiTrap Q column and eluted with a linear gradient of NaCl (0–500 mM) at pH 7.1. Wild-type and variant forms of the δ subunit were purified by loading the lyzate supernatant onto a 5 ml HiTrap SP Sepharose column and eluting with a linear gradient of NaCl (50 mM–500 mM) at pH 7.5. Truncated δ Sepharose column and eluting with a linear gradient of NaCl (50 mM–500 mM) at pH 7.5. Truncated γ subunit (residues 1–373) was purified as described. β Proteins were purified by loading the lyzate supernatant onto a 5 ml HiTrap Q column equilibrated at pH 7.5 and eluted with a linear gradient of NaCl (0–500 mM). Protein concentrations were estimated using UV spectroscopy at 280 nm, with extinction coefficients of 59,600 M⁻¹ cm⁻¹ for δ, 46,230 M⁻¹ cm⁻¹ for δ, 20,340 M⁻¹ cm⁻¹ for truncated γ and 29,300 M⁻¹ cm⁻¹ for the β dimer as calculated from the number of aromatic residues in the protein sequences.  

**Labeling of cysteine-light proteins with fluorescence tags**

Fluorescent maleimide derivatives (fluorescein 5-maleimide and tetramethylrhodamine 5-maleimide) were obtained from Molecular Probes, Inc. Following buffer exchange via gravity-fed PD-10 desalting columns to remove thiol-containing reducing agents present in the purification buffer, proteins were labeled in 100 mM Tris (pH 6.8), 300 mM NaCl, 10% (v/v) glycerol for 4–18 hours with ~5–15 fold molar excess of fluorophores. Labeled proteins were passed through a PD-10 desalting column equilibrated in the same buffer. SDS/polyacrylamide gel analysis of the resulting labeled protein indicates that this step is sufficient for removal of unincorporated fluorophores from the labeling reaction.

Fluorophore concentrations were determined by visible spectroscopy using a Cary 50 Scan spectrophotometer using the extinction coefficients supplied by Molecular Probes Inc. (86,000 M⁻¹ cm⁻¹ for fluorescein 5-maleimide and 491 nm, 100,900 M⁻¹ cm⁻¹ for tetramethylrhodamine 5-maleimide at 541 nm). For protein quantification, the absorbance at 280 nm was corrected by subtracting 18% of the peak absorbance for fluorescein 5-maleimide or 13% of the peak absorbance for tetramethylrhodamine 5-maleimide. The percentage of labeling of each protein was determined by the molar ratio of fluorophore to protein and varied depending on labeling preparation (yields were typically 0.5–0.8 label per protein molecule).

**Assembly of clamp loader complexes**

Clamp loader complexes were assembled by the addition of the δ and δ' subunits in 1.5-fold stoichiometric excess to the truncated γ subunits. The subunits were combined and allowed to associate overnight during dialysis at 4°C against a buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 2 mM DTT, 10% glycerol. To separate clamp loader complex from free δ and δ' subunits and δ/δ' complex, this mixture was loaded onto a 1 ml MonoQ column and eluted with a linear gradient of NaCl (50 mM–500 mM). Because the δ or δ' subunits cannot associate stably with the γ subunits in the absence of the other and the retention time of the multimerized γ subunits alone is very similar to that of the assembled clamp loader,  using an excess of the δ and δ' subunits insures that all protein in the γ-associated peak is pentameric clamp loader containing both the δ and δ' subunits. All clamp loaders used here included truncated γ subunits.

**DNA replication assays**

Activity of wild-type and labeled mutant clamp loaders was assayed by the requirement to load β clamp onto a primed circular ssM13mp18 template in order to observe nucleotide incorporation by the core DNA polymerase III (αεβ subunits). The reaction mixture contained E. coli core (5 nM), β (10 nM); SSB (420 nM tetramer); 30-mer-primed-ssM13mp18 (1.1 nM); dATP, dCTP, dGTP (60 μM), [α-32P]dTTP (20 μM); 1 mM ATP; and 8 mM MgCl₂ in 25 μL of 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 4% glycerol, 40 μg/ml BSA (final volume). Replication was initiated upon addition of either wild-type or labeled clamp loader (20 nM) and incubated at 37°C for ten seconds, 20 seconds, 30 seconds, or 40 seconds. The reaction was quenched with 25 μL of 1% (w/v) SDS, 40 mM EDTA. Quenched reactions were spotted onto DE81 (Whatman) filter circles, then washed and quantified by liquid scintillation counting as described. In the absence of clamp loader, dNTP incorporation at time zero and time 40 seconds was essentially identical.

**Fluorescence measurements and FRET calculations**

Fluorescence measurements were conducted using a Spex Jobin Yvon FluoroMax-3 fluorometer in a 250 μl, 3 mm pathlength microcuvette inside a Peltier temperature-controlled sample chamber at 25°C. Fluorescein (donor) was excited at 460 nm, at which wavelength the amount of direct excitation of the TMR acceptor is negligible. Excitation and emission slit bandpass settings varied between 1 nm and 5 nm depending on the particular concentration of fluorophore. Emission spectra were collected before and after the addition of nucleotide and/or β clamp, and the changes in the spectra were generally complete within the mixing time (~ one minute). The DNA oligonucleotides used for the primer/template DNA experiments were of the following sequences: template strand 5'-GAGCATTCAAGGACTTAGCTACCCCAA-3' and primer strand 5'-TTGTTGGTAGATAATACAGACCTAAGTCC-3'. The efficiency of fluorescence resonance energy transfer (E₂obs) was calculated from the reduction in donor fluorescence intensity using the following equation:

\[
E_{obs} = 1 - (A_D/A_D-A) (F_D-A/F_D)
\]

where F₀ and F₀-A represent the donor fluorescence intensity in a sample with no acceptor, and in a sample with both donor and acceptor, respectively. Both measurements were taken in the same buffer with identical concentrations of nucleotide and β clamp concentrations, and the ratio of F₀-A/F₀ was averaged over a 60 nm range around the fluorescence maximum. A₀ and A₀-A represent the absorbance of each sample at the excitation wavelength, and their ratio is used to correct for differences in fluorophore concentration.

Because acceptor labeling was incomplete, the measured FRET efficiency is a convolution of two populations: one with no acceptor (E = 0), and one containing both donor and acceptor with an effective energy transfer efficiency (Eeff) that was calculated using the following equation:

\[
E_{eff} = E_{obs}/\text{acceptor labeling fraction}
\]
The distance between donor and acceptor was calculated from FRET efficiencies using:

\[ R = R_0(1/E_{\text{ref}} - 1)^{1/6} \]

where \( R \) is the distance between the fluorophores and \( R_0 \), the Forster distance, is the distance at which energy transfer is 50% of the maximum value. A value for \( R_0 \) of 50 Å for the fluorescein–TMR pair was assumed.\(^{38}\)

Acknowledgements

We gratefully recognize the assistance of Xiaoxian Cao for DNA mutagenesis, Holger Sondermann for various biochemical help, Greg Bowman, Steven Kaczmirski, Marijeta Podobnik and Oren Rosenberg for helpful discussions, and Meindert Lamers for the critical reading of this manuscript. This work was supported by an American Cancer Society post-doctoral fellowship to E.R.G., and by grants from the NIH to J.K. (GM45547) and M.O.D. (GM38839).

References


(Received 9 October 2003; received in revised form 19 December 2003; accepted 19 December 2003)