Mechanism of the δ Wrench in Opening the β Sliding Clamp*

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The β sliding clamp encircles DNA and tethers DNA polymerase III holoenzyme to the template for high processivity. The clamp loader, γ complex (γδδ0 ψθ), assembles β around DNA in an ATP-fueled reaction. The δ subunit of the clamp loader opens the β ring and is referred to as the wrench; ATP modulates contacts between β and δ among other functions. Crystal structures of δβ and the γδδ0 minimal clamp loader make predictions of the clamp loader mechanism, which are tested in this report by mutagenesis. The δ wrench contacts β at two sites. One site is at the β dimer interface, where δ appears to distort the interface by via a steric clash between a helix on δ and a loop near the β interface. The energy for this steric clash is thought to derive from the other site of interaction, in which δ binds to a hydrophobic pocket in β. The current study demonstrates that rather than a simple steric clash with β, δ specifically contacts β at this site, but not through amino acid side chains, and thus is presumably mediated by peptide backbone atoms. The results also imply that the interaction of δ at the hydrophobic site on β contributes to destabilization of the β dimer interface rather than acting solely as a grip of δ on β. Within the γ complex, δ′ is proposed to prevent δ from binding to β in the absence of ATP. This report demonstrates that one or more γ subunits also contribute to this role. The results also indicate that δ′ acts as a backboard upon which the γ subunits push to attain the ATP induced change needed for the δ wrench to bind and open the β ring.

Chromosomal replicases of both eukaryotes and prokaryotes derive their high processivity during synthesis from a ring-shaped clamp that encircles DNA and binds to the chromosomal replicase (1–5). The closed circular structure of the clamp necessitates a clamp loader to crack open the ring and place it around the primed DNA in an ATP-driven reaction. In Escherichia coli, the β-clamp is a ring-shaped dimer formed by two crescent-shaped protomers that encircle the duplex (6). Each β protomer consists of three domains, each of which have the same chain fold (7). This gives the β dimer a 6-fold appearance. The β ring binds to the replicative DNA polymerase III core (Pol III) and tethers it to the template for high processivity. The clamp is opened and closed around the DNA by the γ complex clamp loader.

The minimal γ complex clamp loader machine (reviewed in Ref. 5) consists of five different subunits: δ, δ′, and three copies of γ(τ) that are arranged as a circular heteropentamer (Fig. 1A) (8). In addition, two other subunits, χ and ψ, are associated with the clamp loader, but they are not required for clamp loading in vitro (9). In order for this clamp loader to bind two molecules of DNA polymerase III core, two of the γ subunits are replaced by two τ subunits. τ and γ are encoded by the same gene (dnaX); τ is the full-length product, and γ is a truncated version produced by a translational frameshift (10–12). The unique 24-kDa C terminus of τ, absent in γ, binds DNA polymerase III core (13, 14) and DnaB helicase (15, 16), thereby acting to organize the replisome machinery.

The γ(τ) subunits of the γ complex are the only subunits that hydrolyze ATP (1) and therefore constitute the motor of the clamp loading machine. The δ subunit is referred to as the wrench of the clamp loader, since it can open the β dimer at one interface on its own (17–20). The energy for ring opening is not derived from ATP (neither δ nor β bind ATP) but from the energy of protein-protein interaction between δ and β (17). In the absence of ATP, the γ complex does not bind β (18). Study of the δ′ subunit shows that it modulates the ability of δ to bind β even in the absence of other γ complex subunits (17, 19).

Thus, δ′ is proposed to obscure the δ subunit within γ complex from binding to β when ATP is not present (17, 20). However, when ATP binds to the γ subunits, the complex undergoes a conformational change in which it is hypothesized that a portion of δ′ separates from δ, allowing δ to bind and open the β ring (18, 20, 22). Only one δ binds to the δ2 dimer (18). Moreover, δ binds to a monomer mutant of δ (δ′) with 50-fold higher affinity than to δ2 (19). This result indicates that when δ interacts with one member of the δ2 dimer, it uses a portion of its binding energy to perform work (i.e. to part one of the dimer interfaces), thus lowering the observed affinity of δ to δ2. Furthermore, only one δ subunit binds δ2, suggesting that only one of the dimer interfaces is disrupted by δ (17–19). Opening at only one δ2 interface is also consistent with the observation that δ2 cross-linked at only one dimer interface, is efficiently loaded onto DNA by γ complex (17).

The crystal structure of δ in complex with a β monomer mutant (δβ1) combined with the structure of the minimal clamp loader (γδδ0), provides further details and allows predictions about how δ opens β2 (8). Of the three domains of δ, only the N-terminal domain (domain I) interacts with β (Fig. 1B), and it contacts the clamp in two different places. The first site of the δ-β interaction involves hydrophobic contacts between residues Leu73 and Phe74 of δ and a hydrophobic pocket of β located between domains II and III (Fig. 1, B and C). We refer to this as “site 1.” Both Leu73 and Phe74 of δ protrude out to form a hydrophobic plug that fits into the hydrophobic pocket on the surface of β. This hydrophobic interaction is presumed to

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be responsible for the majority of the binding energy. Interestingly, δ residues Leu\(^2\) and Phe\(^4\) are the most highly conserved residues among δ subunits of different bacteria (20, 23). Likewise, alignment of bacterial β subunits shows that residues comprising the hydrophobic pocket in β to which δ binds are highly conserved (20).

There is a second interaction between δ and β that involves δ helix α\(_4\) and β loop α\(_1\)β\(_{2}'\). This contact is referred to here as "site 2" (Fig. 1C). The interaction of δ with β at this site leads to an extensive conformational change of the β loop that is thought to be important for ring opening (20). This five-residue loop of β (residues 274–278) is near the interface and in fact connects to the surface of α helix that contains the two residues (Ile\(^{272}\) and Leu\(^{273}\)) that form the hydrophobic core of the β\(_2\) dimer interface. In contrast, the structure of the δ\(_{2}\) complex shows that the hydrophobic core residues of β are rotated out of position, thereby precluding formation of the dimer interface (Fig. 1C). Thus, it would appear that δ distorts one β\(_2\) interface by altering the conformation of the β 274–278 loop. This distortion at site 2 appears to be the result of a steric clash between δ and β, which pushes on the loop, rather than being due to specific side chain contacts between δ and β. Hence, the site 2 interaction/steric clash presumably requires an input of energy that is obtained from the binding energy at site 1.

The δ subunit destabilizes the interface of β\(_2\) but does not explain how a gap opens up at the β interface for DNA to pass through. The shape of β\(_2\) in the δ\(_2\) structure relative to the β\(_2\) dimer suggests how the ring actually opens (20). Superposition of monomeric β (i.e., of the δ\(_2\) complex) onto dimeric β reveals that the shape of the β monomer is less curved than in the dimer. This change in curvature is produced by rigid body motions between the three domains of β. The largest rigid body motion occurs distant from the δ-binding sites, suggesting that the change is intrinsic to β and that the closed β dimer is under spring tension between domains of β. After disruption of the dimer interface by the δ subunit, release of the spring tension results in the motions between domains that produce the gap at the broken interface (20).

The γ\(_β\)δ' structure fits nicely with biochemical data indicating that β cannot bind γ complex in the absence of ATP and that δ' participates in modulating the δ-β interaction. The γ\(_β\)δ' structure (8) shows that each of the subunits is composed of three domains having the same chain fold and is a member of the AAA\(^-\) family. The five subunits are arranged as a circular heterotetramer (Fig. 1A). The C-terminal domains of all five subunits form a tight circular connection, holding the subunits together. On the contrary, the connections between the N-terminal domains contain a gap between δ and δ' (see Fig. 1A). Docking of β\(_2\) onto δ shows that β\(_2\) does not fit due to steric occlusion by δ and possibly some of the γ subunits as well. This is consistent with the fact that ATP is not present in the structure. It is proposed that as the ATP sites fill, conformation changes in γ are propagated around the pentamer to increase the gap between δ and δ', thereby allowing δ to bind to β for clamp opening (8, 17, 18, 22). In this state, with β and ATP bound to γ complex, a tight affinity for DNA is established (22, 24). Upon recognizing a primed site, the ATP is hydrolyzed and the γ subunits may move δ back into proximity with δ', forcing the β ring off the δ wrench and allowing the β ring to close around the DNA.

The δ structure was the first clamp loader subunit to be solved (25). δ appears to be more rigid than the γ and δ subunits (8). This conclusion derives from the observation that the three domains of δ' in γ complex are oriented nearly the same as in the δ' alone. In contrast, the relative orientations of domain III relative to domains II/II of all three γ subunits are different in γ\(_β\)δ', and the same is true for δ in δ\(_2\)β compared with δ in the γ\(_β\)δ' structure. Consistent with a rigid structure, δ' has additional connections between domains compared with the few connections between the domains in either γ or δ. This rigid conformation of δ' has earned it the title of stator, the stationary part of a machine upon which the other parts move (8). Perhaps the rigid δ' stator serves as an anvil for the β interactive element of δ to strike, pushing β\(_2\) off of the γ complex following ATP hydrolysis. A recent report demonstrates that δ', besides its role as stator, also plays an instrumental role in the motor function of γ complex, as predicted by the structure, by supplying a catalytic arginine into the ATP site at the intersubunit junction δ'/γ\(_1\) (8, 26).

In light of the γ\(_β\)δ' and δ\(_2\)β crystal structures and predictions of how these proteins function, we reexamine here the mechanism of clamp loading by the γ complex, in particular the δ-β interaction, and the role of δ' in rendering the β interacting elements of δ accessible for binding the clamp. The studies contain surprises, but overall the results provide significant advancements in our understanding of how this complex machinery functions.

**EXPERIMENTAL PROCEDURES**

**Materials**

Unabeled deoxyribonucleoside triphosphates were from Amersham Biosciences; radioactive deoxyribonucleoside triphosphates were from PerkinElmer Life Sciences. Proteins were purified as described: α, ε, γ, τ (27), β (7), δ and δ' (28), χ and ϕ (29), θ (30), SSB (31). Core polymerase and γ complex were reconstituted from wild-type and/or mutant subunits and purified as described (30). Samples of purified γ complex were analyzed on a 14% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue G-250. M13mp18 ssDNA was purified as described (32) and prised with a30-mer DNA oligonucleotide as described (27).

**Buffers**

Buffer A is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% glycerol (v/v). Buffer B is Buffer A, except the pH was adjusted to 8.2. Buffer C is 10 mM sodium acetate (pH 7.5), 0.5 mM EDTA, 2 mM DTT. Buffer D is Buffer C, except the pH was adjusted to 6.1. Gel filtration buffer is Buffer A containing 100 mM NaCl, 1 mM ATP, and 10 mM MgCl\(_2\). Reaction buffer is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 4% glycerol (v/v), and 40 μg/ml bovine serum albumin. Tris-Sucrose buffer is 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 10% sucrose.

**Mutant Proteins**

δN62A, P65A and δL73A, F74A—Two different double mutants of δ were constructed. In one, which we refer to here as δN62A, the Asn\(^{62}\) and Phe\(^{74}\) residues located on helix α\(_4\) were mutated to alanines. In the other, referred to as δL73A, residues Leu\(^{73}\) and Phe\(^{74}\) were mutated to alanines. These mutants were constructed by DNA oligonucleotide site-directed mutagenesis of the pl29 expression plasmid (28) and confirmed by sequence.

Expression and purification of δ\(^{N62A}\) and δ\(^{L73A, F74A}\) were performed as follows. Expression plasmids were transformed into competent BL21 (DE3) cells (Novagen). Fresh transformants were grown in 12 liters of LB containing 200 μg of ampicillin/ml to a density of A\(_{600}\) = 0.6 and induced with 1 mM isopropyl-1-thio-D-galactopyranoside. Cells were incubated 4 h with shaking at 37 °C, chilled to 15 °C, and incubated another 20 h with shaking at 15 °C. For cell lysis, cells were brought to a final volume of 300–400 ml with a final concentration of 30 mM spermidine, 100 mM NaCl, and 5 mM DTT in Tris-sucrose buffer. Cells were lysed by two passages through a French Press at 17,000 p.s.i. and insoluble material was removed by centrifugation at 12,000 rpm for 1 h at 4 °C in an SLA1500 rotor. The soluble cell lysate supernatant (Fraction 1) was decanted and treated with 0.21 g/ml ammonium sulfate. After stirring for 30 min at 4 °C, the pellet was collected by centrifugation at 12,000 rpm for 30 min in a SLA1500 rotor. The resulting pellet was resuspended in Buffer B. The protein was diluted to a conductivity of 60 mM NaCl with Buffer B and then applied to a Heparan-agarose column (Bio-Rad) equilibrated in Buffer B. Protein was eluted with a 100–500 mM NaCl gradient in Buffer B. Fractions containing δ were pooled (Fraction II) and diluted with Buffer B to a conductivity equivalent to 100 mM NaCl. Particulate matter was removed by cen-
trifugation at 10,000 rpm for 10 min at 4 °C in an SS34 rotor. The supernatant was applied to a MonoQ column equilibrated in Buffer B. The column was then washed with Buffer B before eluting the protein with a 100–500 mM NaCl gradient in Buffer B. Fractions containing δ were pooled (Fraction III, δ^ββ^-AAA mutant: 45 ml, 1.2 mg/ml; δ^ββ^-AAA mutant: 32 ml, 1.0 mg/ml) and then stored at ~80 °C.

δ^AN—Nucleosides encoding the N-terminal 206 residues of δ were designed using the P-FPLC Superose 12 column (Amersham Biosciences). The β subunit (25 or 30 μm dimer, as indicated) was incubated with 5 or 25 μM (as indicated) γ complex (or mutant γ complex) for 15 min at 15 °C in 200 μl of Buffer A containing 100 mM NaCl in the presence or absence of 1 mM ATP and 10 mM MgCl₂. The mixture was then injected onto a 24-ml Superose 12 column equilibrated in the same buffer at 4 °C. After collecting the first 5.8 ml (void volume), fractions of 155 μl were collected and analyzed in a 14% SDS-polyacrylamide gel.

**Clamp Loading Replication Assays**

The clamp loading activity of wild-type and mutant γ complexes were assayed by their requirement to load β onto a primed circular M13mp18 ssDNA template in order to observe nucleotide incorporation by the core polymerase (αεθ subunits). The reaction mixture contained SSB (470 μM tetramer), primed M13mp18 ssDNA (1.3 nm), core polymerase (5 β subunits, β1 (10–740 nm), γ complex (0–0.64 nm), 60 μm each of dATP, dCTP, and dGTP, 20 μM [α-^32P]dATP, 1 mM ATP, and 10 mM MgCl₂ in 25 μl of reaction buffer (final volume). In the γ complex titration assays, core polymerase (5 nm) and β (10 nm as dimer) were added to the reaction mixture, and replication was initiated upon the addition of either wild-type or mutant γ complex (0–0.64 nm titration). In the β titration assays, 0.64 nm γ complex was used, and DNA synthesis was initiated upon the addition of either wild-type or mutant β (0–740 nm titration). Reactions were incubated at 37 °C for 5 min and quenched upon the addition of 25 μl of SSB, 5 mM EDTA, and 0.8 M NaCl. Quenched reactions were spotted onto DE51 (Whatman) filters and then washed and quantitated by liquid scintillation as described (27).

**RESULTS**

*Leu*3 and *Phe*4 of δ Are Essential for Strong δ-β Interaction—The δβ structure suggests that residues Leu*3* and *Phe*4 of δ are responsible for most of the binding energy between δ and the hydrophobic pocket of site 1 in β. In the δβ structure, Leu*3* and *Phe*4 stick out from a loop to form a hydrophobic plug that fits into the hydrophobic pocket on the surface of β (Fig. 1B) (20). To assess the importance of these residues to clamp loading activity, we mutated them to alanines and purified the δ double mutant (δ^ββ^-AAA) protein from an overproducing strain of *E. coli*. The γ complex was reconstituted using the δ^ββ^-AAA mutant along with the δ1, δ2, δ3, and γ subunits. The fully assembled mutant γ complex was purified from excess unbound subunits on a MonoQ anion exchange column. The subunit ratio of γ (δ^ββ^-AAA) complex is comparable with wild-type γ complex as observed in the Coomassie Brilliant Blue-stained SDS-polyacrylamide gel of Fig. 2A. The γ (δ^ββ^-AAA) complex also remains intact during analysis on a gel filtration column, as shown in the middle panel of Fig. 2C. The ability of δ^ββ^-AAA to assemble into a multisubunit complex with γ, δ1, δ2, δ3, and ψ indicates that the mutant δ subunit is properly folded. This result is also consistent with the crystal structure of γ, δδδ⁰, which shows that neither of these residues are involved in subunit-subunit interaction within γ complex. The major intersubunit connections within γ, δδδ⁰ occur through the δ-domain loops of each of the five subunits of the complex.

Next, the reconstituted mutant γ complex was tested for clamp loading activity by measuring β-dependent stimulation of DNA synthesis by core polymerase (αεθ). Pol III core is unable to extend a primer on an SSB-coated singly primed M13mp18 ssDNA template unless it is coupled to a β clamp (1). The γ (δ^ββ^-AAA) complex showed almost no clamp loading activity in this assay, whereas the wild-type γ complex produced a strong signal (Fig. 2B). This result is consistent with the δβ structure, which shows that both Leu*3* and *Phe*4 of δ are directly engaged in the δ-β interaction. Therefore, the loss of activity of the γ (δ^ββ^-AAA) complex may be explained by a decrease in affinity between the mutant complex and β. If this is the case, the γ (δ^ββ^-AAA) complex should show decreased ability...
to bind β in a gel filtration analysis, and the addition of more β may rescue the activity of \( \gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex.

To examine interaction between \( \gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex and β, the proteins were mixed and analyzed for complex formation on a Superose 12 sizing column equilibrated with buffer containing ATP. Previous studies have shown that ATP is required to promote binding of the β subunit to γ complex (18). As a control, β alone migrates in fractions 33–43 (Fig. 2C, bottom panel). Analysis of a mixture of wild-type γ complex and β is shown in Fig. 2C (top panel). The β subunit binds to the large γ complex and therefore co-elutes with it in fractions 15–29 and resolves from excess unbound β, which elutes in the later fractions. A similar analysis using the \( \gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex mutant shows that the mutant is unable to associate with β under these conditions (Fig. 2C, middle panel). The \( \gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex elutes in fractions 21–29, but the β clamp does not co-migrate with it; instead, the unbound β migrates in the later fractions 33–43, as observed for β alone (Fig. 2C, bottom panel). The experiment was repeated using a 5-fold higher concentration of \( \gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex, but it still did not bind to β (Fig. 2C, right panels). These results indicate that the γ(\(\delta^{LF} \rightarrow \Lambda \Lambda\)) complex-β interaction is affected by mutation of the two hydrophobic residues of δ in the mutant γ complex. Analysis of the polyacrylamide gels of Fig. 2C by laser densitometry showed no detectable β in the peak fractions containing the mutant γ complex. Assuming that this method would have detected as little as 10% the amount of β that comigrates with wild-type γ complex, the affinity of β for the mutant γ complex is at least 10-fold weaker than for wild-type γ complex. Furthermore, study of \(\delta^{LF} \rightarrow \Lambda \Lambda\) mixed with β showed no interaction between them (data not shown). However, the gel filtration analysis and clamp loading replication assays are performed using different protein concentrations. During gel filtration, protein complexes are not at equilibrium, requiring use of a relatively high concentration of protein (i.e. micromolar), and only strong complexes with relatively slow dissociation rates are observed. On the other hand, during the clamp loading replication assay, components exist in equilibrium, and even transient weak complexes can produce a signal. Therefore, it remains possible that β binds transiently to the \(\gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex. In fact, the replication assays described below indicate that they interact, albeit with much less affinity.

The above results indicate that the defect in activity of the \(\gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex is due to the lower affinity of β for the mutant clamp loader. In this case, a high concentration of β may rescue the replication activity. The result of a β titration into the replication assay demonstrates that high concentrations of β indeed bring the activity of the \(\gamma(\delta^{LF} \rightarrow \Lambda \Lambda) \) complex back to wild-type levels (Fig. 2D). Thus, we conclude that the primary effect of the mutation is on the strength of the δ-β interaction.

The δ Residues Asn^{62} and Phe^{65} Do Not Interact with β—The C-terminal portion of the α4 helix of δ contacts the β loop near the interface of the ring and is thought to do so via a steric clash between the proteins (Fig. 1B) (20). If there were specific contact between amino acid side chains of δ and β at site 2, residues Asn^{62} and Phe^{65} of δ (on the α4 helix) are the only side chains of δ that are close enough to interact with β. If these
residues contact β, replacement of δ Asn62 and Phe65 with alanines (δ4) should affect the strength of δ-β interaction. To test this possibility, these two residues were mutated to alanines, and the resulting δ4 mutant was purified and then reconstituted with γ, δ, χ, and ψ to form γ(δ4) complex. As illustrated in Fig. 3A, the subunit stoichiometry of γ(δ4) complex appears similar to that of wild-type γ complex in an SDS-polyacrylamide gel.

Next, we studied the γ(δ4) complex for the ability to bind β and to load β onto DNA. In contrast to γ(δL–ΔΔA) complex, the interaction with β does not appear to be compromised (Fig. 3B). The mutant γ complex remains competent to associate with β as indicated by their coelution in fractions 17–29 (Fig. 3B, middle panel). Densitometric analysis of the polyacrylamide gels shows ~70% of the level of β comigrating with γ(δ4) complex compared with wild-type γ complex, indicating that if γ(δ4) complex binds β less tightly than wild-type γ complex, the difference in affinity is probably less than 2-fold. Furthermore, the γ(δ4) complex is fully active with β in stimulating DNA synthesis by the Pol III core (Fig. 3C). Although, these results support our earlier prediction that the αhelix of δ is involved in a nonspecific clash with β to destabilize the interface, this proposal is further tested below. The results indicate that the interaction at site 2 is not a simple steric clash between proteins and that some modification of the proposal is needed.

**Analysis of the β Loop near the Interface**—The current model for δ action at the β interface is that δ helix αδ collides sterically with β to push on a loop near the β interface, leading to distortion of the interface and ring opening. The loop inβ consists of residues 274–278 and is located adjacent to the αhelix of β at the dimer interface. This αhelix is strained at one end into a 4–10 helix in the β dimer structure. The δβ1 structure suggests that the push on the β loop results in releasing the strained portion of the αhelix (Fig. 1C). The two hydrophobic core residues of the β dimer interface are contained on this αhelix, and when the strain is released these two hydrophobic residues are rotated out of the dimer interface so that they no longer participate in dimer formation.

A prediction of this model is that deletion of the β loop should not only render β inactive and unable to open but should also increase the affinity of δ for β, since it would no longer need to expend binding energy to push on the β loop. To test this prediction, we deleted 4 residues (residues 275–278) of the loop and purified the mutant (βloop) from an overproducing strain of *E. coli*. We wished to delete the maximum number of residues but decided on only four, since one residue is needed to link the αhelix with its upstream secondary structure element. Hence, a more extensive deletion may have affected the structure of β and led to monomerization of the β dimer.

To examine whether the βloop mutant retained its dimeric status, we analyzed the size of the βloop mutant by gel filtration. Fig. 4A shows the SDS-polyacrylamide gel of the gel filtration column fractions for wild-type β dimer, the βloop mutant, and a β mutant that behaves as a monomer (βmonomer) (19). The βloop mutant elutes in fractions 33–42, the same fractions as the wild-type β dimer (Fig. 4A, bottom and top panel, respectively), whereas the β monomer elutes much later, in fractions 39–48 (Fig. 4A, middle panel). Hence, the βloop mutant retains its dimeric structure. However, the βloop mutant was inactive in the clamp loading assay using γ complex and core polymerase on primed M13mp18 ssDNA, consistent with the loop being important for activity (Fig. 4B).
The $\beta^{\text{loop}}$ mutation should result in tighter binding between $\gamma$ complex (or $\delta$) and $\beta^{\text{loop}}$, since we have proposed that $\delta$ pushes on this loop to distort the interface. However, analysis of the $\beta^{\text{loop}}$-$\gamma$ complex interaction by gel filtration showed that instead of binding tighter, the interaction between them was compromised. In Fig. 4C, either wild-type $\beta$ (top panel) or the $\beta^{\text{loop}}$ mutant (middle panel) was mixed with $\gamma$ complex and then analyzed by gel filtration in the presence of ATP. Analysis of the column fractions on an SDS-polyacrylamide gel shows that wild-type $\beta$ clamp co-elutes with the five subunits of the $\gamma$ complex in fractions 12–30 (Fig. 4C, left, top panel), whereas the $\beta^{\text{loop}}$ mutant does not interact, or at best only weakly associates, with the $\gamma$ complex in fractions 21–27 (Fig. 4C, left, middle panel). This result demonstrates that the strength of the $\delta$-$\beta$ interaction is decreased by the $\beta$ loop deletion, not increased as we had expected. Hence, there must be a positive interaction between $\delta$ and $\beta$ at site 2, not just a nonspecific clash. Next, the gel filtration analysis was repeated using a 5-fold greater concentration of $\gamma$ complex (Fig. 4C, right panels). Under these conditions of elevated protein, the $\beta^{\text{loop}}$-$\gamma$ complex interaction is evident. This result is consistent with the $\beta$ loop mediating a positive contact between $\delta$ and $\beta$ and with the fact that without it, a greater concentration of protein is required in order to detect an interaction between the $\beta^{\text{loop}}$ mutant and $\gamma$ complex. The fact that interaction of $\gamma$ complex with the $\beta^{\text{loop}}$ mutant is restored with a 5-fold elevation in the concentration of the $\gamma$ complex suggests that the affinity of $\beta^{\text{loop}}$ for $\gamma$ complex is reduced only about 5-fold or less relative to wild-type $\beta$. The interactions between $\delta$ and $\beta$ at site 2 are probably not as strong as site 1, since the $\gamma$($\delta^{\text{E}}$–$\alpha$) complex displayed no interaction with $\beta$ even at elevated concentration.

To determine whether the defect in replication activity of the $\beta^{\text{loop}}$ mutant is compensated by use of a higher concentration of $\beta$ in the assay, as observed in the experiment of Fig. 2D where additional $\beta$ restored activity to the $\gamma$($\delta^{\text{E}}$–$\alpha$) complex, we performed clamp loading replication assays over a range of $\beta$ concentration. Although some activity (10–20%) was restored upon titrating more of the $\beta^{\text{loop}}$ mutant into the replication assay with core and $\gamma$ complex (Fig. 4D), full replication could not be restored as had been observed by titrating $\beta$ into site 1-mutated $\gamma$($\delta^{\text{E}}$–$\alpha$) complex. Hence, the $\beta$ loop is important to its ability to be loaded onto DNA, and activity is only partially restored by adding $\beta^{\text{loop}}$ in large excess. The residual activity of $\beta^{\text{loop}}$ when added in excess may be explained if the site 1 interaction contributes to destabilization of the interface, leading to the low clamp loading activity observed here for the $\beta^{\text{loop}}$ mutant. Alternatively, the single residue of the loop that remains in the $\beta^{\text{loop}}$ mutant is sufficient to provide some activity deriving from $\delta$-$\beta$ interaction at site 2.

In summary, the results are consistent with predictions from the structure, except the decrease in affinity of $\gamma$ complex for the $\beta^{\text{loop}}$ mutant indicates that a positive interaction at site 2 is lost. This implies that $\delta$-$\beta$ interaction at this site is not just a steric clash between $\delta$ and $\beta$ at site 2. The affinity at this site is probably mediated via backbone atoms instead of side chains, as implied by the retention of $\beta$ binding and clamp loading activity by the $\gamma$($\delta^{\text{E}}$) complex, as well as by the lack of homology between prokaryotic $\beta$ clamps in the position of the loop.

Role of the $\delta$ Stator—The N-terminal domain (domain I) of $\delta$ is thought to block interaction of $\delta$ with $\beta$ in the $\gamma$ complex in the absence of ATP (17, 20). ATP promotes $\gamma$ complex-$\beta$ interaction, presumably due to a conformation change in $\gamma$ complex that pulls domain I of $\delta$ away from domain I of $\delta'$, allowing $\delta$ to bind and open the $\beta$ ring (18, 20). Based on these findings, one may predict that a $\gamma$ complex mutant containing a $\delta'$ deletion that lacks domain I would bind to $\beta$ even in the absence of ATP. To test this prediction, we deleted the first 206 residues of $\delta$($\delta^{\text{E}}$), corresponding to domains I and II, and cloned, overexpressed, and purified the C-terminal domain III of $\delta'$ (referred to here as $\delta^{\text{E}}$)N).

The $\delta^{\text{E}}$N mutant was first tested for its ability to assemble into a $\gamma$ complex with the other subunits. The $\gamma$($\delta^{\text{E}}$)N complex is indeed formed upon mixing all of the subunits together, and the mutant $\gamma$($\delta$)N complex is also stable to the ion exchange purification step (Fig. 5A). The $\gamma$($\delta$)N complex also remains intact during analysis by gel filtration (Fig. 5C), as previously reported (33). Reconstituted $\gamma$($\delta^{\text{E}}$)N complex retained 10–15% clamp loading activity compared with wild-type $\gamma$ complex in the $\beta$-dependent stimulation of pol III core assay (Fig. 5B). Further, the addition of wild-type $\delta'$ (0–16 ng) into a replication mixture containing 2 ng of $\gamma$($\delta^{\text{E}}$)N complex did not affect the DNA replication activity (data not shown). The $\gamma$($\delta$)N complex lacks $\delta$ domain I and thus may be expected to bind $\beta$ even in the absence of ATP, since $\delta$ should no longer need to be pulled away from domain I of $\delta'$. To test this, we examined the $\gamma$($\delta$)N complex for interaction with $\beta$ by gel
filtration in the absence of ATP. However, the result in Fig. 5C (left panel) demonstrates that the β dimer does not bind γδΔN complex. These results indicate that other subunits of γ complex, in addition to the δ′ subunit, are responsible for blocking the β interacting element of δ′. Although not shown here, γδΔN complex was also incapable of binding to β in the absence of ATP. Therefore, γ must contribute to blocking δ′ from binding to β.

Next we tested the ability of ATP to promote the binding of β to γδΔN complex. Surprisingly, the results in Fig. 5C (right panel) show that β does not appreciably bind to γδΔN complex even in the presence of ATP (middle panel; compared with wild-type γ complex in the top panel). Overall, this set of experiments with γδΔN complex gave results that were the opposite of those expected. Whereas we thought it would bind β with or without ATP, it does not bind to β at all, even with ATP present. One could explain this result if δ′ were known to bind β tightly and the δ′ deletion no longer bound β. However, the δ′ subunit has no detectable interaction with β, unlike γ, which binds β weakly, and δ, which binds β tightly (34). Further, under the protein concentrations used here, the δ subunit alone would bind β if it were not blocked by other subunits. Hence, γ must block β binding to γδΔN complex, and the γ block is not relieved in the presence of ATP. Overall, these results indicate that the δ′ stator, which does not bind ATP, is required to promote the ATP-dependent conformation change of γ complex needed for its interaction with β.

DISCUSSION

This report utilizes specific mutants of β and γ complex to test predictions of the γ complex clamp loader mechanism based on the γδδ′ and δβ1 structures. In some cases, the results did not meet expectations and led to a rethinking of how this complicated machinery works. A summary of the prediction, results, and new implications is discussed below.

Site 1 δ-β Interaction—The structure of δβ1 shows that the δ wrench binds β in two places. Site 1 is predicted to be where most of the grip of δ on β is attained. The main feature of this site consists of a conserved hydrophobic pocket in β, located between domains II and III, and two conserved hydrophobic residues on δ, Leu73 and Phe74. If the prediction is true, then mutation of δ residues 73 and 74 to alalanines should greatly diminish the affinity of β for δ and γδΔN complex. This prediction was nicely upheld in the current study. Furthermore, the γδδ′ complex was inactive with β at typical concentrations of β in clamp loading replication assays. As
expected, high concentrations of β, which should force the interaction of β with the γ(δεΔN) complex by mass action, restores activity to the γ(δεΔN) complex essentially to wild-type levels.

**Site 2 δ-β Interaction**—The second site at which δ contacts β is near the dimer interface and is thought to be the site where work is performed to crack the interface. Presumably, the energy for this work is derived from the site 1 interaction of δ to β. This proposal is consistent with the biochemical observation that δ binds a β monomer mutant 50-fold tighter than the β dimer (19). It is hypothesized that δ binds the β monomer tightly because it does not need to expend any of its binding energy to part a β dimer interface. Binding of δ to the β dimer is weaker than to a β monomer, because some of the binding energy is expended to perform the work of cracking the dimer interface open.

The structure of β at site 2 contains a loop that juts out from the end of the interfacial α helix, which contains the hydrophobic core residues of the β dimer interface. In the dimer, this α helix is strained to adopt a 4–10-helix structure in order that two hydrophobic residues are pointing into the interface to form the hydrophobic core. In the δβ2 structure, the interfacial α helix of β is no longer strained, and the two hydrophobic residues are turned away from the interface, preventing a proper interface structure from forming. More striking yet is the position of the loop. The β loop is severely bent due to what appears to be a steric collision with δ helix α₅. Since the loop is connected to the β interfacial helix, it appears from the δβ2 structure that bending the loop applies a force to the strained helix that then causes the hydrophobic core residues to pop out of the interface and relieves the stress in the 4–10 portion of the helix. The δ residues Asn²⁶ and Phe⁷⁴ are the only side chains close enough to play a role in specific binding to β. Thus, the observed interaction appears to be a steric clash between δ and β at site 2, implying that δ acts like a crowbar to get a grip on one site (site 1) and push nonspecifically at the other (site 2) to crack the interface.

The first mutant we studied to assess this model was one in which we replaced the δ residues Asn²⁶ and Phe⁷⁴ with Ala residues. The results upheld the nonspecific steric clash model. The γ complex containing δ N62A/F65A (referred to as the γ(δ⁴⁵) complex) had no significant departures from wild-type γ complex in either β binding or clamp loading activities. We then went on to study the effect of deleting the β loop. In the steric collision crowbar model of δ action, δ pushes on the loop at the β interface and distorts it. The energy for the steric clash/push is obtained from the binding energy of δ to site 1 of β. This idea predicts that elimination of the β loop should lead to enhanced affinity between γ complex (or δ) and the βloop mutant but an inability of the βloop mutant to be loaded onto the DNA. However, when the β loop was deleted, we obtained our first unexpected result.

Eliminating the β loop decreased the affinity between γ complex and the βloop mutant, implying that δ normally has a positive interaction with the β loop instead of an interaction that requires an expenditure of binding energy. If this interaction is not mediated by amino acid side chains, as study of the γ(δ⁴⁵) complex mutant suggests, then it must be mediated by interactions between the peptide backbones of δ and β. However, the βloop mutant was nearly inactive in the clamp loading replication assay, and the activity was only partially rescued by the addition of a large excess of βloop mutant to the assay. Furthermore, the loss of the β loop did not decrease the affinity to γ complex by much, since interaction with the βloop mutant was reestablished by increasing the γ complex concentration only 5-fold. Hence, in outline, the proposal that δ derives most of its binding energy to β from site 1 and distorts the loop in site 2 to destabilize the interface is upheld in this study. Moreover, the results indicate that site 2 is not simply a steric clash of δ with β but rather an interaction between peptide backbone atoms of δ and β.

The βloop mutant retains about 15% activity compared with wild-type β when supplied in excess to the assay. How can β open if the loop required for opening is missing? One possibility is that the site 1 interaction provides a source of interface instability, since the hydrophobic pocket of β is strategically located between two of the three domains. If δ residues Leu⁷³ and Phe⁷⁴ were to act as a wedge, they might increase the angle at which these domains are oriented, pulling domain III away...
from the interface. Yet another possibility is that no outside push by δ is even required to open β. In this model, as described earlier (20), the β dimer may rapidly alternate between open and closed states, and δ may simply bind β in the open form and stabilize it in the open state. However, even in this model, δ would still need to stabilize the open form of the β ring, which also amounts to an input of energy. Hence, direct destabilization of the interface of closed β or stabilization of an open β ring requires energy. The current study demonstrates that this energy is derived mainly from interaction of δ at site 1 of β but that energy for this is also contributed by the δ-β interaction at site 2.

Action of δ as a Stator—Biochemical studies have demonstrated that γ complex requires ATP to bind δ, indicating that one or more subunits of γ complex block the interaction of β with δ when ATP is not present. The δ′ subunit and β compete for binding to δ, implying that the δ′ subunit is involved in preventing access of β to δ in the γ complex (17). Further, δ can remove β rings from DNA by virtue of δ′ opening the β ring, but δ′ prevents δ in this action, consistent with δ′ preventing β-δ interaction (34). The crystal structure of γδ′δδ′ shows a gap between δ′ and δ, and modeling of β2 onto δ′ indicates that β2 does not fit on δ without clashing with δ′ (8). Presumably, ATP binding to γδ alters the conformation of γδδ′ to allow β2 to dock onto δ for ring opening. It has been suggested that an increase in the gap between δ and δ′ is powered by ATP binding to γδ (8). ATP hydrolysis, triggered by DNA binding, could reverse the conformational change narrowing the gap between δ and δ′, thereby squeezing β2 from δ and freeing the ring to close around DNA.

The δ′ subunit is rigid, as described in the Introduction, and has sometimes been referred to as an anvil to describe a possible role in which β gets pushed, or hammered, off δ by drawing β2-δ′ close to the rigid δ′ stator in γ complex. The anvil role proposed for δ′ implied that deleting the N-terminal domain of δ′, which is situated directly across the gap from the β inter-active site on δ, may result in a γ complex that can bind β without need for ATP. In other words, δ′ would no longer block β from binding δ. To ask whether δ′ blocks β binding in γ complex, we cannot simply omit δ′ altogether, because the γδ′δψ complex is not stable without δ′. Biochemical studies have shown that the C-terminal domain of δ′ is all that is needed for δ′ to assemble into a stable complex with γ (33). Hence, we produced the C-terminal domain of δ′ (δ′ΔN), used it to reconstitute γδΔN complex, and then asked whether it binds β without ATP. However, the γδΔN complex did not bind β in the absence of ATP, and therefore δ′ is not the sole subunit that blocks β from binding δ in γ complex. We obtained similar results in the absence of the χ and ψ subunits (γδδ′ΔN complex). This result demonstrates that one or more of the γ subunits also modulate δ-β contact in γ complex. In fact, this modified view is consistent with a recent report in which γ was shown to inhibit δ-mediated unloading of β rings from DNA, like δ′, indicating that γ modulates δ-β contact (34). Presumably, both γ and δ′ conspire in their actions to control access of δ to the β clamp.

Another proposed role for the rigidity that δ′ supplies to γ complex is one in which δ′ acts as a backbone to direct the ATP-induced conformational changes in the γ subunits (5,8). Elimination of the δ′ backbone in the γδΔN complex may disable the ATP-dependent conformation change required to promote binding of β. Indeed, the γδΔN complex does not bind β even in the presence of ATP. Therefore, the results using the γδΔN complex indicate that the primary role of the stator may be to act as a backbone to direct the conformation changes induced by γ upon binding ATP as indicated in the scheme of Fig. 6. Of course, other, as yet unimagined roles for δ′ may also explain the results, and further studies will be needed for a comprehensive understanding of the role of δ′. In this regard, our earlier study showed that an Arg residue contained within a conserved SRC motif in domain I contributed to ATP catalysis, as proposed by the structure (26). However, γ complex reconstituted with the δ′ R158A mutant still bound to β in response to ATP, and thus the clamp loading defect was at a later step in the mechanism compared with γδΔN complex.

Clamps and Clamp Loaders in Eukaryotes—The clamp loading machineries from bacteria, bacteriophage T4/RB69, archaeobacteria, and eukaryotes are related by structure, function, and evolution. Evolutionary relationships between these assemblies are evident from the primary sequences of their subunits, which share amino acid motifs associated with the AAA+ family of ATPases (35–37). In particular, the eukaryotic clamp loader, replication factor C (RFC) consists of five different subunits, each of which is homologous with each other and with E. coli γ and δ′ (36, 37). Atomic force microscopy and electron microscope studies of the human RFC clamp loader (37) reveal a pentameric ring arrangement of the five RFC subunits with nucleotide-dependent conformational changes and form structures similar to that of E. coli γδδ′ (5). In the human clamp loader, at least two subunits (p140 and p40) and probably others bind the PCNA clamp, similar to δ and γ subunits binding to β. Yeast RFC 1 (human p140) has two conserved adjacent hydrophobic residues between RFC box IV and V, the same location as the conserved Leu73 and Phe74 in δ that bind the 1 hydrophobic pocket of β. PCNA also has a similar architecture to β, except that PCNA monomers contain only two domains and therefore trimmerize to form a ring. Moreover, it has been observed that the p21 cell cycle regulator protein binds PCNA in a similar location as that of δ to β (39). Namely, hydrophobic residues of p21 bind to a hydrophobic pocket in PCNA located between two domains.

Like γ complex, RFC interaction with PCNA is strengthened by ATP (21). Therefore, one RFC subunit might act like the δ′ stator to act as a backbone for ATP-induced changes in the motor subunits. Three RFC subunits are similar to the γ trimer in that they each contain both a P-loop and SRC motif (yeast RFC 2,3,4 and human p36, 37, and 40). If RFC 1 is the wrench, and RFC 2,3,4 is the motor-like γ, this leaves RFC 5 to act as the δ′ stator. In both yeast and humans, RFC 5 (human p88) contains the SRC motif but lacks a consensus P-loop, and thus it may be analogous to the δ′ stator, which also has an SRC that donates a catalytic Arg to the neighboring γ but lacks a P-loop and does not bind ATP itself (26). These several similarities between prokaryotic and eukaryotic clamp loaders suggest that the findings reported here on γ complex and β may generalize to eukaryotic systems.

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Mechanism of the $\delta$ Wrench in Opening the $\beta$ Sliding Clamp

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