ORDERED ATP HYDROLYSIS IN THE $\gamma$ COMPLEX

CLAMP LOADER AAA+ MACHINE

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ABSTRACT

The γ complex couples ATP hydrolysis to the loading of β sliding clamps onto DNA for processive replication. The γ complex structure shows that the clamp loader subunits are arranged as a circular heteropentamer. The three γ motor subunits bind ATP, the δ wrench opens the β ring, and the δ’ stator modulates the δ-β interaction. Neither δ nor δ’ bind ATP. This report demonstrates that the δ’ stator contributes a catalytic arginine for hydrolysis of ATP bound to the adjacent γ1 subunit. Thus, the δ’ stator contributes to the motor function of the γ trimer. Mutation of arginine 169 of γ, which removes the catalytic arginines from only the γ2 and γ3 ATP sites, abolishes ATPase activity even though ATP Site 1 is intact and all three sites are filled. This result implies that hydrolysis of the three ATP molecules occurs in a particular order, the reverse of ATP binding, where ATP in Site 1 is not hydrolyzed until ATP in Sites 2 and/or 3 is hydrolyzed. Implications of these results to clamp loaders of other systems are discussed.
INTRODUCTION

DNA polymerase III holoenzyme, the chromosomal replicase of *E. coli*, contains a clamp loading machine within its multicomponent structure (reviewed in (1)). The clamp loader couples ATP hydrolysis to the assembly of circular β clamps onto primed DNA sites. The circular β clamp, formed from two crescent shaped protomers, binds to the DNA polymerase III core (αεθ), tethering it to template DNA for highly processive synthesis. There are at least two molecules of DNA polymerase III core within the holoenzyme architecture, held together by one clamp loader.

The γ complex clamp loader consists of several different subunits; three γ (τ) subunits, and one each of δ, δ’, χ and ψ (2-4). The χ and ψ subunits play roles in the primase-to-polymerase switching process (5) and they also interact with SSB (6-7), but are not essential for clamp loading. The crystal structure of the minimal clamp loader, γ₃δ₁δ’₁, shows that the five subunits are arranged as a circular heteropentamer (4). 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 truncated by a translational frameshift (8-10). The N-terminal 47 kDa of τ contains the sequence of the γ subunit, thus explaining how γ and τ can replace one another in clamp loading action with δ and δ’ (11). The extra 24 kDa of C-terminal sequence unique to τ is responsible for binding DNA polymerase III core (12-13); these sequences also bind the DnaB helicase (14-15). Thus, a single clamp loader cross links two DNA polymerases and holds the hexameric helicase into the replisome (reviewed in (6)).

The γ (τ) subunits of the γ complex constitute the motor of the clamp loading machine, as they are the only subunits that hydrolyze ATP; neither δ nor δ’ bind or hydrolyze ATP (1). The δ subunit forms the main attachment to the β clamp and it can open the β ring single-handedly (16-18). δ is sometimes referred to as the wrench, or crowbar, of the clamp loader since it can open β on its own (19). The δ’ subunit modulates the ability of δ to bind β (16-18). In the absence of ATP, δ’ obscures the δ subunit within γ complex from binding to β (4,16). However, with ATP bound to the γ subunits, δ is pulled away from δ’, allowing δ to bind and open the β ring (4,20). In this state, with β and ATP...
bound to γ complex, a tight affinity for DNA is established (21-22). Upon recognizing a primed site, the ATP is hydrolyzed resulting in the dissociation of γ complex from β, leaving β to close around the DNA whereupon it may associate with the polymerase component of the holoenzyme.

β also interacts with several other proteins besides DNA polymerase III core. These include ligase, MutS, UvrB, DNA polymerases I, II, IV and V, and possibly many other proteins involved in DNA repair (23-26). These additional roles of β in other processes besides replication may account for the presence of γ complex in *E. coli* that lacks τ altogether, presumably freeing it for action at sites distinct from the replication fork.

The structure of γδδ′ reveals that the ATP sites of the γ subunits are located at subunit interfaces (see Fig. 1) (4). Site 1 is at the δ′/γ₁ interface, Site 2 is at the γ₁/γ₂ interface, and Site 3 is at the γ₂/γ₃ interface. The Site 3 γ₃ subunit is located directly next to the δ wrench. The numbering of these sites is thought to reflect the order in which they become filled with ATP (4). The major pentameric contacts occur via the C-terminal domains of the five subunits. The ATP binding sites of γ are located in the N-terminal domains. The N-terminal domain of δ is also where the β interactive element is located (19), although proximity of δ′ to δ blocks access of δ to β (4). Combining several biochemical findings with the structure of γδδ′, suggests that as the ATP sites fill, conformation changes in γ are propagated around the pentamer to pull the δ wrench away from the δ′ stator so that δ can bind to β for clamp opening. The apparent rigidity of δ′, compared to γ and δ which have a flexible joint for motion in clamp loading, has earned δ′ the term “stator”. ATP hydrolysis presumably reverses the conformational changes in γ and δ induced by ATP binding to γ, thus bringing the N-terminal domain of δ back into proximity to the δ′ stator. This effectively pushes β off of δ, allowing the β ring to close around DNA.

The subunits of γδδ′ are members of the large AAA+ family (27). As their name implies (ATPases Associated with a variety of cellular Activities) these proteins are generally ATPases and they function in a wide diversity of cellular processes. The structure of homohexameric AAA+ proteins NSF and p97 (membrane fusion), RuvB (branch migration) and HslU (proteosome), reveals
an Arg residue that reaches over the interface to the ATP site of the neighboring subunit (28-31). This Arg is thought to be analogous to the “arginine finger” of GAP, which plays a catalytic role in hydrolysis of GTP bound to Ras by stabilizing the accumulating negative charge in the transition state (32). It is proposed that the use of this Arg residue in catalysis provides a means of intersubunit communication that coordinates nucleotide hydrolysis around the ring.

The γ complex has many similarities to the homohexameric AAA+ proteins, but also has several important differences. The largest differences are its heterooligomeric composition, use of five subunits instead of six, and presence of two subunits which do not bind ATP. Like the homohexamers, the γ complex subunits are arranged in a ring and the γ subunit ATP sites are located at interfaces where the Arg of one subunit is in proximity to ATP modeled into the subunit adjacent to it (Fig. 1). This Arg residue is embedded in an SRC motif that is conserved in clamp loading subunits of T4 phage, eubacteria, archaea and eukaryotes. The δ’ subunit also contains an SRC motif and the Arg residue is proximal to ATP Site 1 of γ1. In each case, the Arg needs to move a few angstroms to be near enough to exert an influence on the bound ATP.

The contribution of these potential arginine fingers to ATP binding, hydrolysis and clamp loading is one subject of this report. The results demonstrate that these SRC motif arginine residues in both γ and δ’ are not required for ATP binding, however they are important to catalysis. The finding that δ’ contributes a catalytic arginine residue to an ATP site in the γ trimer motor demonstrates that the δ’ stator also functions as a component of the motor of the clamp loader. This conclusion has implications for analogies between γ complex and clamp loaders of other systems (see Discussion). Further, the findings indicate that there is an ordered sequence to hydrolysis. Mutation of the arginine in γ removes the catalytic arginine in Sites 2 and 3, but not the arginine contributed by δ’ to Site 1. However, this mutation prevents hydrolysis of ATP in all three sites. This result indicates there is a sequential order to hydrolysis of the three ATP molecules, with ATP in Sites 2 and/or 3 being hydrolyzed before ATP in Site 1.
MATERIALS AND METHODS

Materials—Unlabeled deoxyribonucleoside triphosphates, Pharmacia LKB Biotechnology Inc.; radioactive nucleoside triphosphates, DuPont-New England Nuclear. Proteins were purified as described: α, ε, γ, τ (33), β (34), δ and δ’ (35), χ and ψ (36), θ (37), SSB (38). Core polymerase (37), and γ complex (11) were reconstituted from pure subunits and purified as described. Mutant subunits were purified by the same methods as wild-type proteins. The γ complex containing mutant subunits was reconstituted and purified using the same procedure as wild-type γ-complex. Samples of purified complex were analyzed on a 14% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue G-250 and each lane was scanned by laser densitometer (Molecular Dynamics). βPK is β containing a 6 residue C-terminal kinase recognition site (39), and was labeled to a specific activity of 10 dpm/fmol with $^{32}$P-ATP using the recombinant catalytic subunit of cAMP-dependent protein kinase produced in E. coli (a gift from Dr. Susan Taylor, Univ. of California at San Diego). The following oligonucleotides were synthesized and purified by Integrated DNA Technologies: 62 mer, 5’-GGG TAG CAT ATG CTT CCC GAA TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA CTG GGA AAA CCC TGG CGT TAC CCA ACT T-3’; 44 mer, 5’-GGG TTT TCC CAG TCA CGA CGT TGT AAA ACG AGT GAA TTC-3’. To form the synthetic primed template, the oligonucleotides were mixed in 50 µl of 5 mM Tris-HCl, 150 mM NaCl, 15 mM sodium citrate (final pH 8.5), then incubated in a 95°C water bath which was allowed to cool to room temperature over a 30 min interval. M13mp18 ssDNA was purified as described (40) and primed with a 30 mer DNA oligonucleotide as described (33). Bio-gel A-15m and P6 resins were purchased from Bio-Rad.

Buffers—Buffer A is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% glycerol (v/v). Gel Filtration Buffer is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 10 mM MgCl$_2$, 100 µg/ml BSA, and 4% glycerol (v/v). 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 BSA. ATPase Buffer is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, and 10% glycerol (v/v).

**Equilibrium Gel Filtration**—Analysis of ATP binding to wild-type and mutant γ complexes was performed by equilibrium gel filtration as described (41). Wild-type and either γ (R169A) γ complex or δ’ (R158A) γ complex (8.3 µM γ complex in 60 µL; 6.6 µM γ (R169A) complex in 150 µL; 6.6 µM δ’ (R158A) γ complex in 150 µL) were incubated in Gel Filtration Buffer + 100 mM NaCl containing α-32P ATP at the indicated concentration (0.1-10 µM) for 15 min. at 25°C. Samples were then applied to a 5 mL Biogel P-6 column (Bio-Rad) at 25°C pre-equilibrated in Gel Filtration Buffer + 100 mM NaCl having the same concentration of α32P-ATP as the respective sample. Thirty-five fractions of 240 µL each were collected, and 100 µL of each fraction was analyzed by liquid scintillation to determine the total amount of ATP ([ATP]_TOTAL, see below). 50 µL of the peak fractions were also analyzed for total protein concentration by the Bradford assay (Bio-Rad) using γ complex as a standard. Scatchard analysis from equilibrium gel filtration data was as described (42).

**Gel Filtration Analysis of γ complex•β Interaction**—Ability of wild-type and mutant γ complex to associate with β was analyzed by gel filtration on a FPLC Superose 12 column (Pharmacia). β (30 µM, 480 µg) was incubated alone or with γ complex (25 µM, 1.25 mg) for 15 min. at 15°C in 200 µL Buffer A + 100 mM NaCl containing 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.

**ATPase Assays**—Wild-type and mutant γ complexes were tested for ATPase activity in the presence of the synthetic primed template, with or without β. ATPase assays contained 50 nM γ complex, 1 mM α32P ATP, 200 nM β dimer (when present), and 500 nM synthetic primed template DNA in a final
volume of 60 µL ATPase Buffer. The synthetic primer/template DNA is linear, and thus allows β to slide off the ends after it is loaded. Thus β is continuously recycled during these assays as demonstrated previously (43). Reactions were brought to 37°C and initiated upon addition of γ complex. Aliquots of 5 µl each were removed at intervals (0-10 min.) and quenched with an equal volume of 0.5 M EDTA (pH 7.5). 1 µL of each quenched aliquot was spotted on a polyethylenemine cellulose TLC sheet (EM Science) and developed in 0.6 M potassium phosphate buffer (pH 3.4). The TLC sheet was dried and α-32P ATP and α-32P ADP were quantitated using a PhosphorImager (Molecular Dynamics).

**Clamp Loading**—Clamp loading was measured by separating 32P-βPK on DNA from free 32P-βPK using BioGel A15m, a large pore resin that excludes large DNA substrates but includes protein. 32P-βPK (13.3 nM as dimer) was incubated for 10 min. at 37°C either alone or with mutant or wild-type γ complex (10.7 nM) in 75 µL of Reaction Buffer containing primed M13mp18 ssDNA (13.3 nM), SSB (3.2 µM as tetramer), 1 mM ATP, and 10 mM MgCl2. The reaction was applied to a 5 mL BioGel A15m column (Bio-Rad) equilibrated in Gel Filtration Buffer + 50 mM NaCl at 25°C. Thirty-five fractions of 180 µl each were collected and 100 µL was analyzed by liquid scintillation. 32P-βPK bound to the DNA elutes early (fractions 11-15) while the free 32P-βPK elutes later (fraction 17-28). The amount of β in each fraction was determined from its known specific activity.

**Replication Activity Assays**—Activity of wild-type and mutant γ complexes was assayed by the 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 core polymerase (5 nM), β (10 nM as dimer), SSB (420 nM tetramer), primed M13mp18 ssDNA (1.1 nM), 60 µM each of dATP, dCTP, and dGTP, 20 µM α-32P TTP, 1 mM ATP, and 10 mM MgCl2 in 25 µL Reaction Buffer (final volume). Replication was initiated upon addition of either wild-type or mutant γ complex (0-1.28 nM titration) and incubated at 37°C for 5 min. Reactions were quenched upon
addition of 25 μL 1 % SDS, 40 mM EDTA. Quenched reactions were spotted onto DE81 (Whatman) filters, then washed and quantitated by liquid scintillation as described (33).
RESULTS

Reconstitution of γ (R169A) complex.

Arginine 169 is located in the highly conserved SRC motif of the γ subunit. To assess the importance of γ arginine 169 to clamp loading activity we mutated it to alanine and purified the γ (R169A) protein from an overproducing strain of E. coli. To study the effect of this mutation on clamp loader activity, we reconstituted the γ complex using γ (R169A) with δ, δ', χ and ψ. Our previous studies have shown that fully assembled γ complex is stable to ion exchange chromatography on an FPLC MonoQ column, where it elutes much later than free subunits (11). The γ (R169A) mutant was mixed with an excess of δ, δ', χ and ψ, incubated 30 min. at 15°C, then applied to a MonoQ column followed with a gradient of NaCl. The result, in Fig. 2A, demonstrates that the γ (R169A) mutant forms a "γ (R169A) complex" in which all five subunits co-elute in fractions 41 - 49, while the excess subunits elute much earlier. As demonstrated later in this report (Fig. 4), the γ (R169A) complex also remains intact during analysis on a gel filtration column. The subunit ratio of γ (R169A) complex is comparable to wild-type γ complex as observed in the Coomassie Blue stained SDS polyacrylamide gel of Fig. 2B. This ability of γ (R169A) to assemble into a multisubunit complex with δ, δ', χ and ψ demonstrates that the γ (R169A) mutant is properly folded. It also provides reconstituted γ (R169A) complex for the studies to follow.

The γ (R169A) complex binds three molecules of ATP with similar affinity as the wild-type.

Next, we determined whether γ (R169A) complex binds ATP, and if so whether it binds ATP with similar stoichiometry and affinity compared to wild-type γ complex. To address these issues we used the equilibrium gel filtration technique. In this analysis a gel filtration column is equilibrated with a known concentration of $^{32}$P-ATP. The γ complex is incubated with the same concentration of $^{32}$P-ATP as present in the column buffer, and then is applied to the column. Fractions are collected and the amount of protein and $^{32}$P-ATP in each fraction is determined. Protein bound $^{32}$P-ATP is carried around the beads resulting in a peak of $^{32}$P-ATP that elutes early, followed later by a trough which has less $^{32}$P-ATP than the column buffer due to its displacement from the buffer by the protein.
This information can be used to calculate the $K_d$ value for ATP binding to the complex. However, a more accurate assessment of the $K_d$ value can be obtained by repeating the experiment at a variety of ATP concentrations (the column is equilibrated at different ATP concentrations), followed by plotting the data as a Scatchard plot. This detailed analysis also carries the advantage of providing the stoichiometry of ATP bound to the complex.

The results, in Fig. 3, show that $\gamma$ (R169A) complex binds three molecules of ATP with similar affinity as wild-type $\gamma$ complex ($K_d$ 1-2 µM). The data for both wild-type and $\gamma$ (R169A) complex fall on a relatively straight line indicating that the three sites bind ATP with similar affinity. This conclusion is also supported by a study of a monomeric $\gamma$ subunit (missing the C-terminal oligomerization domain) which binds ATP with a $K_d$ value of 1.36 µM, determined by isothermal calorimetry (44).

Overall, these results indicate that the two ATP sites that carry alanine residues in place of arginine 169 (Sites 2 and 3) still bind ATP, and that this arginine residue contributes little, if any, to the binding affinity of ATP to $\gamma$ complex.

**$\gamma$ (R169A) complex binds $\beta$.**

Previous studies have demonstrated that ATP binding to $\gamma$ complex induces the conformation change that leads to the binding of the $\beta$ subunit (21). This predicts that the $\gamma$ (R169A) complex, which binds ATP, should be capable of binding to $\beta$. To test this prediction, we analyzed a mixture of $\gamma$ (R169A) complex and $\beta$ for complex formation on a Superose 12 sizing column equilibrated with buffer containing ATP. $\beta$ alone migrates in fractions 37-43 (Fig. 4A). Analysis of a mixture of wild-type $\gamma$ complex and $\beta$ is shown in Fig. 4B; $\beta$ subunit co-elutes with the large $\gamma$ complex in fractions 22-31 and resolves from unbound $\beta$ which elutes in the later fractions. A similar analysis using $\gamma$ (R169A) complex, shown in Fig. 4C, demonstrates that the mutant $\gamma$ complex is also capable of associating with $\beta$. The amount of $\beta$ that comigrates with the $\gamma$ (R169A) complex is nearly the same compared to wild-type $\gamma$ complex indicating that it is capable of binding $\beta$, although its affinity for $\beta$ may be somewhat decreased by the mutation. ATP binding, not hydrolysis, powers all the steps of clamp
loading except the final stage of dissociating from the β•DNA complex, allowing β to close around the DNA (21). This last step requires ATP hydrolysis. Next, the mutant γ complex was studied for ability to hydrolyze ATP.

The γ R169 is essential to catalysis.

We next analyzed γ (R169A) complex in three different assays, each of which require ATP hydrolysis. The first activity to be tested was DNA-dependent ATPase activity of γ complex, followed by clamp loading and finally β-dependent DNA synthesis by core DNA polymerase.

The γ complex requires the presence of DNA for significant ATPase activity (45). The β subunit stimulates γ complex ATPase activity provided a primed DNA, not ssDNA, is present (45). In the experiments of Fig. 5A we examined the γ complex ATPase activity using primed DNA with and without β. Whereas wild-type γ complex hydrolyzes approximately 217 molecules of ATP per min in the presence of the primed template, the γ (R169A) complex shows no detectable ATPase activity (i.e. detection limit ~ 5 ATP hydrolyzed/min/γ complex). The β subunit stimulates the ATPase activity of wild-type γ complex in the presence of primed DNA as illustrated in Fig. 5A. However, β does not provide detectable ATPase activity by γ (R169A) complex in the presence of the primed template (Fig. 5A), even when a very large excess of β is present (2 µM, data not shown).

The absence of catalytic activity predicts that the mutant γ complex will be incapable of loading a β clamp onto primed DNA. To assay β clamp loading activity directly we radiolabeled β with $^{32}$P using a derivative of β that carries an N-terminal tag with a kinase site. The $^{32}$P-β was incubated with γ complex and primed M13mp18 ssDNA (SSB coated), then the reaction was analyzed for assembly of $^{32}$P-β onto the primed DNA by gel filtration on a BioGel A15m column. This resin has large pores which include proteins, but exclude the large M13mp18 primed ssDNA (see agarose gel analysis). As a result, $^{32}$P-β bound to DNA elutes early and resolves from free $^{32}$P-β that is not bound to DNA. A control reaction using wild-type γ complex is shown in Fig. 5B. Most of the $^{32}$P-β elutes with the DNA in fractions 11 - 16. An agarose gel analysis, shown in Fig. 5B, confirms that the large SSB coated DNA substrate alone elutes in these same fractions (11-16). However, repeating this analysis using
mutant γ complex did not result in assembly of $^{32}$P-β on the DNA, and instead the $^{32}$P-β eluted later, in fractions 17-33. A control reaction lacking the DNA template confirmed that free $^{32}$P-β elutes in fractions 17-33 (Fig. 5B). Analysis of γ complex migration shows that it elutes in the included fractions in the same position as free β, consistent with the large pore size of the A15m resin (not shown, but as in Fig. 5 of ref. 16). Hence, the γ (R169A) complex is inactive for clamp loading action, consistent with its lack of ATPase activity.

Finally, γ (R169A) complex and β were tested for ability to support DNA synthesis by core polymerase. The core Pol III is incapable of extending a primer around an SSB coated primed M13mp18 ssDNA template unless it is coupled to a β clamp, and thus provides another measure of clamp loading activity. Use of the wild-type γ complex yielded a strong signal in this assay, but as expected, when mutant γ complex was used in this assay no activity was detected, consistent with the inactivity of mutant γ complex in ATPase and clamp loading assays (Fig. 5C). These results support and extend those of an earlier analysis in which several conserved residues of γ were mutated (46). Mutation of γ R169 resulted in loss of replication and ATPase activity, and failed to complement a conditional lethal dnaX gene in vivo, although that study did not demonstrate that the mutant γ was properly folded, or that it retained ability to form a stable complex with δ and δ', bind ATP and associate with β.

**The δ' SRC motif**

The above results strongly support an essential role of the γ arginine 169 in catalysis. However, the results imply something further. Mutation of γ arginine 169 only eliminates this catalytic residue at ATP Sites 2 and 3. The putative catalytic arginine of ATP Site 1 is supplied by δ', not γ, and therefore this site should remain competent for ATP hydrolysis, even in the γ (R169A) complex. Even though γ (R169A) complex retains one intact ATP site, the results show that it has lost essentially all of its ATPase activity. This implies that the ATP bound to Sites 2 and/or 3 must first be hydrolyzed before ATP in Site 1 is hydrolyzed. Alternatively, Site 1 binds ATP but is simply not catalytic. A non-catalytic Site 1 would explain why the γ (R169A) complex has no ATPase, but would
not explain why the SRC motif is broadly conserved in prokaryotic δ′ subunits. To test whether arginine 158 in the SRC motif of δ′ is important to catalysis, this arginine was mutated to alanine, and the δ′ (R158A) mutant was purified and reconstituted into γ complex for analysis. If ATP Site 1 is not hydrolytic, the δ′ (R158A) γ complex should have wild-type levels of ATPase and clamp loading activity.

The δ′ (R158A) mutant was mixed with γ, δ, χ and ψ to reconstitute the “δ′ (R158A) γ complex”. The complex was stable to ion exchange chromatography yielding purified reconstituted δ′ (R158A) γ complex with similar subunit stoichiometry as wild-type γ complex (Fig. 6A). ATP binding analysis by equilibrium gel filtration demonstrated that the δ′ (R158A) γ complex retained ability to bind three ATP molecules with similar affinity as wild-type γ complex (Fig. 6B; Kd ~ 0.8 µM). The δ′ (R158A) γ complex also remained capable of forming a complex with β in the presence of ATP (Fig. 6C). In contrast to the γ (R169A) complex, the δ′ (R158A) γ complex retained some activity in the catalytic assays requiring that ATP be hydrolyzed. The δ′ (R158A) γ complex retained approximately 30% of the DNA dependent ATPase activity of wild-type γ complex, although β no longer stimulated the ATPase as it did wild-type γ complex (Fig. 6D). Also, the DNA synthetic activity of δ′ (R158A) γ complex was about 20-30% active compared to wild-type γ complex (Fig. 6E). Hence, the δ′ (R158A) γ complex retains some clamp loading activity, but is nevertheless significantly compromised compared to wild-type γ complex.

The above results demonstrate that ATP Site 1 is catalytic, since δ′ (R158A) γ complex is significantly less active than wild-type γ complex. If Site 1 were only used for ATP binding, the δ′ (R158A) γ complex would have been expected to be fully active in the catalytic assays. Hence arginine 158 of δ′ is important to catalytic activity of γ complex, consistent with conservation of the SRC motif among prokaryotic δ′ subunits.
DISCUSSION

The δ′ stator contributes a catalytic arginine to the clamp loader motor: The γ complex clamp loader has been proposed to consist of three main components (4,6): the δ wrench (opens β), the γ trimer motor (hydrolyzes ATP), and the δ′ stator (modulates β interaction with δ) (reviewed in (47)). The γ and δ subunits appear to have a flexible joint between the C-terminal domain (domain III), and the N-terminal domains (domains I/II). In contrast, the three domains of the δ′ stator appear to be held in a rigid conformation, thus the term stator, the stationary part of a machine upon which the other parts move (4,44). The C-terminal domains of all 5 subunits form a tight closed circular connection, holding the subunits together. However, the N-terminal domains have an interruption between δ and δ′. The size of this gap modulates the ability of β to interact with the δ wrench. The rigid δ′ stator is proposed to function as an anvil, and when ATP is hydrolyzed, the γ subunits move δ close to δ′, forcing the β ring off the δ wrench, allowing the β ring to close.

This report demonstrates that δ′, besides its role as stator, also plays an instrumental role in the motor function of γ complex by supplying a catalytic arginine into ATP Site 1 of γ complex. A catalytic role for this arginine was suggested by its proximity to ATP modeled into ATP Site 1 of the γ complex structure (4). Further, this arginine is embedded in an “SRC” motif that is highly conserved among clamp loading subunits of prokaryotes, eukaryotes and archaeabacteria. The E. coli δ′ subunit is a member of the AAA+ family and has the same chain fold as γ, yet δ′ does not bind ATP (48). The P-loop of δ′ has been modified through evolution and the N-terminus blocks the nucleotide binding site. However, there are examples of prokaryotic δ′ subunits that contain a consensus P-loop (i.e. Aquifex aeolicus (49)). Whether these δ′ subunits bind ATP is not known. However, even if these δ′ subunits bind ATP they may not hydrolyze it for lack of a catalytic arginine in the neighboring δ subunit. Perhaps noncatalytic ATP provides rigidity to these δ′ subunits without needing the extra connections between Domains III and Domains I/II observed in the E. coli δ′ stator.

The catalytic role played by δ′ in clamp loading ATPase action may explain why the δ′ sequence is highly conserved in prokaryotes compared to the sequence of δ. The δ subunit is the main
subunit responsible for opening the β clamp, but it has no catalytic role (16,19). Simple maintenance of protein-protein contacts, with no catalytic role to preserve, has apparently allowed the δ sequence to drift considerably. The catalytic role played by δ′ may be responsible for the much greater conservation of the δ′ sequence.

**An ordered hydrolysis model for clamp loading:** This report demonstrates that mutation of arginine 169 in γ, which removes the arginine of the SRC motif near ATP Sites 2 and 3, abolishes ATPase activity even though three ATP molecules still bind the mutant γ complex and ATP Site 1 remains intact. This result suggests that Site 1 can not hydrolyze ATP until after ATP in Sites 2 and/or 3 is hydrolyzed. Conversely, if ATP must be hydrolyzed in Sites 2 and/or 3 before ATP is hydrolyzed in Site 1, then an ATP Site 1 mutant may not block hydrolysis of ATP in Sites 2 and 3. Indeed, this expectation is largely upheld in this study. The δ′ (R158A) γ complex retains significant ATPase activity indicating that ATP in Sites 2 and/or 3 can be hydrolyzed, even when Site 1 is missing the catalytic arginine. The δ′ (R158A) γ complex also retains some clamp loading activity allowing it to support processive DNA synthesis. Thus, hydrolysis of ATP in Sites 2 and 3 would appear to be sufficient, although not optimal, for clamp loading.

Ordered hydrolysis of ATP starting at Sites 3 (or 2) and ending with Site 1 is the opposite order that predicted for ATP binding. Study of the γδδ′ structure (4) suggested that the sites may fill starting at Site 1 and ending with Site 3. A model encompassing an ordered binding and a reverse order of hydrolysis, as the current study suggests, is illustrated in Fig. 7. As ATP binds to Site 1, 2 and then 3, the gap between the δ and δ′ subunits is proposed to increase in order to accommodate interaction of δ with the β dimer. The δ subunit wrench then cracks one interface of the β ring, allowing the spring tension between the domains of the β ring to relax, thereby opening the ring for DNA strand passage. Upon association of a primed template through the open β ring, the arginines of the SRC motifs in γ and δ′ align for ATP hydrolysis. The current study indicates that ATP hydrolysis occurs first at Site 3 (or 2) before hydrolysis at Site 1. This order may be achieved via proper positioning of the catalytic Arg residues. Specifically, hydrolysis at Site 3 may be required in
order for the arginine in Site 2 to become properly aligned, and hydrolysis in Site 2 may be required for the arginine in Site 1 to be aligned. The threading of a primed site through β may align the arginine in Site 3 to start the hydrolysis cycle (discussed further, below).

The proposed order of ATP hydrolysis in the model proceeds first from Site 3, then 2 and finally to Site 1. However, it is also possible that Site 2 fires first. We would like to construct different γ complexes containing all the permutations of single and double ATP site mutants, but are prevented from this strategy by the fact that Sites 2 and 3 are formed solely by identical γ subunits.

**Generalization of these results to other clamp loaders.** The studies of this report on the *E. coli* clamp loader have implications for subunit function in clamp loaders of other organisms. The eukaryotic RFC heteropentamer is composed of five different subunits, three of which contain both the SRC motif and consensus P-loops, suggesting they may act similarly to the γ trimer motor (yeast RFC 2•3•4 and human p36•37•40) (50). Indeed, these complexes contain DNA-dependent ATPase activity (51-52). One RFC subunit (yRFC5 and human p38) contains the SRC motif, but lacks a consensus P-loop, and thus is analogous to the δ' stator, and may contribute a catalytic arginine for ATP hydrolysis in the RFC 2, 3, 4 motor. The yRFC1 subunit (human p128) forms a strong attachment to the PCNA clamp (53) and thus may be analogous to the δ wrench. Like *E. coli* δ, yRFC1 lacks an SRC motif, but unlike δ it contains a consensus P-loop suggesting it binds ATP. Mutation of this P-loop is without significant effect on yRFC activity, indicating that this fourth ATP site in the eukaryotic clamp loader may be coupled to some other process (54).

Studies of P-loop mutants of yRFC2, 3, and 4 show that mutation of either RFC 2 or 3 greatly reduce ATPase activity and clamp loading function of RFC, while mutation of RFC4 has much less of an effect on these activities (50). These results are similar to the current study of γ complex. Namely, δ' (R158A) γ complex is partially active, while γ (R169A) complex is completely inactive. Thus, it seems quite possible that the RFC clamp loader may also hydrolyze ATP in an ordered sequence around the circular pentamer, as proposed here for γ complex, where some sites must first hydrolyze ATP before ATP in other sites can be hydrolyzed.
The five subunit clamp loader of bacteriophage T4 has two different subunits, four copies of gp44 and one gp62 subunit. Biochemical studies demonstrate that ATP is hydrolyzed to load the T4 gp45 clamp onto DNA; stoichiometry measurements range from 1-4 ATP per gp45 clamp loading event (55-58). The gp44 tetramer is an ATPase, contains both the SRC motif and P-loop, and is homologous to *E. coli* γ and δ’. The gp62 is similar to δ in that it has neither the SRC motif nor P-loop, and its sequence has diverged from γ/δ’. At first glance it would seem that the T4 clamp loader has done away with the stator, and indeed it may have. However, keeping in mind that gp62 has no SRC motif, it seems likely that one gp44 subunit (i.e. adjacent to gp62) will be incapable of hydrolyzing ATP even if it binds ATP. Thus, one gp44 subunit may serve a similar role as δ’ in modulating contact between the gp44/62 clamp loader and the gp45 clamp, at the same time as providing a catalytic arginine residue for ATP hydrolysis in the neighboring gp44 subunit.

The clamp loader of archaebacteria has been studied, but less intensively than the clamp loaders of *E. coli*, T4, yeast and humans. Generally, archaebacterial clamp loaders (called RFC) consist of two subunits, RFC large and RFC small (59). The stoichiometry of these subunits is not certain, with reports ranging from 1:4, like T4, to 3:2 and even 4:2 (60-62). The crystal structure of *Pyrococcus* RFC small subunit shows that its basic unit is a trimer, presumably the equivalent to the γ trimer motor, in which each subunit contains both a nucleotide binding site and a closely juxtaposed arginine from the neighboring subunit (63). The *Pyrococcus* RFC large subunit contains a consensus P-loop motif and thus may bind nucleotide, but lacks the SRC motif. The lack of a SRC motif in the RFC large subunit suggests that if there are two of these subunits in the clamp loader, then one of these will be unable to hydrolyze ATP and thus may function in an analogous fashion as the δ’ stator. Alternatively, if only one RFC large subunit is present, adjacent RFC small subunit will not hydrolyze bound ATP, thereby acting as a stator. In this second scenario, the stator would also contain an SRC motif, like δ’.

**Possible role of the SRC arginines in clamp loading fidelity.** The γ complex is a very poor ATPase without a DNA effector. The crystal structure indicates that γ R169 and δ’ R158 are not quite close
enough to function with ATP, they must move an extra one or two angstroms to have an effect on ATP hydrolysis (4). Hence, it is tempting to speculate that misalignment of these arginines may underlie the very weak ATPase of γ complex, and their proper positioning may be used as a regulatory mechanism.

DNA stimulates the γ complex ATPase activity, and thus may bring the SRC motif Arg/ATP site pairs into a more favorable alignment for hydrolysis. Curiously, the β subunit only stimulates the γ complex ATPase when a primed template is used as an activator; ssDNA and duplex DNA stimulate γ complex in the absence of β, but do not give more activity when β is added (11). Consistent with this observation, γ complex does not load β onto ssDNA, even though ssDNA stimulates ATPase activity (64). Furthermore, the ATPase cycle is tightly coupled to clamp loading, as only 2-3 ATP are hydrolyzed for each β clamp that is loaded onto a primed template (16,21). Finally, the head-to-tail architecture of the β dimer generates two distinct “front” and “back” faces, only one of which functions with the DNA polymerase and thus it must be oriented correctly on DNA to interface with the polymerase (34,39). What system of checks and balances does γ complex have to ensure that these criteria have been met? It seems possible that the catalytic arginines, and their juxtaposition to the ATP sites, may act as a fidelity mechanism to ensure that β is only loaded when primed DNA is threaded through β, and only when β is oriented correctly for function with polymerase. Perhaps when these different criteria are met, the catalytic SRC motif arginine residues are brought into register for ATP hydrolysis to propel loading of the β clamp onto DNA.
FIGURE LEGENDS

Figure 1. Architecture of γ complex ATP sites. Panel A Orthogonal views of the γ ATP binding domain. R169 is located far away from the phosphate binding loop (P-loop) on the same polypeptide chain, preventing formation of a functional intramolecular ATP site. Panel B Cartoon of the circular pentamer of γ complex looking down the center of the ring from the C-terminus. Three ATP sites are created at subunit interfaces. Each site is highlighted by the P-loop of a γ subunit and the Sensor 1 Arg in the SRC motif from the adjacent δ’ (ATP Site 1) or γ (Sites 2 and 3) subunit. Panel C ATP Site 1: A magnified view of the δ’/γ1 N-terminal domain interface with an ATP molecule modeled against the P-loop of γ1 based on the NSF D2 crystal structure (31). The δ’ Sensor 1 R158 (green residue) is in close proximity to the γ-phosphate of ATP, suggesting a catalytic function. ATP Sites 2 and 3: the γ1/γ2 and γ2/γ3 interfaces, respectively, have a similar architecture to ATP Site 1. The γ Sensor 1 R169 is positioned proximal to the ATP molecule modeled into the neighboring γ. It should be noted that ATP modeled into Site 2 clashes with some residues of γ1, and thus a conformation change is needed to make this site accessible to ATP.

Figure 2. Reconstitution of γ (R169A) complex. Panel A Gradient elution of reconstituted γ (R169A) complex from a Mono-Q column. Note the excess free χψ (fractions 17-21), and δδ’ (fractions 31-33) subcomplexes that elute early in the gradient compared to the full γ (R169A) complex (fractions 41-49) made with limiting γ (R169A) subunit. Panel B Reconstituted wild-type γ complex and γ (R169A) complex have comparable subunit stoichiometries as analyzed in a 14% SDS polyacrylamide gel stained with Coomassie Blue.

Figure 3. The γ (R169A) complex binds three ATP molecules with similar affinity as wild-type γ complex. Wild-type γ complex (A) and γ (R169A) complex (B) were analyzed for ATP binding affinity and stoichiometry by equilibrium gel filtration as described in “Materials and Methods.” The
concentration of ATP (diamonds) and γ complex (black area curve) were measured in column fractions for a series of experiments performed at different concentrations of ATP. The Scatchard plot at the top is derived from the series of column analyses (each data point is one column analysis). The 10 µM ATP analysis is included in the Scatchard plot, but is not shown in the profile below the plots. The line is the least squares fit to the data. Panel A Wild-type γ complex analysis yields a K_d of 0.9 µM and stoichiometry of 3.2 ATP/γ complex. Panel B The γ (R169A) complex analysis yields a K_d value for ATP binding of 2.10 µM with a stoichiometry of 3.2 ATP/γ (R169A) complex.

Figure 4. ATP promotes β binding in the γ (R169A) complex. The interaction between β and γ complex was analyzed by gel filtration on a Superose 12 column to separate free β (fractions 37-43) from β bound to γ complex (fractions 22-31). Panel A β alone in the presence of ATP. Panel B β and wild-type γ complex with ATP. Panel C β and the γ (R169A) complex with ATP.

Figure 5. Analysis of catalytic activity by γ (R169A) complex. Wild-type and γ (R169A) complex clamp loaders were tested in three assays that each require hydrolysis of ATP. Panel A Steady-state ATPase assays in the presence of synthetic primed template + and − β. Squares and diamonds are wild-type γ complex plus and minus β, respectively. Circles and open triangles are γ (R169A) complex plus and minus β, respectively. Panel B In the clamp loading assays, 32P-β was incubated with wild-type γ complex (triangles) or γ (R169A) complex (squares) in the presence of SSB coated singly primed M13mp18 ssDNA, then DNA-associated 32P-β was separated (fractions 11-16) from free 32P-β (fractions 17-33) on a BioGel A15m column. The open circles are a control in which only the DNA substrate was omitted from the reaction. Above the plot is an ethidium bromide-stained neutral agarose gel analysis which demonstrates that the large DNA substrate alone elutes in fractions 11-16. Panel C Wild-type γ complex (triangles) or γ (R169A) complex (squares) was titrated into replication reactions containing β, core, SSB-coated singly primed M13mp18 ssDNA, and 32P-dNTPs.
Incorporation of $^{32}$P-dTTP indicates that the $\beta$ clamps are loaded onto DNA for processive replication by core polymerase.

**Figure 6. The $\delta^\prime$ (R158A) $\gamma$ complex is impaired in clamp loading and ATPase activity.**

Reconstituted $\delta^\prime$ (R158A) $\gamma$ complex was analyzed for ATP binding, $\beta$ interaction, ATPase activity and clamp loading. *Panel A* The reconstituted $\delta^\prime$ (R158A) $\gamma$ complex has a similar ratio of subunits as wild-type $\gamma$ complex. *Panel B* Equilibrium gel filtration demonstrates that the $\delta^\prime$ (R158A) $\gamma$ complex binds 2.7 ATP molecules with a $K_d$ value of 0.8 $\mu$M. *Panel C* The $\delta^\prime$ (R158A) $\gamma$ complex binds $\beta$ in a Superose 12 gel filtration analysis in the presence of ATP and magnesium. *Panel D* Steady-state ATPase assays show that the $\delta^\prime$ (R158A) $\gamma$ complex (diamonds, $-\beta$; open squares, $+\beta$) has reduced ATPase activity compared to wild-type $\gamma$ complex ($\sim 35\% - \beta$, and $\sim 15\% + \beta$). The activity of wild-type $\gamma$ complex is shown for comparison. (triangles, $-\beta$; closed circles, $+\beta$). *Panel E* DNA replication assays reflect the level of clamp loading activity by the $\delta^\prime$ (R158A) $\gamma$ complex (diamonds) compared to wild-type $\gamma$ complex (triangles).

**Figure 7. Sequential ATP binding and hydrolysis during the clamp loading operation.** *A* In the absence of ATP, the $\gamma$ complex adopts a conformation that does not bind $\beta$ due to proximity between $\delta$ and $\delta^\prime$. In going from A to D, ATP binds to $\gamma_1$, $\gamma_2$ and then $\gamma_3$ resulting in a conformation change that separates $\delta$ from $\delta^\prime$ and creates a docking site for $\beta$ binding to $\delta$ and the $\gamma$ subunits. In diagram E, primed template is bound by $\gamma$ complex-$\beta$-ATP. Upon sensing that the primed DNA passes through the central cavity of $\beta$, ATP is hydrolyzed sequentially, first at Site 3 (diagram F), then Site 2 (diagram G) and finally Site 1 (diagram H), bringing $\delta$ back in proximity to $\delta^\prime$, closing the $\beta$ ring around DNA and ejecting $\gamma$ complex from $\beta$. Upon ADP release, the $\gamma$ complex reassumes the ground state, ready for another round of clamp loading (diagram A).
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A. γ subunit ATP Binding Domain

B. ATP Site 1, ATP Site 2, ATP Site 3

C. ATP Site 1, ATP Site 2, ATP Site 3

Figure 1
Figure 2

A. MonoQ Reconstitution

B. SDS PAGE
Figure 3

A. Wild-Type γ Complex

\[ K_d = 0.9 \, \mu M \]
\[ n = 3.2 \]

\[ \bar{V} = \frac{[\text{ATP}_{\text{FREE}}]}{[\gamma \text{ comp}]} \]

\[ \bar{V} = \frac{[\text{ATP}_{\text{BOUND}}]}{[\gamma \text{ comp}]} \]

B. γ(R169A) Complex

\[ K_d = 2.1 \, \mu M \]
\[ n = 3.2 \]

\[ \bar{V} = \frac{[\text{ATP}_{\text{FREE}}]}{[\gamma \text{ comp}]} \]

\[ \bar{V} = \frac{[\text{ATP}_{\text{BOUND}}]}{[\gamma \text{ comp}]} \]
Figure 4

A. $\beta$ alone

B. Wild-Type $\gamma$ complex

C. $\gamma$(R169A) complex
Figure 5

A. ATPase Activity

B. Clamp Loading

C. DNA Synthesis
Ordered hydrolysis in the γ complex clamp loader AAA+ machine
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