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Mechanism of β Clamp Opening by the δ Subunit of *Escherichia coli* DNA Polymerase III Holoenzyme*

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The β sliding clamp encircles the primer-template and tethers DNA polymerase III holoenzyme to DNA for processive replication of the *Escherichia coli* genome. The clamp is formed via hydrophobic and ionic interactions between two semicircular β monomers. This report demonstrates that the β dimer is a stable closed ring and is not monomerized when the γ complex clamp loader ($\gamma_3\delta_1\delta_1\chi_1\psi_1$) assembles the β ring around DNA. δ is the subunit of the γ complex that binds β and opens the ring; it also does not appear to monomerize β. Point mutations were introduced at the β dimer interface to test its structural integrity and gain insight into its interaction with δ. Mutation of two residues at the dimer interface of β, I272A/L273A, yields a stable β monomer. We find that δ binds the β monomer mutant at least 50-fold tighter than the β dimer. These findings suggest that when δ interacts with the β clamp, it binds one β subunit with high affinity and utilizes some of that binding energy to perform work on the dimeric clamp, probably cracking one dimer interface open.

The DNA polymerase III (Pol III)[1] holoenzyme is primarily responsible for replicating the 4.4-megabase *Escherichia coli* genome (1, 2). Pol III holoenzyme performs this task with high speed and accuracy with the help of ten component subunits. These are α (the DNA polymerase (3)), ε (the proofreading 3′-5′ exonuclease (4)), and θ (unknown function) that form the DNA polymerase III core (5); β (the sliding clamp (6, 7)); and the multisubunit DnaX complex ($\gamma\tau\delta\delta'\chi\psi$) that functions as the clamp loader (8–10) and contains at least two subunits of the τ "organizer" that binds two core polymerases (11–13) and connects to the DnaB helicase at the replication fork (14, 15).

Rapid and processive DNA synthesis by Pol III holoenzyme is dependent on the interaction between the α subunit of the core polymerase and the β clamp (6). β is a ring-shaped protein that encircles double-stranded DNA and can slide freely along its length (6, 7). By itself, core polymerase can extend a primer by only a few nucleotides before dissociating from DNA (16). When β is bound to the polymerase and topologicaly linked to the primer-template, it serves as a mobile tether to keep the enzyme associated with DNA, facilitating replication of several thousand nucleotides at a time. Similar mechanisms for processive DNA synthesis by replicative polymerases have been discovered in a variety of other organisms (reviewed in Refs. 2, 17, 18, and 19), including eukaryotic DNA polymerase δ (tethered to DNA by the PCNA sliding clamp (20, 21)) and bacteriophage T4 DNA polymerase, gp43 (tethered by the gp45 sliding clamp (22)).

The crystal structure of β shows it to be a ring-shaped dimer formed by the head-to-tail interaction of two semicircle-shaped monomers (7). A continuous β-sheet forms a scaffold around the outer surface of the ring that supports 12 α-helices lining the inside of the ring. The central cavity is about 35 Å in diameter, which is large enough to encircle double-stranded DNA as well as one or two layers of water molecules. Moreover, although the inside of the β ring is positively charged, it lacks specific contact with DNA, allowing β to form a stable topological link with the DNA and yet slide freely along the duplex. At the two identical dimer interfaces, a continuous β-sheet formed by hydrogen bonding between β strands from each monomer stabilizes the ring structure in addition to a small hydrophobic core formed by packing of Ile^{272} and Leu^{273} of one monomer with Phe^{106} and Leu^{108} on the other monomer. Charged amino acids at the interface are also in position to form six ion pairs (these interactions are detailed in Fig. 4). These numerous and potentially strong interactions between the two β subunits presumably underlie the highly stable dimeric structure of β and its ability to remain bound to DNA with a half-life of over 100 min (23, 24). Yet the closed circular clamp must be opened frequently during DNA replication for assembly on DNA to initiate processive replication as well as for disassembly of the β ring from DNA when replication is complete.

The γ complex clamp loader ($\gamma\delta\delta'\chi\psi$) assembles β clamps on primer-template DNA (where they can be used by the polymerase) and can also remove clamps from DNA when necessary (23–26). The process of clamp assembly requires that the γ complex open the β clamp, guide DNA into the central cavity, and facilitate closure of the clamp around DNA. Crystal structure analysis,[2] and a recent biochemical study (27) reveals that the γ complex contains three copies of γ; the other subunits (δ, δ′, χ, ψ) are each present in a single copy (10, 13). The δ subunit of γ complex binds to β and destabilizes or opens the dimer interface (28, 29). The γ subunits are the only ones that hydrolyze ATP (30–32). The δ′ subunit is homologous to γ and

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[1] The abbreviations used are: Pol III, DNA polymerase III; PCNA, proliferating cell nuclear antigen; ssDNA, single-stranded DNA; SPR, surface plasmon resonance; HA, hemagglutinin; ^{3}H- and ^{32}P-β, ^{3}H- and ^{32}P-labeled β subunit, respectively; $^{ha}\beta$ and β^{pk}, subunit β with HA epitope tag and protein kinase tag, respectively; RFC, replication factor C.

[2] The crystal structure of $\gamma\delta\delta'$ complex has been solved (D. Jeruzalmi and J. Kuriyan, personal communication). The stoichiometry is $\gamma_3\delta_1\delta'_1$, and the five subunits form a pentameric ring.

appears to play a role in modulating the access of δ to β (10, 33, 34). In the absence of ATP, the affinity between the γ complex and β is low compared with the affinity between the δ subunit and β (28). Clamp assembly initiates when ATP binds the γ subunits and induces a change in conformation of the γ complex that results in ability of δ to bind β (28, 29, 32). The δ′ subunit appears at least partially responsible for modulating the access of δ to β, since a previous study indicated that δ′ and β compete for interaction with δ (29). The ATP-induced conformational change of γ complex may entail removing a surface of δ′ from δ, allowing δ to bind and open the β clamp. In the presence of a nonhydrolyzable ATP analogue, the clamp loader-β complex binds primer-template DNA with high affinity (32, 36). Interaction of γ complex with DNA, especially primed template, triggers ATP hydrolysis and is stimulated by the presence of β (29, 32, 36, 37). ATP hydrolysis is coupled to closure of the clamp around DNA and γ complex turnover. The χ subunit of γ complex binds to SSB and helps coordinate the switch between the primase, clamp loader, and polymerase proteins at the primer template (38, 39), and ψ enhances the stability of the γ complex; however, these two proteins are not absolutely essential for clamp assembly (40–43).

Although all three subunits, γ, δ, and δ′, are required for loading β onto DNA, the single δ subunit appears to be the predominant contact between β and the γ complex (28). It remains possible that weaker interactions between β and the other γ complex subunits exist.[3] However, our previous studies demonstrated that δ alone can open and remove β clamps from circular DNA molecules with nearly the same efficiency as γ complex ($k_{\text{unloading }\gamma^-\text{complex}} = 0.015\ \text{s}^{-1}$; $k_{\delta\ \text{unloading}} = 0.011\ \text{s}^{-1}$) (24). We were therefore curious as to how the δ subunit generates the leverage required to part the apparently tightly closed β dimer interfaces. Previous studies indicate that β opening at just one interface is sufficient to allow passage of DNA into (or out of) the central cavity (29). Experiments herein measure the exchange of labeled β subunits as they are utilized by the γ complex, and the results support the conclusion that the dimeric clamp is not split apart into monomers but rather stays intact during clamp assembly, presumably opening at only one interface for entry of DNA. In the simplest possible mechanism, the clamp loader could prompt clamp opening merely by perturbing one of the dimer interfaces and transiently reducing its stability.

Study of the δ-β interaction in this report provides insight into how the δ and γ complex might open the β ring. We demonstrate here that the β ring retains its dimeric structure when bound by one δ subunit. Furthermore, we have mutated two hydrophobic residues in the β dimer interface to produce a stable monomeric version of β. Only one δ subunit binds the β monomer, which is surprising, given the one δ/two β stoichiometry of the wild type δ-β complex. This suggests that the binding site of δ on the β ring is located primarily on one of the two β subunits. The affinity of δ for the β monomer mutant is about 50-fold greater than for the β dimer, implying that the binding energy of δ to a single β subunit of the dimer is harnessed to perform work, namely to force open one of the dimer interfaces. The δ subunit binds β at the carboxyl terminus, which lies in the vicinity of the dimer interface (44). Therefore, it is conceivable that δ binding to one β protomer disrupts the contacts in a nearby dimer interface that hold the ring closed.

EXPERIMENTAL PROCEDURES

Nucleotides, DNAs, and Buffers—Radioactive nucleotides were purchased from PerkinElmer Life Sciences. Unlabeled nucleotides were purchased from Amersham Pharmacia Biotech. M13mp18 ssDNA was prepared by phenol extraction of purified M13mp18 phage that had been banded twice in CsCl gradients (45) and primed with a 30-nucleotide primer (Life Technologies, Inc.) as described (46). Buffer A contained 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA (pH 8.0), 100 mM NaCl, and 10% glycerol. DNA replication buffer contained 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 40 μg/ml bovine serum albumin, 5 mM dithiothreitol, 8 mM $MgCl_2$, 4% glycerol, 0.5 mM ATP, 60 μM dGTP, and 60 μM dCTP. Surface plasmon resonance (SPR) buffer contained 10 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20.

Proteins—Proteins were purified as described: α, ε, γ (46), δ, δ′, χ, ψ (33), θ (47), and SSB (46). γ complex and Pol III* (a subcomplex of Pol III holoenzyme lacking the β subunit) were reconstituted from individual subunits and purified as described in Refs. 9 and 13, respectively. Mutant β proteins were constructed using DNA oligonucleotide site-directed mutagenesis. Various N-terminal tagged versions of β (described below) were purified according to the previously described protocol for wild type β (7). Radiolabeling of tagged β with ^{32}P was performed using [α-^{32}P]ATP and cAMP-dependent protein kinase to a specific activity of ~100 cpm/fmol as described (48). The catalytic subunit of cAMP-dependent protein kinase produced in *E. coli* was a gift from Dr. Susan Taylor (University of California, San Diego). ^{3}H-β was labeled by reductive methylation as described (48).

Gel Filtration Analysis of β, δ, L273A-β, and I272A/L273A-β—The β, L273A-β, and I272A/L273A-β proteins (3 μM as dimer) were sized by gel filtration (at 4 °C) on an FPLC HR 10/30 Superose 12 column (Amersham Pharmacia Biotech) equilibrated with Buffer A. The proteins were incubated in a final volume of 200 μl of Buffer A for 15 min at 15 °C and then applied to the column. After collecting 6-ml, 170-μl fractions were collected, and 25-μl aliquots of the indicated fractions were analyzed by SDS-polyacrylamide gel electrophoresis (15% gels); proteins were visualized by Coomassie Blue staining. For size standards, α (130 kDa), bovine serum albumin (66 kDa), and δ (39 kDa) were analyzed similarly.

Interaction between δ and β was analyzed by incubating 9 μM δ with 12.5 μM wild type β (as dimer) or 25 μM I272A/L273A-β, (as monomer) for 15 min at 15 °C in a final volume of 200 μl of Buffer A, followed by gel filtration chromatography and SDS-polyacrylamide gel electrophoresis analysis as described above.

DNA Replication Assays—Singly primed M13mp18 ssDNA (20 fmol), 0.8 μg of SSB, 75 fmol of Pol III*, and 750 fmol of β (wild type and mutant concentrations are calculated as monomer) were incubated at 37 °C for 2 min in 25 μl (final volume) of DNA replication buffer (this buffer contains ATP, dCTP, and dGTP). DNA synthesis was initiated upon the addition of the remaining two deoxyribonucleoside triphosphates (60 μM dATP, 20 μM dTTP (final concentrations), and 1 μCi of [α-^{32}P]dTTP). After 20 s, reactions were quenched with 25 μl of 40 mM EDTA and 1% SDS. Aliquots (20 μl) of the quenched reactions were analyzed by electrophoresis on a 1% TBE-agarose gel, and the radiolabeled DNA was visualized on a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). Synthesis was quantitated by spotting 20-μl aliquots of the reaction on DE81 filters, followed by liquid scintillation counting as described (49).

SPR Analysis of β-δ Interaction—The δ subunit (10 μl of 0.6 μM) was immobilized on a carboxymethylated dextran matrix-coated sensor chip (CM5; Biacore) by carbodiimide coupling in 10 mM sodium acetate (pH 5.5). SPR analysis was performed at 23 °C by injecting 15 μl of β or I272A/L273A−β (0.25 and 1.23 μM; concentrations for both are given as monomer) in SPR buffer, at a flow rate of 5 μl/s. After each analysis was complete, the chip surface was regenerated by injecting 10 μl of 0.1 M glycine (pH 9.5) over the chip, which releases bound β with no significant effect on the binding capacity of the immobilized δ protein.

The kinetic constants for interaction between δ and β were determined by nonlinear curve fitting, using the BIAevaluation 2.1 software. The rate of dissociation (k_{off}) was calculated by fitting the curves to a single exponential decay described by Equation 1,

$$R = R_0 e^{-k_{\text{off}}(t-t_0)} \quad \text{(Eq. 1)}$$

where R_0 represents the response and t_0 represents the time at the start of the dissociation phase. The association rate (k_{on}) was calculated using the binding model A + B= AB and Equation 2,

$$R = R_{\text{eq}}(1 - e^{-k_{\text{on}}C + k_{\text{off}}})(t - t_0) \quad \text{(Eq. 2)}$$

where R_{eq} is the response at steady state, C is the concentration of β, and t_0 is the time at the start of the association phase. The dissociation constant (K_d) for interaction between β and δ was calculated as $k_{\text{off}}/k_{\text{on}}$.

Protomer Exchange Assay—The two β mutants for this assay were

[3] Weak interaction between γ and β and between χ and β can be detected by surface plasmon resonance (A. Yuzhakov and M. O'Donnell, unpublished observations).

constructed by placing the β gene into either the pHKEp vector or the pHKEp^{mut} vector (50). Both of these vectors place a 34-amino acid tag onto the N terminus of the protein. The tags contain a protein kinase site (to label the protein with ^{32}P) and either a functional (pHKEp) or a nonfunctional (pHKEp^{mut}) hemagglutinin (HA) epitope. The nonfunctional epitope was formed by replacing two amino acids; YPYDVPDYA was changed to YPYDVPAAA. After expression and purification, one β contains a functional HA epitope ($^{ha}\beta_2$) and the other β contains a nonfunctional HA epitope, which we use in this report in the phosphorylated form and refer to as ^{32}P-β_2. The β with the mutated HA-epitope was labeled with ^{32}P (^{32}P-β) as described (48). Titrations of these β variants showed that they were as active as wild type β in replication assays with Pol III* on SSB-coated M13mp18 ssDNA primed with a single oligonucleotide. Monoclonal antibody to the HA epitope was purchased from BabCo, and Protein A-Sepharose 4B was from Zymed Laboratories Inc. The HA antibody was conjugated to Protein A beads by incubation for 15 min at 25 °C in 400 μl of 20 mM Hepes (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol.

Spontaneous protomer exchange was measured (*i.e.* no other proteins besides β) in 50-μl reactions containing 2 pmol of ^{32}P-β_2 and 2 pmol of $^{ha}\beta_2$ in 20 mM Hepes (pH 7.5), 150 mM NaCl, and 10% glycerol. Reactions were incubated at 37 °C for 0, 1, 2, 4, 6, or 8 h before the addition of 50 μl of HA antibody-conjugated beads and placed at 4 °C for a further 30 min. Beads were pelleted, washed three times with 1 ml of 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM EDTA, 0.1% SDS, and 0.1% Triton X-100; resuspended in Eco-Lume (ICN); and counted in a scintillation counter. Control experiments were performed similarly except that either no antibody was conjugated to the beads or the $^{ha}\beta_2$ was not added to the reaction.

To measure the effect of γ complex on β protomer exchange during clamp assembly onto DNA, 250 fmol each of $^{ha}\beta_2$ and ^{32}P-β_2 were incubated for 5 min at 37 °C with 500 fmol of γ complex and 1.8 pmol of nicked pBS DNA in 70 μl of 20 nM Tris-HCl (pH. 7.5), 0.1 mM EDTA, 4% glycerol, and 8 mM $MgCl_2$. The reaction was then applied to a 5-ml A15 M gel filtration column equilibrated with the same buffer plus 0.15 M NaCl. Fractions of six drops each were collected, and those containing β on DNA were identified by scintillation counting and pooled (420 μl), and then the DNA was linearized upon treatment with 700 units of *Bam*HI for 3 min at 37 °C to release β. To confirm that linearization was complete within this time, an aliquot (20 μl) was removed, quenched with 20 μl of 1% SDS, 40 mM EDTA, and then analyzed in a native agarose gel. Then 50 μl of HA antibody beads were added to the reaction, and incubation was continued for a further 30 min at 4 °C. The beads were pelleted; washed three times with 1 ml of 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM EDTA, 0.1% SDS, and 0.1% Triton X-100; resuspended in Eco-Lume (ICN); and counted in a scintillation counter. In a control experiment, the above procedure was repeated except that heterodimeric β was used in the assembly reaction by first preincubating 250 fmol of each β in one tube for 5 h at 37 °C before adding to the reaction containing γ complex and DNA.

Nickel Column Affinity Assay for δ-β_2 Complex—Reactions contained 67.5 pmol of ^{3}H-β_2 (wild type β labeled by reductive methylation), 1.7 nmol of $^{his}\beta_2$, which contained a six-residue histidine tag on a 23-residue N-terminal leader (β was cloned into the pHK vector in Ref. 50), and 6.6 nmol of δ (where present) in 200 μl of 20 mM Tris-HCl (pH 7.5), 10% glycerol, 8 mM $MgCl_2$, and 100 mM NaCl. A control reaction utilized 1.7 nmol of unlabeled wild type β_2 in placed of the $^{his}\beta_2$ derivative. Reactions were assembled on ice and then shifted to 37 °C, and aliquots of 20 μl were removed at 2 and 24 h of incubation. Upon removal of an aliquot, NaCl was added to a final concentration of 0.5 M, and the reaction was applied to a 1-ml nickel chelate column (HiTrap; Amersham Pharmacia Biotech) equilibrated in 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 8 mM $MgCl_2$, and 10% glycerol. The column was washed with 5 ml of the same buffer and then eluted with 3 ml of 20 mM Tris-HCl (pH 7.9), 1 M imidazole, 8 mM $MgCl_2$, and 10% glycerol. Fractions of 1 ml were collected. The flow-through (wash) and bound (elution) fractions were analyzed by liquid scintillation counting and analyzed in a 10% SDS-polyacrylamide gel to confirm the presence of δ with β in the bound fractions. The typical yield of ^{3}H-β_2 off the column was greater than 85%.

RESULTS

β Is Not Monomerized during Assembly onto DNA—We have shown previously that the β clamp is a tight dimer and remains a dimer even when diluted to a concentration of 50 nM (23). Nonetheless, it is possible that γ complex dissociates the β dimer into monomers using the energy of ATP hydrolysis and then reassembles the β dimer onto DNA in a second step. To test this possibility, we constructed two chemically distinct β mutants; one was phosphorylated and contained a protein kinase tag (^{32}P-β^{pk}), and the other had a hemagglutinin epitope tag ($^{ha}\beta$). If the γ complex monomerizes β dimers and reassembles them onto DNA, then it should act upon a mixture of ^{32}P-β_2 and $^{ha}\beta_2$ to form ^{32}P-β-^{ha}β heterodimers on DNA.

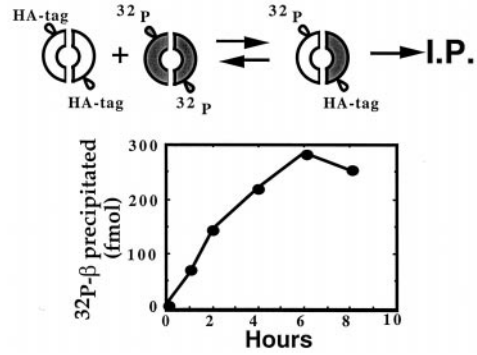

FIG. 1. **Time course of β protomer exchange.** Two chemically distinct species of β, one containing a hemagglutinin epitope and one labeled with ^{32}P, were mixed together to initiate formation of heterodimers as indicated. After various times of incubation, aliquots were withdrawn, and beads to which antibody to the hemagglutinin tag were attached were added. Heterodimeric β consists of a ^{32}P-β protomer attached to a ^{ha}β protomer that should be trapped by the hemagglutinin beads. Radioactivity in the pellet, representing heterodimeric β, is plotted with respect to time. *I.P.*, immunoprecipitation.

As a prerequisite for an experiment of this type, it is important that the ^{32}P-β_2 and $^{ha}\beta_2$ mixture does not undergo spontaneous protomer exchange to form heterodimers during the time of the experiment. The time course for spontaneous heterodimer formation was measured in the experiment of Fig. 1 by mixing equal amounts of ^{32}P-β_2 and $^{ha}\beta_2$, followed by removal of aliquots at time intervals and immunoprecipitation of the mixture using Protein A beads to which an antibody to hemagglutinin is attached. Initially, ^{32}P-β will not be precipitated, since it lacks the epitope. But as protomer exchange occurs, the ^{32}P-β-^{ha}β heterodimer will be formed, which should result in the appearance of radioactivity in the pellet. The result, shown in Fig. 1, demonstrates that the time scale of spontaneous subunit exchange is on the order of hours ($t_{1/2} \sim$ 2 h). As the clamp-loading reaction only requires 5 min, spontaneous protomer exchange during the reaction should be nearly negligible. Control reactions not shown here have been performed that demonstrate requirements for both the antibody and the presence of the ^{ha}β to detect radioactivity attached to the beads in the pellet. In both controls, the pellets lacked radioactivity above background levels (0.5 fmol of ^{32}P-β_2).

Next, we examined how the γ complex loads a mixture of these two β variants onto DNA to determine whether it catalyzes protomer exchange during the clamp assembly process (*i.e.* whether γ complex breaks β dimers apart and reassembles them onto DNA as illustrated in the scheme of Fig. 2). To test this possible action, ^{32}P-β_2 and $^{ha}\beta_2$ were mixed together, and γ complex was added immediately along with ATP and circular plasmid DNA containing a single nick to initiate clamp assembly. After 5 min, the reaction was applied to a gel filtration column to separate clamps that had been assembled on DNA from those remaining in solution. Following this, the isolated β-DNA complex was treated with *Bam*HI to rapidly linearize the DNA, allowing the clamps to slide off DNA into solution. Then the reaction was analyzed for heterodimer formation by immunoprecipitation using the hemagglutinin antibody beads (see the scheme in Fig. 2).

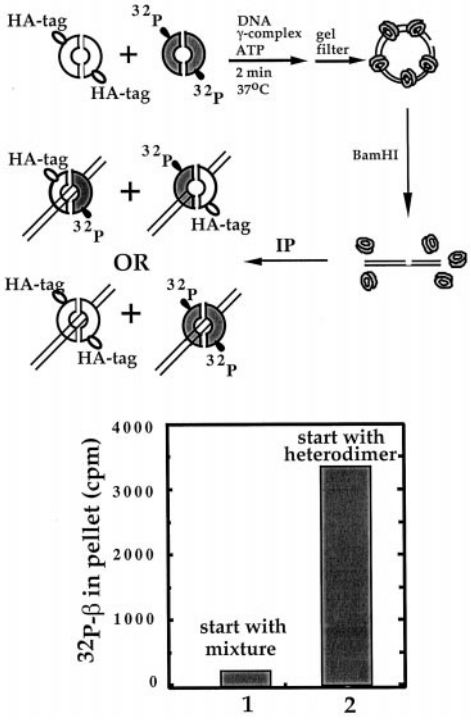

FIG. 2. **γ complex does not monomerize β during clamp assembly onto DNA.** The γ complex and ATP were used to assemble a mixture of ^{32}P-β_2 and $^{ha}\beta$2 onto nicked circular plasmid DNA. As indicted, the β-DNA complex was isolated from free β and then linearized to release β into solution. Hemagglutinin antibody bound to beads was used to quantitate heterodimers that were assembled onto DNA by the γ complex. *Lane 1*, ^{32}P-β_2 and $^{ha}\beta_2$ were mixed immediately prior to their transfer to DNA by γ complex; *lane 2*, ^{32}P-β_2 and $^{ha}\beta_2$ were premixed and incubated 5 h to form heterodimers before being transferred to DNA by γ complex.

The results of this experiment (Fig. 2, *lane 1*) demonstrate that very little heterodimeric β is formed in the reaction, indicating that γ complex does not catalyze β monomerization during clamp loading. In control reactions not shown here, we confirmed that γ complex loads approximately equal amounts of ^{32}P-β_2 and ^{32}P-$^{ha}\beta_2$ on DNA, and both variants of β were as active as wild type β in replication assays with PolIII*. In another control experiment ^{32}P-β_2 and $^{ha}\beta_2$ were premixed for 6 h to form the ^{32}P-β-$^{ha}\beta$ heterodimer prior to use by γ complex in assembly onto DNA. The result, shown in Fig. 2, *lane 2*, demonstrates that the experimental strategy is functional in detecting heterodimers that are assembled on DNA. Thus, it would appear that γ complex does not monomerize β but probably only opens one interface of the ring during the clamp opening process. This conclusion is consistent with a previous finding that showed that γ complex was capable of assembling a β dimer onto DNA that was cross-linked at one interface by a disulfide bond (*i.e.* indicating that γ complex does not need to open both interfaces to assemble β onto DNA (29)).

In the study of Fig. 3 we designed another experiment to examine the oligomeric state of β during clamp assembly, this time while it is in complex with δ, the clamp-opening subunit of the γ complex. Previous studies indicated that one δ monomer binds to the β dimer, consistent with the single copy of δ in γ complex (28). The δ subunit is capable of removing β rings from circular DNA (24, 29) and thus must either destabilize one interface or perhaps transiently dissociate β into monomers. In either case, one may expect δ to accelerate the rate of protomer exchange. We examined these possibilities in a variation of the protomer exchange assay. The assay utilized a hexahistidine-tagged β_2 ($^{his}\beta_2$) and tritiated wild type β_2 (^{3}H-β_2). The ^{3}H-β_2 was mixed with a 25-fold molar excess of $^{his}\beta_2$ in the presence or absence of a 4-fold molar excess of δ (over total β), and then the mixture was analyzed at either 2 or 24 h for heterodimer formation by nickel chelate chromatography. Homodimeric ^{3}H-β_2 should not bind to the column (flow-through fraction), and heterodimeric ^{3}H-β-$^{his}\beta$ should be retained (bound fraction) and detected by elution from the nickel chelate column, followed by scintillation counting.

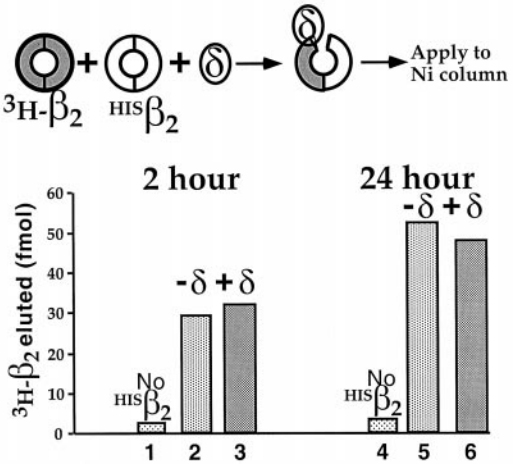

FIG. 3. **δ does not monomerize β_2.** A mixture of ^{3}H-β_2 and a histidine-tagged β ($^{his}\beta$) were mixed in the presence or absence of a 4-fold excess of δ subunit (over total β). At either 2 h (*lanes 2* and *3*) or 24 h (*lanes 5* and *6*), aliquots were removed and loaded onto nickel-chelate columns. After washing the columns, bound protein was eluted with buffer containing 1 M imidazole. Since ^{3}H-β lacks a His tag, ^{3}H-β in the bound fraction represents ^{3}H-β-$^{his}\beta$ heterodimers. Controls in which wild type β_2 was substituted for $^{his}\beta$ are shown in *lanes 1* and *4*.

The results of this experiment, shown in Fig. 3, illustrate that similar amounts of heterodimer are formed within 2 h in the presence or absence of δ, indicating that δ does not appreciably speed up protomer interchange. Also, the fact that ^{3}H-β is retained on the column in the presence of δ supports the δ_1-β_2 stoichiometry, since if δ monomerized β_2, heterodimer would not be present for retention on the column. As a control, wild type β_2 was substituted for $^{his}\beta_2$, which should form a ^{3}H-β-β^{wt} heterodimer, but should not bind the nickel chelate column. The result of this control showed that ^{3}H-β was not retained on the column, as expected (not shown).

How Does δ Open One Interface of the β Ring?—The experiments described above demonstrate that the γ complex does not catalyze the exchange of β protomers during the clamp loading operation. The results also demonstrate that δ does not monomerize the β dimer. These results support and extend earlier studies that indicate that only one interface of the β dimer ring is cracked open during assembly onto DNA. The δ subunit is the clamp-opening subunit of γ complex. How does δ open an interface of the β ring? To gain insight into how δ performs its ring opening task, we mutated β to form a stable β monomer. Initially, we set out to determine whether δ mainly binds only one protomer of the β dimer, in which case δ should still bind a β monomer about as well as a β dimer. Alternatively, δ may need to associate with elements on both protomers of β_2 in order to establish a firm grip on the β ring. The results of this line of investigation were unexpected and provided significant insight into the clamp opening function of δ.

To form a stable monomer of β, we utilized the crystal structure to design site-specific mutations that would destabilize the dimer interface. The crystal structure of the dimeric β clamp revealed a small interface between the two β subunits that, despite its size, has an abundance of potentially strong interactions (see the *diagrams* in Fig. 4, *A* and *B*) (7). These inter-

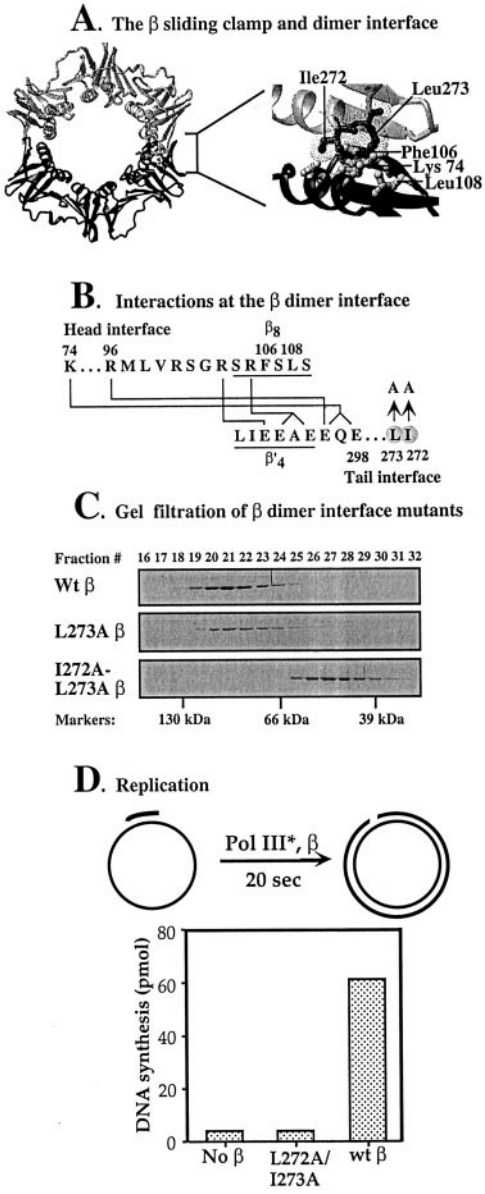

FIG. 4. **Mutation of the β interface to form a stable β monomer.** *A*, the crystal structure of the β dimer shows that it is a ring-shaped structure, with a 35-Å central hole, wide enough to encircle double-stranded DNA (7). The β dimer interface, in particular the amino acid residues forming the hydrophobic core, is highlighted on the *right*. *B*, the dimer interface consists of two β sheets, β_8 and β'_4 (one from each subunit), that form an antiparallel sheet across the dimer interface and neighboring amino acid contacts that stabilize the clamp structure. The *lines* connect amino acid residues predicted to form six ion pairs across the interface. The hydrophobic core residues, Phe^{106}, Leu^{108}, Ile^{272}, and Leu^{273}, are indicated, and *gray circles* highlight Ile^{272} and Leu^{273}, which were mutated to Ala in this study. *C*, analysis of wild type β and mutants of β on a sizing column followed by SDS-polyacrylamide gel electrophoresis. The results show that wild type β (*top panel*) and L273A−β (*middle panel*) elute as dimers (81.2 kDa). In contrast, the I272A/L273A-β (*bottom panel*) elutes as a smaller, monomeric protein (40.6 kDa), indicating that the double mutation severely disrupts the β dimer interface. *D*, quantitation of DNA synthesis by Pol III* on SSB-coated singly primed M13mp18 ssDNA in the presence of either no β, I272A/L273A-β, or wild type β.

actions facilitate the formation of a highly stable circular clamp that maintains its dimeric structure even at low nanomolar concentrations. In particular, a small hydrophobic core of four amino acid residues (Phe^{106}, Leu^{108}, Ile^{272}, and Leu^{273}) at the dimer interface appears to play an important role in the stability of the clamp structure. Initially, we constructed three single residue mutants in which Ala was substituted in place of either Phe^{106}, Leu^{108}, or Leu^{273} (we could not obtain the I272A mutant). Each of these point mutants migrated as a dimer in gel filtration analysis and retained 70–100% activity with PolIII* (not shown). However, a double mutant, I272A/I273A, behaved as a monomer and lacked replication activity (explained below). The experiments to follow focus on the double mutant and compare it with wild type β.

In Fig. 4*C*, wild type β and the β mutants, L273A-β and I272A/L273A-β, were examined by gel filtration to determine their oligomeric state. Fig. 4*C* shows the SDS-polyacrylamide gel electrophoresis analysis of column fractions from the gel filtration analysis of wild type β, L273A-β, and I272A/L273A-β, in the *top*, *middle*, and *bottom panel*, respectively. Wild type β elutes as a dimer in peak fraction 21, as does the L273A-β mutant (calculated mass = 81.2 kDa). In contrast, the double mutant I272A/L273A-β migrates more slowly through the column, indicative of a smaller size, and elutes as a monomer (calculated mass = 40.6 kDa). The gel filtration experiments were performed with 3 μM β (as dimer). Therefore, even at high protein concentration, I272A/L273A-β is unable to form a stable dimer.

It has long been presumed that the circular structure of the β dimer is required for its action as a DNA polymerase processivity factor. There are, however, single subunit processivity factors that do not appear to encircle DNA, particularly the herpes simplex virus UL42 protein, which in fact is structurally similar to the eukaryotic PCNA clamp but does not oligomerize into a ring (51). To determine if a monomeric form of β can serve as a processivity factor, the monomeric I272A/L273A-β mutant was tested for DNA replication activity with PolIII* using primed M13mp18 ssDNA as substrate. The result, in Fig. 4*D*, demonstrates that the monomeric β mutant is inactive with PolIII*. The dimeric single mutants (L273A, L108A, and F106A) retained 70–100% the activity of wild type β (not shown). Thus, a β monomer that does not form a circular clamp is not capable of tethering Pol III* to DNA for processive DNA replication.

δ Binds the β Monomer with Higher Affinity than the β Dimer—Only one copy of the δ subunit is present in the γ complex, consistent with the stoichiometry of one δ to two β in the δ-β complex. The stoichiometry of only one δ subunit per β dimer invokes the question of whether δ interacts with both β protomers or can stably attach to one β protomer, perhaps somehow preventing a second δ from binding the other β protomer (*e.g.* by steric occlusion). Interaction of δ with the β monomer was tested in Fig. 5 by mixing δ with an excess of either wild type β_2 or the momeric β_1 mutant, followed by gel filtration analysis on a sizing column. The elution profiles of the proteins were analyzed by SDS-polyacrylamide gel electrophoresis. As expected from previous studies, Fig. 5*A* shows that δ and wild type β_2 form a stable complex with an apparent molecular mass of 111 kDa, consistent with the $\delta_1\beta_2$ complex observed in our previous study (38.7-kDa δ + 2 × 40.6-kDa β = 119.9 kDa) (28). Fig. 5*B* shows that δ and the I272A/L273A-β mutant also interact, forming a smaller $\delta_1\beta_1$ complex that migrates at an intermediate position between the $\delta_1\beta_2$ complex (Figs. 5*A*) and the free I272A/L273A-β monomer (Fig. 5*E*). This result reveals that the binding site for δ on the β clamp resides within one monomer and demonstrates that δ need not bind both subunits of the dimer to form a stable contact with the clamp.

The gel filtration analysis revealed that δ can bind a single β protomer, but the possibility remained that the affinity of δ for β may be affected by disruption of the dimeric structure. In particular, we noticed that during gel filtration δ trails as free

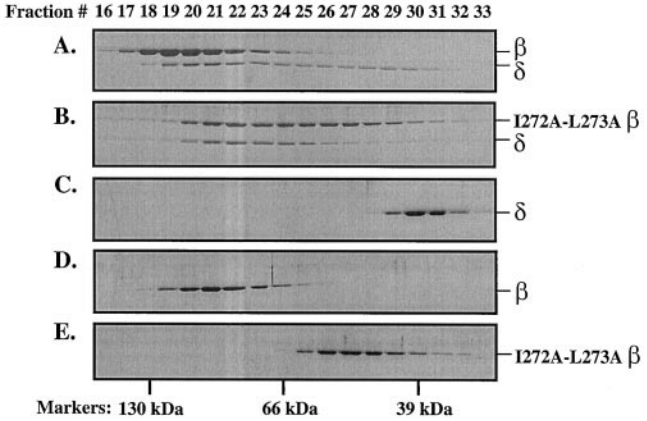

FIG. 5. **δ binds the β monomer mutant.** δ-β interaction was analyzed by gel filtration on a sizing column, and the proteins were visualized in column fractions by SDS-polyacrylamide gel electrophoresis and Coomassie staining. The complex of the wild type β dimer with δ (*A*) elutes faster than free δ (*C*) and free β (*D*), indicative of its large size (81.2 + 38.7-kDa $\delta_1\beta_2$ complex). *B*, the monomeric I272A/L273A-β protein also interacts stably with δ, forming a $\delta_1\beta_1$ complex that elutes in an intermediate position between the $\delta_1\beta_2$ complex (*A*) and either δ (*C*) or free β monomer (*E*). The elution positions of molecular weight standards are shown at the *bottom* of the gel.

protein from a complex with wild type β (fractions 24–31 in Fig. 5*A*), whereas in Fig. 5*B* most of the δ appears in complex with the β monomer, suggesting that δ may bind β_1 tighter than β_2. Next, we used the SPR technique to examine more closely the relative affinity between δ and the β dimer *versus* the I272A/L273A-β monomer mutant (Fig. 6). The δ subunit was immobilized on a sensor chip, and a solution of β in buffer (at different concentrations) was passed over it. The increase in mass (response units) resulting from interaction between δ and β was measured over time; this is the association phase from which the association rate (k_{on}) can be calculated. Next, buffer lacking β was passed over the δ-β complex on the chip, and the resulting decrease in mass over time provides information from which the dissociation rate (k_{off}) can be calculated. Fig. 6, *A* and *B*, shows sensorgrams of the interaction between δ and two different concentrations of β and I272A/L273A-β, respectively.

Kinetic analysis of the SPR data yielded the association (k_{on}) and dissociation (k_{off}) rate constants, from which the equilibrium dissociation constant for the δ-β interaction could be calculated ($K_d = k_{off}/k_{on}$). The parameters, summarized in Table I, reveal that δ binds the β monomer mutant with substantially higher affinity than the wild type β protein. The average K_d value for interaction between δ and β is about 0.46 μM (average of values determined at 0.25 and 1.23 μM β concentrations), which is ~57-fold higher than the K_d for the interaction between δ and I272A/L273A-β (average K_d = 0.0075 μM). The tighter interaction between δ and the β monomer is particularly striking because the β monomer has only one potential δ binding site, in contrast to the β dimer.

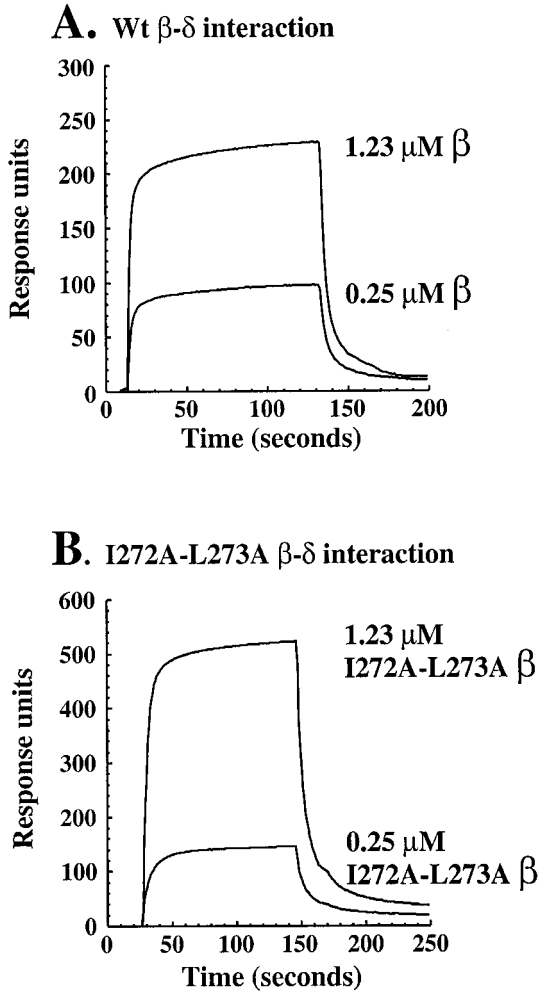

FIG. 6. **δ binds the β monomer tighter than the β dimer.** The sensorgrams of wild type β (*A*), and I272A/L273A-β (*B*) were obtained by measuring the increase in response units when a 0.25 or 1.23 μM solution of either β (as dimer for wild type β, as monomer for mutant β) was passed over δ immobilized on a sensor chip. The sensorgrams were analyzed for kinetic and equilibrium parameters of the δ-β interaction as described under "Experimental Procedures" (summarized in Table I). The K_d values indicate that δ binds I272A/L273A-β with ~80-fold higher affinity than the wild type β dimer.

TABLE I
Kinetic and equilibrium constants describing the interaction between β and δ

Protein	Concentration	k_{off}	k_{on}	K_d
	μM	s^{-1}	$\mu M^{-1}\ s^{-1}$	μM
Wild type β	0.25	0.011	0.14	7.8×10^{-2}
	1.23	0.24	0.28	8.5×10^{-1}
I272A/L273A-β	0.25	7.6×10^{-4}	0.39	1.9×10^{-3}
	1.23	3.2×10^{-3}	0.24	1.3×10^{-2}

DISCUSSION

The β Subunit Remains Dimeric during Clamp Loading—This study has examined whether γ complex monomerizes β during the clamp loading operation but could detect no evidence for splitting of β dimers during their assembly onto DNA. Consistent with retention of the β dimeric state, the δ subunit does not appear to monomerize β_2 or to significantly increase the rate of protomer exchange among β dimers. Hence, it seems likely that the δ subunit opens only one interface of the β dimer during clamp loading, consistent with the ability of γ complex to load a β dimer onto DNA that has been cross-linked via a disulfide bond across one of the two interfaces (29).

The δ subunit can open β and remove it from DNA but cannot load β onto DNA. The γ and δ′ subunits of the γ complex, along with δ, may orient DNA inside the open ring. The δ′ subunit, possibly also assisted by γ, must also sever the δ-to-β contact, allowing the ring to close around DNA. Release of the γ complex and closure of the β clamp around the DNA are tied to ATP hydrolysis and are probably coordinated with sensing the appropriate structure of DNA.

The Critical Role of Hydrophobic Interface Contacts in the β Clamp Structure—Two β monomers contact each other in a head-to-tail fashion at two small, identical interfaces to form the ring-shaped, dimeric β clamp (Ref. 7; see also Fig. 4). A

central feature of the β clamp structure is the continuous layer of sheet around the entire molecule, including the dimer interfaces. Further, particular hydrophobic amino acid side chains, contributed by each monomer, pack to form a small hydrophobic core within each interface. There are also six potential ion pairs formed at the interface, which may further strengthen the dimer. Earlier studies demonstrated that the β clamp retains its dimeric structure even when it is highly dilute (23). It is possible that the β clamp may "breathe" by alternate opening and closing of one or the other interface. However, the observed long lifetime of β on circular DNA when topologically linked to it (23, 24) indicates that if there is breathing at the interfaces, opening a wide distance (*i.e.* to slip off DNA) is a rare occurrence.

This study confirms an important role of the hydrophobic residues at the interface in maintaining a stable β clamp structure. Although β retained its dimeric status upon mutation of only one hydrophobic residue at the interface,[4] mutation of two of the amino acids at the hydrophobic core, Ile^{272} and Leu^{273}, to Ala destabilizes the two interfaces to such an extent that the β dimer exists as a stable monomer in solution.

The δ-to-β Binding Energy Opens the β Clamp—In this study, we show that δ binds the β monomer about 50-fold tighter than the β dimer. Moreover, the tight δ-β monomer complex has a 1:1 stoichiometry, indicating that δ has a binding site for only one β protomer. Thus, in the δ_1-β_2 complex, δ probably binds only one of the β subunits. The apparent higher affinity of δ for the β monomer mutant compared with wild type β also indicates that some of the binding energy of δ to a β protomer is put into performing work on the β dimer, thus lowering the observed affinity. Given that upon binding of δ to β, the dimer opens, the work of the "lost" binding energy is probably utilized to part one of the β dimer interfaces.

The amount of work to open one interface can be calculated to be ~2.4 kcal, assuming a difference of 57-fold in the equilibrium binding constants for δ binding to either β_1 or to β_2 (since β does not monomerize, there is no entropy component, and the free energy represents work). It is interesting to note that β remains a dimer well below 50 nM (23), and thus the free energy for dissociation to monomers is in excess of 10 kcal. These calculated free energies imply that the amount of work required to open one interface (*i.e.* ~2.4 kcal) is far less than the free energy to open the second interface (*i.e.* the full 10 kcal needed for β to monomerize). These results imply that the β dimer is constructed in such a way as to ensure preservation of a dimeric structure, even after one interface has been pried open.

The γ Complex Mechanism—The high stability of the wild type β dimeric clamp explains the need for a clamp loader/unloader protein during DNA replication. The γ complex serves this function by binding and opening the β clamp when it must be loaded onto primer-template DNA or unloaded from a newly replicated duplex (25, 45). The γ complex utilizes energy from ATP binding and hydrolysis to perform its function. However, to our surprise, we found in earlier studies that γ complex opens the clamp simply on binding ATP and that energy from ATP hydrolysis is not necessary to crack the β dimer interface open (29, 32). The ATP binding energy is not utilized for opening the clamp but rather to expose the δ subunit in the γ complex (28, 29), which then opens one interface of the β dimer, prior to ATP hydrolysis. In fact, free δ protein appears to open the clamp almost as well as the ATP-using γ complex, as evidenced by the fairly similar rates at which they catalyze unloading of β from DNA ($k_{\gamma^-\text{complex unloading}} = 0.015\ s^{-1}$; $k_{\delta^-\text{unloading}} = 0.0115\ s^{-1}$) (24). These results are consistent with the above conclusions, that the binding energy of the interaction between δ and β is sufficient to open the clamp.

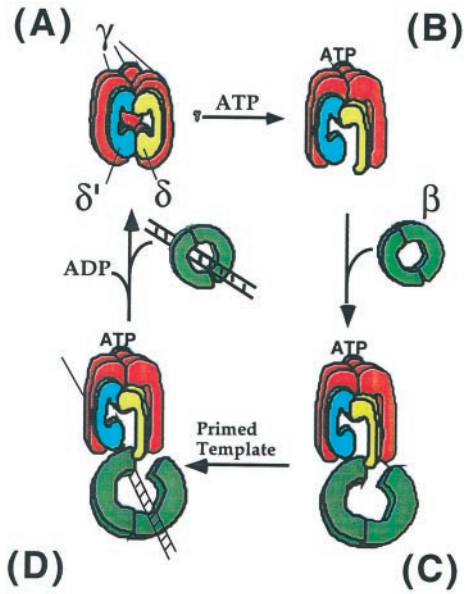

FIG. 7. **Scheme of γ complex action.** The five subunits of γ complex needed for clamp loading, $\gamma_3\delta_1\delta'_1$, are shown as related C-shaped proteins. *A*, The area on δ that binds β is in contact with δ′ to indicate that it is blocked for β binding. *B*, ATP binding to γ subunits induces a conformational change pulling δ′ from δ, so that δ can bind to β. *C*, the δ subunit contacts one protomer of β, and the binding energy of this interaction is placed into wedging open one interface of the β ring. *D*, γ complex/β locate a proper DNA structure for loading β, which triggers ATP hydrolysis, leading to dissociation of the γ complex and leaving β to close around DNA.

The scheme in Fig. 7 illustrates our current view of the γ complex mechanism. Only the γ, δ, and δ′ subunits are shown, since previous studies have demonstrated that the χ and ψ subunits of the γ complex are not essential for clamp loader action (40). The stoichiometry of γ in the γ complex has recently been demonstrated by crystal structure analysis of γδδ′ to be three per complex,[2] which is also consistent with the conclusions of a recent biochemical study (27). δ and δ′ are each present in single copy (10, 13). The δ′ subunit is composed of three domains organized in a C-shape (52). The crystal structure shows that the γ subunits are three domain proteins like δ′, consistent with their known homology to δ′ (10, 33, 34). Although the δ subunit shares no recognizable homology to γ and δ′, the crystal structure of δ has recently been solved, and it has a domain structure similar to that of δ′.[5] The affinity of the γ complex for β is quite reduced in comparison with the affinity of δ for β, indicating that δ is sequestered when it is in the γ complex. But in the presence of ATP, the affinity of γ complex for β is enhanced, suggesting that δ becomes more available to bind β. This is illustrated in Fig. 7, going from *diagram A* to *B*, as a conformational change that increases the exposure of δ for β. The illustration is consistent with previous studies that indicate that ATP induces a conformation change in the γ complex (28, 32) and that δ′ competes with β for δ (29). These earlier observations indicate that δ′ binding to δ may partially occlude δ in the γ complex and that, upon binding ATP, γ may relieve this occlusion via a conformational change. Hence, *diagram B* shows a separation between δ′ and δ, due to an ATP-induced conformation change in γ.

Upon interaction of δ with β (Fig. 7, *diagram C*), the ring

[4] J. Stewart and M. O'Donnell, unpublished data.

[5] The crystal structure of δ in complex with a monomer of β has been solved (D. Jeruzalmi and J. Kuriyan, personal communication). δ has the folding pattern of δ′.

opens. This report demonstrates that δ binds only one protomer of the β dimer in performing this ring-opening action. Further, as described above, δ binds the β monomer tighter than the β dimer, indicating that the binding energy between δ and one β protomer is used to perform work on the dimer, to open or destabilize one interface. The remaining single interface of an open β dimer is stronger when β is in the open conformation, thereby preventing decay to monomers.

Events in proceeding from *diagram C* to *D*, where the β ring is closed around DNA, are relatively unknown. Presumably, primed template is recognized and positioned within the open ring, at which time the ATP is hydrolyzed. Hydrolysis is stimulated by β and primed template and is associated with dissociation of γ complex from DNA, leaving the β ring closed around the duplex (29, 32, 37). At this time, we propose that the energy of ATP hydrolysis is utilized to pull δ off of β, allowing β to close. Particular roles of γ complex subunits in DNA recognition, orientation of DNA inside β, and ring closure await further study.

Comparison with the Eukaryotic PCNA Clamp and RFC Clamp Loader—The eukaryotic clamp, the PCNA ring, has essentially the same shape and structure as β, except that each monomer is composed of only two domains, and therefore PCNA trimerizes to form a six-domain ring (20, 21). PCNA, like β, is highly stable on DNA, exhibiting a half-life of ~24 min for spontaneous dissociation from circular DNA at 37 °C (23). The eukaryotic clamp loader, RFC, is composed of five different subunits, but each are homologous to γ/δ′ and thus are probably shaped and arranged like the five subunits of the *E. coli* $\gamma_3\delta\delta'$ clamp loader (34, 53). Given these striking similarities, it seems likely that the internal workings of RFC and the mechanism by which it opens PCNA will be quite similar to the *E. coli* γ complex and β. Thus, one subunit of RFC may contact one protomer of PCNA and through the energy of this protein-protein interaction may force the ring open. Multiple RFC subunits appear to bind PCNA, making it seem different from the γ complex. However, we have recently determined that the γ and χ subunits of γ complex bind β, albeit much weaker than δ.[3] Perhaps these other β and PCNA interactive subunits function in positioning β on DNA, aid δ in ring opening, or function in the ring closure step. These and many other possible functions for additional β interactive subunits must await future studies.

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