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Clamp loaders and replication initiation

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Clamp loaders are ATP-driven multiprotein machines that couple ATP hydrolysis to the opening and closing of a circular protein ring around DNA. This ring-shaped clamp slides along DNA, and interacts with numerous proteins involved in DNA replication, DNA repair and cell cycle control. Recently determined structures of clamp loader complexes from prokaryotic and eukaryotic sources have revealed exciting new details of how these complex AAA+ machines perform this essential clamp loading function.

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Introduction

Cellular chromosomal replicases are multiprotein machines that bind DNA tightly for high processivity during replication (reviewed in [1,2]). Their tight grip on DNA derives from a sliding clamp protein, a ring-shaped homooligomer that encircles DNA and slides freely along the duplex. Representative structures of these clamps are shown in Figure 1a for *Escherichia coli* β and human PCNA [3,4]. Whereas the prokaryotic clamp is a homodimer, eukaryotic PCNA is a homotrimer. The clamps have a similar appearance, as their architecture is based on a domain of common structure. The monomeric units contain either two or three domains to yield a six-domain ring of strikingly similar dimensions.

The sliding clamps are required at primer–template junctions, where they bind to the polymerase and continuously hold it to the DNA during chain extension (Figure 1b). Clamps are repeatedly placed at new RNA-primed sites on the lagging strand, where they serve as markers of efficient polymerase association for the numerous initiation events during discontinuous replication [5]. The task of placing the clamp at a primed site is accomplished by a multiprotein clamp loader, which recognizes the junction of single-stranded (ss) and double-stranded (ds) DNA in a structure-specific fashion.

Despite the symmetrical appearance of the clamp, the two flat faces of the ring are structurally distinct. The polymerase associates with the face from which the C termini protrude in both prokaryotic and eukaryotic systems [6,7]. Therefore, the clamp loader must correctly orient the clamp on the 3′ terminus of the primed site for interaction with the DNA polymerase. Interestingly, clamp loaders from both prokaryotic and eukaryotic systems interact with the same face of their respective clamp as the DNA polymerase; thus, the clamp loader must depart from the clamp for the polymerase to function [8,9].

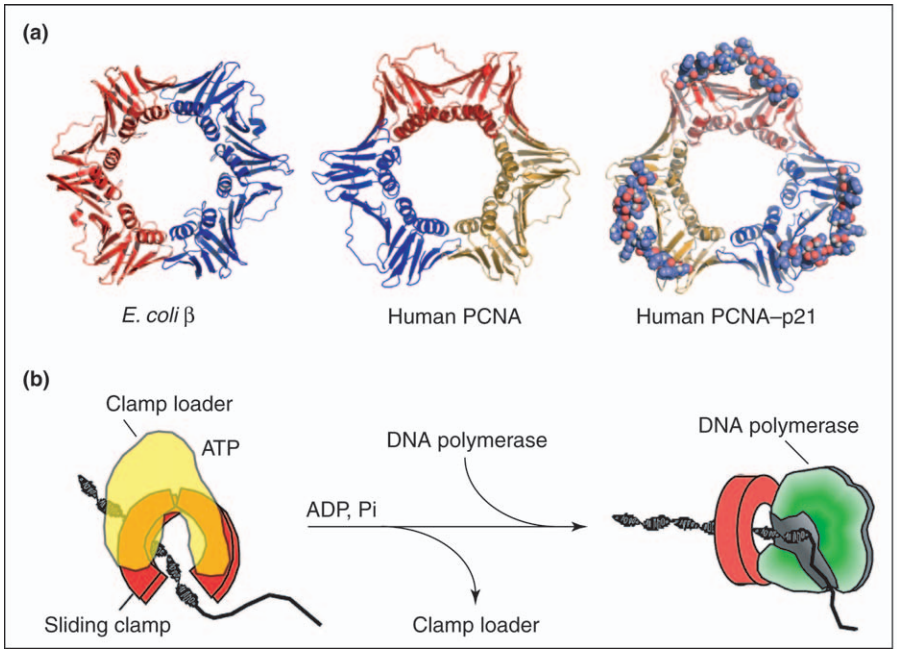
The first detailed view of how proteins bind clamps was provided by the structure of PCNA bound to a peptide of the p21 cell cycle regulator [4]. p21 binds the C-terminal face of PCNA in a hydrophobic pocket between the two domains (Figure 1a). It is now apparent that many different polymerases, regulators and repair proteins bind PCNA and use the same binding pocket [10–13]. An analogous binding pocket exists in β [14–16] and gp45, the T4 clamp [17].

This review provides an overview of clamp loader structure and function as it is currently understood in both prokaryotes and eukaryotes. Overall, they are strikingly similar and lessons learned from one generally apply to the other.

The *E. coli* clamp loader

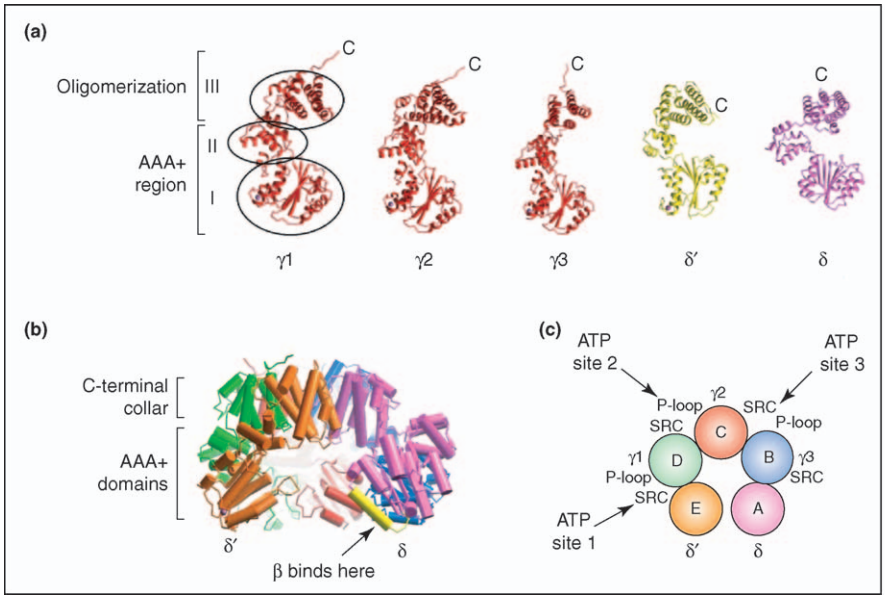
Clamp loader subunits, both prokaryotic and eukaryotic, are members of the AAA+ family [18]. The first crystal structure of an AAA+ protein was that of the *E. coli* δ′ clamp loader subunit, which has a similar chain fold to other clamp loader subunits, as illustrated in Figure 2a [19–21]. Recent structures of intact clamp loaders reveal five core subunits arranged in a circle, as illustrated in Figure 2b for the *E. coli* $\gamma_3\delta\delta'$ clamp loader [22]. The subunits of the $\gamma_3\delta\delta'$ clamp loader are designated A–E in Figure 2c for convenience in comparing it to the eukaryotic RFC clamp loader [23•]. The *E. coli* γ complex consists of the $\gamma_3\delta\delta'$ complex and also two additional small subunits (χ and ψ), which are not required for clamp loading. The structure of the χ–ψ subassembly has been determined and is unrelated to that of the other subunits [24]. Each of the γ, δ and δ′ subunits consists of three domains (Figure 2a). The ATP-binding AAA+ region of

Figure 1



Interplay between the sliding clamp, clamp loader and DNA polymerase. **(a)** Ribbon representations of *E. coli* β (left; PDB code 2POL), human PCNA with bound p21 removed (middle) and human PCNA bound to a C-terminal peptide of p21 (right; PDB code 1AXC for both PCNA structures shown). p21 binds the C-terminal face of the PCNA ring and serves as the prototype for how proteins bind to sliding clamps. Adapted with permission from [3,4]. **(b)** The clamp loader uses ATP to load the sliding clamp on DNA. Hydrolysis ejects the clamp loader from the clamp, enabling DNA polymerase to access the clamp. Adapted with permission from [50].

Figure 2



The *E. coli* γ complex clamp loader. **(a)** Individual subunits of $\gamma_3\delta\delta'$ are displayed side by side. The three-domain construction of each subunit is illustrated for γ1. Adapted with permission from [51]. **(b)** The $\gamma_3\delta\delta'$ minimal clamp loader structure (side view; PDB code 1JR3). Adapted with permission from [22]. Major intersubunit contacts are mediated by the C-terminal domains of each subunit. The AAA+ domains of the pentamer have a gap between δ (purple) and δ′ (orange). The yellow-colored helix in δ interacts with β [14]. **(c)** The A–E nomenclature of the subunits. ATP binds only the three γ subunits, at sites located at subunit interfaces.

homology is located in the two N-terminal domains, whereas the C-terminal domain forms the major inter-subunit connections that hold the pentamer together.

Oligomers of AAA+ proteins typically consist of six identical subunits that form a symmetrical circular disk [25,26]. However, the circular pentameric clamp loaders contain a gap instead of a sixth subunit (Figure 2b). The C-terminal domains form a tight uninterrupted 'collar', but there is a prominent gap between the AAA+ domains of δ and δ′ (subunits A and E). Recent experiments indicate that the gap remains open throughout the reaction and is present to allow DNA entry into the open clamp (described later) [27].

Most if not all of the $\gamma_3\delta\delta'$ subunits interact with β [28]. However, the tightest contact to β occurs through the δ subunit, which acts as a wrench to destabilize the β dimer interface [14,29]. To bind the clamp, the γ complex must first bind ATP, which promotes a conformational change [30]. The $\gamma_3\delta\delta'$ crystal structure lacks nucleotide and thus is in the inactive conformation for clamp binding. Only the γ subunits bind ATP, as the ATP sites of the δ and δ′ subunits are degenerate. Typical of AAA+ oligomers, the ATP sites are positioned at subunit interfaces [25,26]. In particular, an arginine finger residue located in a conserved SRC motif reaches across the interface to participate in the ATP site of the adjacent subunit. The strategic location of ATP sites at subunit interfaces of AAA+ oligomers may enable global conformational changes in response to ATP binding and hydrolysis [25,26]. Site-specific mutants of the arginine finger of γ and δ′ confirm its importance in ATP sensing and hydrolysis [31,32].

ATP sites 1 and 3 in $\gamma_3\delta\delta'$ appear open and accessible to ATP binding, but ATP site 2 is 'squeezed' shut by amino acid sidechains from subunit B that occlude the ATP-binding pocket in subunit C. Interestingly, the structure of $\gamma_3\delta\delta'$ in the presence of ATPγS shows that only sites 1 and 3 are occupied; ATP site 2 remains unfilled and the complex retains nearly the same conformation as the unliganded structure, suggesting that β may be required to assist the binding of the third ATP [33•].

The eukaryotic clamp loader

The eukaryotic clamp loader, replication factor C (RFC), was first identified by its requirement in SV40 replication *in vitro* [34]. RFC consists of five non-identical subunits, four of which have a consensus P-loop [35]. Four of the RFC subunits are similar in size to the $\gamma_3\delta\delta'$ subunits, whereas the A subunit (RFC1) is over twice as large and contains N- and C-terminal extensions. The structure of *Saccharomyces cerevisiae* RFC in complex with PCNA and ATPγS (Figure 3a) yields insight into the nature of the ATP-induced conformational change and reveals how the clamp loader recognizes a primer–template junction [23•,36].

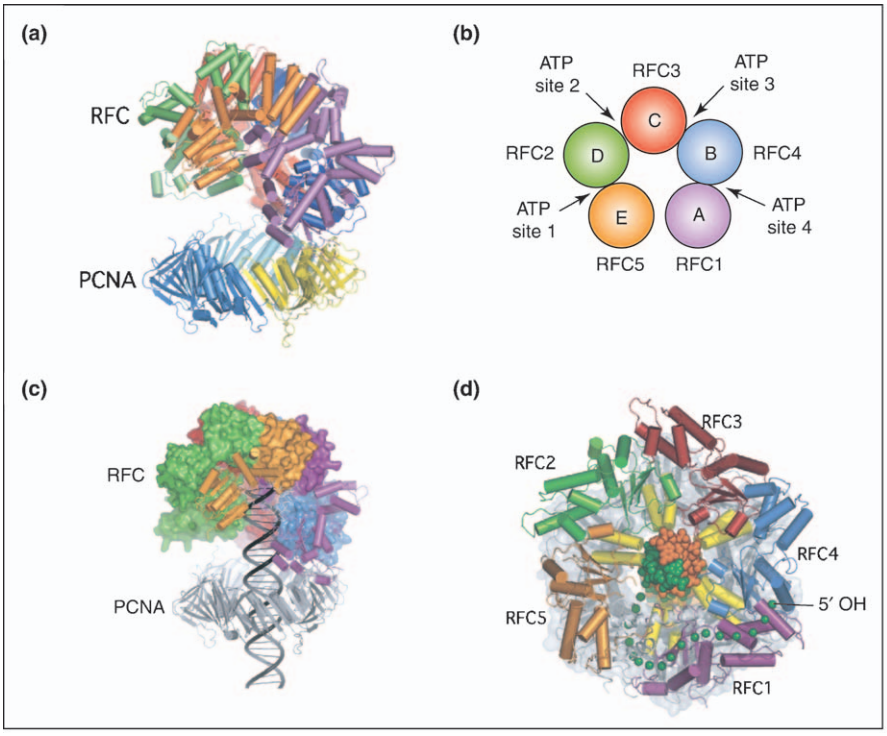
The RFC pentamer contains four ATP sites (Figure 3b). The additional ATP site in RFC (relative to $\gamma_3\delta\delta'$) is in RFC1 (subunit A). Biochemical studies of RFC P-loop mutants indicate that the RFC1 ATP site is not required for clamp loading and its role remains unclear [37]. The RFC structure unexpectedly shows five bound nucleotides, even though RFC5 (in position E, corresponding to *E. coli* δ′) lacks a consensus P-loop. The gap between subunits A and E is present, and the C-terminal extension of RFC1 folds back and packs against subunit E.

The orientations of the five ATPγS in the RFC–PCNA structure are related by a set of similar screw operations about a central axis; this results in a spiral architecture with a pitch that closely matches B-form DNA [23•,36]. Furthermore, the RFC AAA+ domains define a central cavity into which duplex DNA may snugly fit and the size of the footprint corresponds to earlier footprinting studies of the RFC–DNA complex [38] (Figure 3c). Hence, the conformation induced by nucleotide binding, perhaps assisted by the clamp, brings the subunits into a helical arrangement to fit around DNA and track the duplex (Figure 3d). A helical disposition of subunits within an oligomer that encircles DNA is also observed in certain proteins based on the RecA ATPase fold, including T7 helicase, Rho and RecA [39–41].

The PCNA clamp is attached to the A, B and C subunits; the D and E subunits lift off the plane of the ring and the PCNA ring is closed (Figure 3a). In common with the *E. coli* γ complex, biochemical studies have demonstrated that ATPγS induces RFC to bind PCNA and open its ring for DNA binding; hydrolysis is required for RFC to eject from PCNA, thereby enabling DNA polymerases to function with the sliding clamp [42]. Hence, it is somewhat surprising that the PCNA ring is closed in the RFC–PCNA–ATPγS structure. This dilemma may be explained by the fact that the structural study utilized an RFC mutant in which the arginine fingers in the SRC motifs of subunits A–D were mutated to SQC to prevent the hydrolysis of ATPγS, which could occur during the time-frame of crystal growth. The SRC→SQC mutations in subunits A–D of the RFC–PCNA–ATPγS structure may tilt the equilibrium towards a form in which PCNA is closed, thus severing the connection between PCNA and subunits D and E. This form is proposed to represent an intermediate in which PCNA is closed around DNA, just upstream of the ATP hydrolysis and RFC ejection step [23•]. Hydrolysis of ATP would complete the detachment of RFC from PCNA and DNA (explained further in the section below and Figure 4).

Figure 3d shows a cut-away view looking down on the top of an RFC–PCNA complex in which the C-terminal domains that form the clamp loader collar have been removed and the DNA has been modeled through PCNA

Figure 3



The *S. cerevisiae* RFC clamp loader. **(a)** Structure of yeast RFC bound to PCNA and ATPγS (PDB code 1SXJ). The five bound ATPγS are not shown in the figure. Subunit coloring indicated in (b–d). **(b)** Cartoon of the subunit arrangement and ATP sites of the RFC pentamer. **(c)** Model of DNA positioned within the RFC–PCNA structure. The C-terminal collar acts as a screw cap that blocks the continued threading of DNA through the structure. **(d)** Top view of the RFC–PCNA–DNA model, with the C-terminal collar. PCNA is in gray, and DNA is in green and orange. The helices that track DNA are highlighted in yellow. Green spheres indicate a possible exit path for template ssDNA from the central chamber. Panels (a,c,d) adapted with permission from [23•].

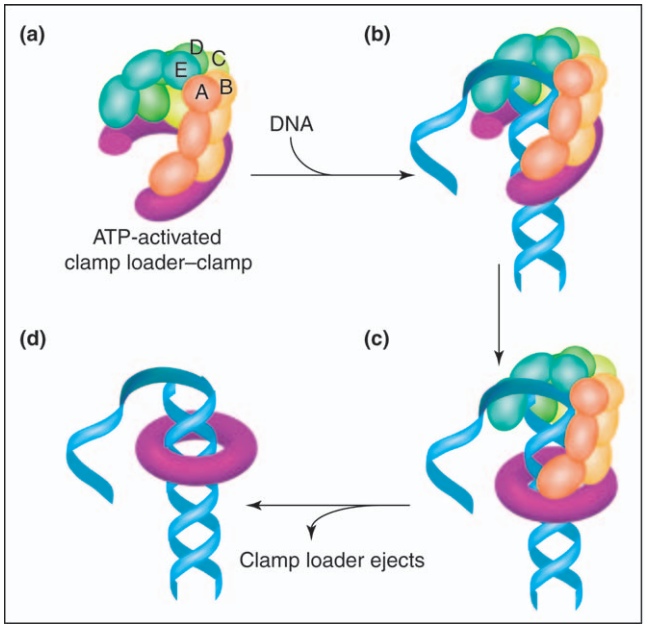
and into the central chamber of RFC. Each subunit contains a pair of helices for which the positive dipole is directed toward the central chamber. Minor groove interactions have been demonstrated to mediate the interaction of protein with DNA in non-sequence-specific fashion [43]; when DNA is modeled inside the RFC chamber, it can be positioned such that these helices track the minor groove (yellow helices in Figure 3d). Conserved positively charged and polar residues exist on and near these helices, several of which are also conserved in prokaryotic clamp loader subunits. As a test for DNA binding inside the clamp loader, advantage has been taken of the prokaryotic clamp loader, which contains three identical copies of γ [44•]. Thus, any given mutation in γ is repeated three times in $\gamma_3\delta\delta'$. Biochemical studies of $\gamma_3\delta\delta'$ with mutated putative DNA-binding residues in γ and/or δ′ support the central chamber as the anatomical locus of DNA binding [44•].

Primed template recognition and insertion of DNA into the clamp

Clamp loaders must open the clamp, recognize a primed template and then insert the DNA through the center of the clamp. Studies in the T4 system indicate that the gp45 clamp may open to form a left-handed helix [45]. Recent molecular simulation studies of yeast PCNA indicate that, upon destabilizing the clamp interface, the PCNA ring springs open into a right-handed spiral [46•]. EM reconstruction studies of an archaeal RFC–PCNA–DNA–ATPγS complex indicate that PCNA has a open lock-washer appearance, enabling it to extensively dock on the helical surface of the RFC pentamer [47•]. These observations are summarized in Figure 4a, wherein the open clamp docks underneath the clamp loader and adopts a complementary spiral to that of the AAA+ domains of the clamp loader subunits. This helical open form allows the clamp to interact with all the clamp loader subunits.

DNA may enter the central chamber of the clamp loader through subunits A and E (e.g. Figure 4b). The tight pentameric contacts comprising the collar provide no opening, thus presenting an obstacle to the fit of a rigid duplex straight through the clamp–clamp loader complex. Instead, a flexible joint in DNA is needed to enable the DNA to bend out of the gap between subunits A and E. The ssDNA portion of a primed template should be sufficiently flexible to permit this bend.

Figure 4



Clamp opening and DNA recognition. **(a)** The open clamp is illustrated as a right-handed lock-washer that fits on the helical surface defined by the five AAA+ domains of the ATP-activated clamp loader. **(b)** Primed DNA enters through the gap in the open ring and the slot between the A and E clamp loader subunits. Template ssDNA provides a flexible joint for DNA exit from the central chamber through the slot in the side of the clamp loader. **(c)** The ring snaps shut around DNA, severing the tie between the clamp and the D and E clamp loader subunits. **(d)** ATP hydrolysis ejects the clamp loader from the clamp–DNA complex, leaving the clamp topologically linked to the DNA. Adapted with permission from [2].

When the clamp closes around DNA, it will become a planar ring and therefore may detach from some subunits of the clamp loader spiral (Figure 4c). This proposed intermediate may correspond to the structure captured in the RFC–PCNA–ATPγS crystal, in which subunits A, B and C interact with the closed clamp, while the ATP sites are poised for hydrolysis [23•]. Hydrolysis ejects the clamp loader, thus enabling DNA polymerase to access the clamp (Figure 4d).

Concluding remarks

Important new questions arise from recent studies on prokaryotic, eukaryotic and archaeal clamp loaders. For example, the nature of the ATP-induced conformational change in the clamp loader needed for clamp and DNA binding remains uncertain. Is the clamp also required for this conformational change? Is the clamp actively 'cracked open' by the clamp loader? Alternatively, the clamp may spontaneously breathe and the clamp loader may trap the clamp in the open form. Little is known about how the clamp loader reads the polarity of a primer–template junction. Does it recognize the 3′ or 5′ terminus at a primed site? Perhaps instead it reads the polarity of the template strand. There exist alternative RFC complexes in which subunit A (RFC1) is replaced by another subunit. For example, in response to DNA damage, RFC1 is replaced by a Rad protein (Rad24 in *S. cerevisiae*, Rad17 in humans) [48]. Rad–RFC loads the clamp (the 911 clamp, which is different from PCNA) onto the 5′ end of a primed site and thus recognizes the opposite side of a single-stranded/double-stranded junction compared to RFC–PCNA [49•]. Is the polarity of the primed site recognized by the A subunit, which is replaced in alternative RFCs?

Although not covered in this review, both prokaryotic and eukaryotic clamps interact with a wide variety of DNA polymerases, repair factors and cell cycle regulators. How these various factors coordinate their action on the clamp can be added to the list of the many exciting questions that remain for future studies.

Acknowledgements

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- •• of outstanding interest

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