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Assembly and Disassembly of DNA Polymerase Holoenzyme

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The complex task of genomic replication requires a large collection of proteins properly assembled within the close confines of the replication fork. The mechanism and dynamics of holoenzyme assembly and disassembly have been investigated using steady state and pre-steady state methods as opposed to structural studies, primarily due to the intrinsic transient nature of these protein complexes during DNA replication. The key step in bacteriophage T4 holoenzyme assembly involves ATP hydrolysis, whereas disassembly is mediated by subunit dissociation of the clamp protein in an ATP-independent manner.

Abbreviations

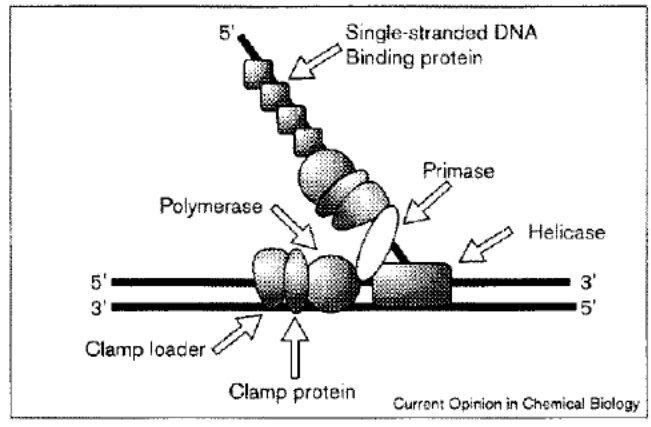
FRET	fluorescence resonance energy transfer
PCNA	proliferating cell nuclear antigen
RF-C	replication factor C

Introduction

In order to replicate genomic DNA quickly and accurately, a large collection of proteins is assembled at a strand-separated region of DNA known as a replication fork. These proteins minimally include a DNA polymerase which is responsible for catalyzing replication, a single-stranded DNA-binding protein which is responsible for holding open the replication fork, a DNA helicase, which unwinds the DNA region to be replicated, and a DNA primase which provides the priming sequences needed for lagging strand synthesis (Figure 1, Table 1). This large ensemble of replicative proteins requires intricate coordination, not only for proper function during the synthesis of both leading and lagging DNA strands during DNA replication but also during assembly and disassembly of these protein complexes at the appropriate time. The similarities in composition and function among DNA replicases and their components suggest a unified mechanism for achieving proper coordination of the macroprocesses of assembly, DNA elongation, and disassembly of the replication fork.

The bacteriophage T4 DNA polymerase holoenzyme is derived from the phage DNA polymerase (the product of gene 43) and its accessory proteins (the products of genes 44, 45 and 62) [1,2]. The DNA polymerase possesses a 3′→5′ polymerization activity, as well as a 3′→5′ exonuclease activity required for the maintenance of fidelity during replication, and has been extensively characterized kinetically [3,4]. As is the case for most replicases, however, the T4 polymerase alone incorporates nucleotides in a distributive manner, as opposed to the highly processive manner required for genomic replication *in vivo* [5–7]. The processivity of the T4 DNA polymerase is increased by its interaction with replicative accessory proteins, which essentially prevent the polymerase from rapidly dissociating from the DNA. In this way, these proteins afford an increase in the overall rate and processivity of both DNA synthesis and 3′→5′ nucleotide excision catalyzed by the polymerase [5,8,9].

Figure 1



Model of bacteriophage T4 proteins at a DNA replication fork. The two holoenzymes are formed from the clamp-loader, the clamp protein, and the DNA polymerase; the helicase–primase constitutes the primosome which is responsible for movement of the replication fork and lagging strand Okazaki fragment priming. Although qualitatively similar in function, specific differences among the three well-defined replication systems are further addressed within the text.

The 44/62 protein, generically referred to as the clamp-loader, places the sliding clamp, the 45 protein, onto DNA in an ATP-dependent manner [9]. The polymerase associates with the loaded clamp protein to form the stable holoenzyme on primer/templates. Because the 44/62 protein and the 45 protein are functionally similar to the γ complex [7] and the β subunit [10], respectively, in *Escherichia coli*, and to the replication factor C (RF-C) complex [11] and proliferating cell nuclear antigen (PCNA) [12] in eukaryotic systems, we have used the relatively simple T4 replication system as a paradigm for understanding the roles and activities of the clamp-loader and clamp protein during assembly and disassembly of the processive holoenzyme complex. This review summarizes our current

knowledge of the molecular mechanism and dynamics of holoenzyme assembly and disassembly, focusing primarily on these well-defined pathways in the bacteriophage T4 DNA replicative complex.

Holoenzyme assembly

Holoenzyme assembly proceeds by at least two ordered events: loading of the clamp protein by the clamp-loader, and rapid association of the polymerase with the loaded clamp protein to form the holoenzyme. Figure 2 presents a putative mechanism for T4 holoenzyme assembly, whereby clamp-loading onto the primer/template precedes interaction with the polymerase. The general features of T4 holoenzyme assembly appear to be species independent.

The crystal structures of the sliding clamp proteins from eukaryotes (PCNA), *E. coli* (β-clamp), and T4 (45 protein) have revealed a common ring-shaped structure with an interior diameter large enough to circumscribe double-stranded DNA (Table 1) [13,14]. The clamp proteins share several structural features, including a sidedness whereby only one face of the ring (the so-called rough face) apparently mediates protein–protein interactions [15••,16•] and the ability to slide on DNA [17], which is necessary for processive DNA synthesis. Consequently, primer/templates have been used that prevent translational dissociation from DNA by way of the presence of an upstream template biotin-streptavidin complex and a downstream fork strand that simulates a replication fork [18]. Differences, however, do exist among the clamps with regards to quaternary structure (PCNA and the 45 protein are homotrimers whereas the β-clamp is a homodimer) and in their affinity for DNA (β-clamp >PCNA >45 protein; DJ Sexton, BF Kaboord, AJ Berdis, TE Carver, SJ Benkovic, unpublished data); [19••]).

Although it has not been directly demonstrated, the clamp proteins probably confer enhanced stability upon the holoenzyme through their concatenation with DNA. Under certain conditions, a fraction of either the 45 protein or PCNA can be assembled on linear DNA substrates in the absence of the clamp-loader [20,21]. Stoichiometric clamp-loading on DNA, however, always requires the ATP-hydrolysis-coupled activity of a dedicated clamp-loader protein complex. Clamp-loaders from eukaryotes, *E. coli* and T4 are multi-protein ATPase complexes (Table 1). The T4 clamp-loader is comprised of one 62 subunit and four 44 subunits, which contain the ATP-binding sites [22,23]. Each 44/62 complex non-cooperatively binds four molecules of ATP [24••]. There appears to be some discrepancy as to the composition of the *E. coli* clamp-loader (DnaX or γ-complex), which varies from $\gamma_4\delta_1\delta'_1\chi_1\psi_1$ to $\gamma_2\delta_1\delta'_1\chi_1\psi_1$ [25,26]. The γ-subunit of the *E. coli* clamp-loader binds two molecules of ATP per complex, which is consistent with the latter composition [27]. Recently, the eukaryotic clamp-loader from both humans and yeast (RF-C) has been reconstituted from its five purified subunits [28,29].

While the exact functions of the individual clamp-loader subunits remain largely unknown, the overall role of the clamp-loader is to chaperone the clamp to the primer/template and catalyze ring opening. ATP binding invokes a conformational change in the 44/62 complex that allows the 62 component of the complex to interact with the 45 protein [23]. This interaction occurs even in the absence of DNA to produce a stoichiometric 45·44/62 complex [30••,31••]. A similar conformational change occurs in the *E. coli* γ-complex, which allows its δ protein component to interact with the β-clamp [32]. Indeed, cross-linking experiments have demonstrated the ability of the 44/62 complex to chaperone the 45 protein to the primer/template [33]. This chaperone activity is dependent upon ATP binding but not hydrolysis, as ATP-γ-S also mediates the interaction of the 44/62 protein with both DNA and the 45 protein [31••,33].

Table 1

Comparison of DNA replication systems.

Bacteriophage T4	*Escherichia coli*	Eukaryotes	Function
43 protein	DNA polymerase II	DNA polymerase α	Leading-strand DNA synthesis
43 protein	DNA polymerase III	DNA polymerase δ	Lagging-strand DNA synthesis
44/62 complex	Subcomplexes of γ, δ, δ′, χ, Ψ, τ subunits	RF-C complex	DNA-dependent ATPase; stimulates DNA polymerase
45 protein	dnaN protein (β subunit)	PCNA	Stimulates ATPase activity of 44/62, γ-complex of RF-C; increases processivity of DNA polymerase; enhances primer binding of DNA polymerase
61 protein	DnaG protein	pol α-primase	RNA-primer synthesis required for initiation of lagging-strand DNA synthesis
41 protein	DnaB protein	*Dna2* protein	Unwinds duplex DNA in 3′→5′ direction; ATP-dependent
32 protein	single-stranded binding protein	RF-A complex (=RP-A=SSB)	Single-stranded DNA binding protein; binds to DNA co-operativity

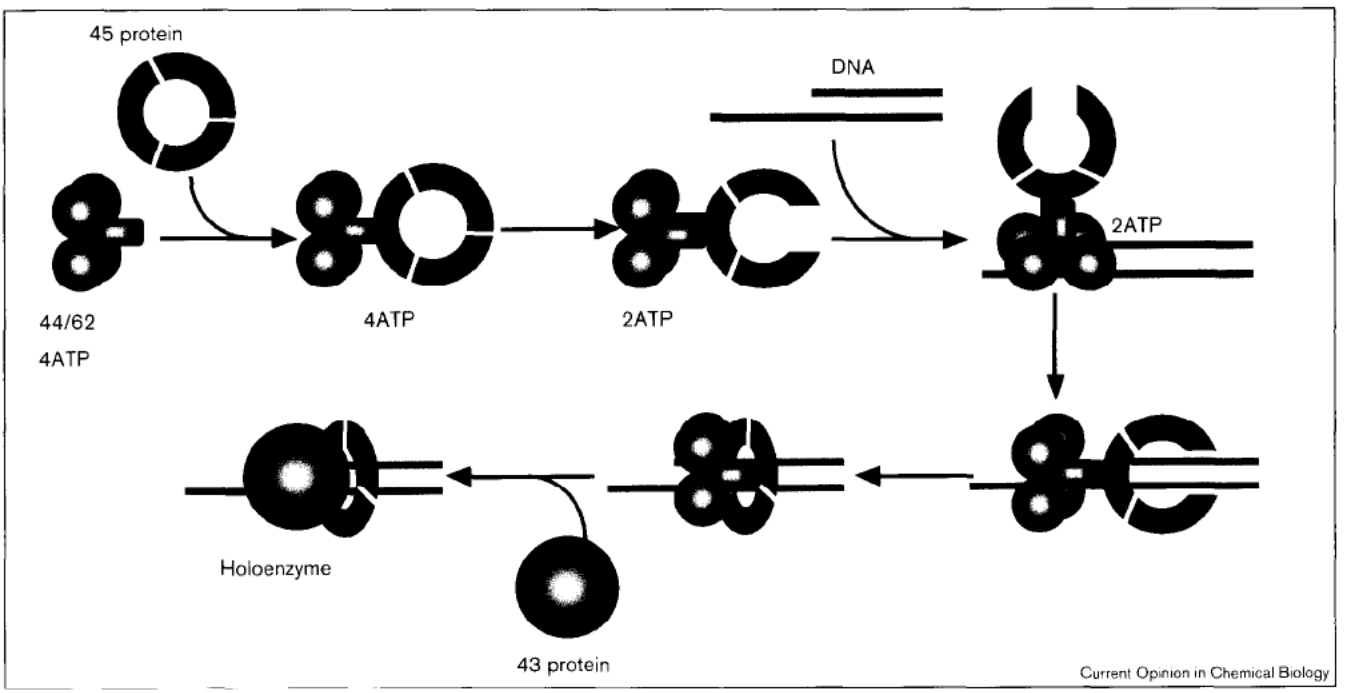
Although ATP binding to the clamp-loader is sufficient to enable initial association of the clamp with the primer template, ATP hydrolysis is required to load the 45 protein on the DNA strand, thereby stimulating T4 polymerase processivity [34,35]. Similar requirements for ATP hydrolysis hold for the *E. coli* and eukaryotic systems [36,37]. Presumably, ATP hydrolysis by the clamp-loader is coupled to a conformational change in the clamp protein, such as a ring-opening event. The mechanism whereby the energy of ATP hydrolysis is coupled to clamp-loading is not known, although a body of biochemical evidence suggests that the clamp-loader is a molecular motor with characteristics similar to the actin–myosin pair [38••].

For the T4 system, the effect of varying the order of holoenzyme component addition on the rate of holoenzyme formation indicates that clamp-loading occurs before polymerase association (DJ Sexton, BF Kaboord, AJ Berdis, TE Carver, SJ Benkovic, unpublished data). Pre-steady state analysis of the ATPase activity of the 44/62 clamp-loader revealed that all four bound ATP molecules are hydrolyzed during the clamp-loading process, with rate constants in the range 1–15 s^{-1} [24••,39••]. Furthermore, ATP hydrolysis appears to occur sequentially during clamp-loading: two molecules of ATP are consumed at a rate of ~14 s^{-1}, then another two with a rate of ~1 s^{-1} (Figure 2; DJ Sexton, BF Kaboord, AJ Berdis, TE Carver, SJ Benkovic, unpublished data). The lower pre-steady state rate of ATP hydrolysis is very similar to the rate of holoenzyme assembly (~1 s^{-1}), suggesting that ATP hydrolysis is somehow coupled to the rate-limiting assembly step [39••,40••].

As expected, the ATP hydrolysis-coupled events are accompanied by conformational changes in the 45 protein. Site-specific conjugation of the 45 protein to an environmentally sensitive fluorescent probe revealed an ATP-hydrolysis-dependent conformational change within the 45·44/62 complex that occurred at a rate comparable to that of ATP hydrolysis (~14 s^{-1};) [31••]. In the presence of DNA, several conformational changes were observed in the fluorescently labeled 45 protein, one of which occurred at the limiting rate for holoenzyme assembly (1 s^{-1}; DJ Sexton, BF Kaboord, AJ Berdis, TE Carver, SJ Benkovic, unpublished data). The rate-limiting step in clamp-loading in the *E. coli* system may also involve an ATP-hydrolysis-coupled conformational change [41••].

Once clamp-loading is complete, the T4 polymerase rapidly associates under diffusion control to form the holoenzyme, in which the 45 protein interacts with the carboxyl terminus of the polymerase (Figure 2);

Figure 2



Proposed mechanism of bacteriophage T4 DNA polymerase holoenzyme assembly. The ATP-bound 44/62 complex is shown interacting first with the 45 protein, then with DNA. Two of the four bound ATPs are hydrolyzed prior to interaction with DNA. The resulting 45·44/62·DNA complex undergoes a conformational change, yielding a DNA·45·44/62 complex, followed by hydrolysis of remaining bound ATP. After both sets of ATP are hydrolyzed, the 45·44/62·DNA complex is poised for rapid association with the T4 polymerase (the 43 protein) and departure of the catalytic 44/62 complex, thus completing holoenzyme formation.

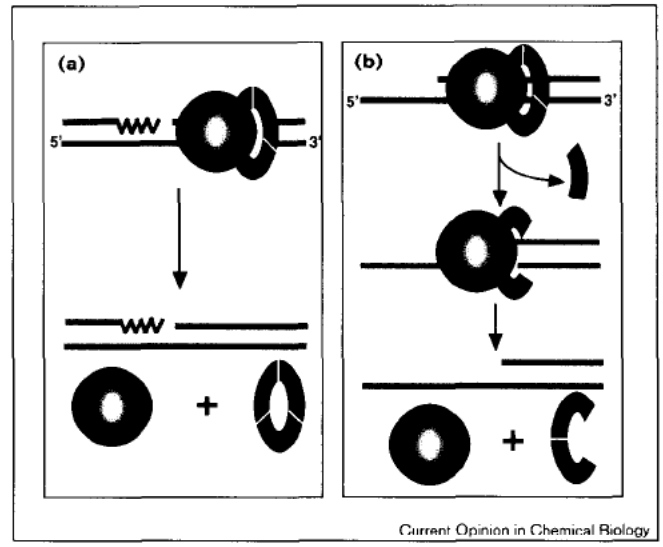
(DJ Sexton, BF Kaboord, AJ Berdis, TE Carver, SJ Benkovic, unpublished data) [42••]. The polymerase can conceivably interact with either a 45·DNA or a 45·44/62·DNA complex, depending on whether or not the clamp loader has already dissociated. The 45·44/62·DNA complex is probably preferred, as the 44/62 complex functions as a molecular matchmaker for the introduction of the T4 polymerase into the holoenzyme [43,44]. Because the T4 clamp is loaded onto DNA catalytically, the clamp loader is not part of the final leading-strand holoenzyme complex [39••,40••]. In the *E. coli* and eukaryotic systems, however, one clamp-loader remains associated between the leading- and lagging-strand holoenzyme dimer [26,45]. There are no reports for such an asymmetric complex in T4.

Holoenzyme disassembly

Although highly processive DNA synthesis is desirable for rapid completion of the leading strand, this mode of synthesis is not amenable for replication of the lagging strand. Specifically, the lagging-strand holoenzyme must be recycled after completion of one Okazaki fragment to move to the next fragment. This recycling problem can be alleviated by the proper disassembly of the lagging-strand holoenzyme, but this process must occur in a highly regulated fashion so as not to perturb continuous leading-strand DNA synthesis. Because ATP hydrolysis by the clamp loader is required for assembly of the holoenzyme, the clamp-loader should also participate in the ATP-dependent disassembly of the holoenzyme. However, the implication that this protein is not stably associated with the leading-strand holoenzyme means that the protein does not participate in disassembly, which must be mediated via an alternative mechanism.

In the search for a plausible mechanism of lagging-strand holoenzyme dissociation, kinetic experiments were performed that examined the stability of the holoenzyme complex once it encountered a completed Okazaki fragment (Figure 3a) [46]. Utilizing a defined primer/template designed to mimic an *in vivo* RNA primer, the kinetic consequence of the holoenzyme encountering this structure after processive DNA synthesis was examined by measuring the frequency at which the complex stalled or dissociated from the elongated DNA after encountering this structure. Measurements indicated that the holoenzyme preferred to dissociate upon encountering the hairpin rather than stall or perform strand-displacement synthesis, suggesting that the 5′-OH of the DNA segment is a signal that triggers the decomposition of the complex. To define further the molecular mechanism of this decomposition event, kinetic studies utilizing defined primer/templates containing either a DNA or RNA segment were recently performed (Figure 3a; TE Carver, DJ Sexton and SJ Benkovic, unpublished data). These studies not only confirmed the frequency at which the holoenzyme dissociates, but they also uniquely demonstrated that the rate of the holoenzyme decomposition is stimulated 30-fold by the presence of the RNA block ($k_{dis} = 0.3\ s^{-1}$ versus $k_{off} = 0.01\ s^{-1}$), as required by the discontinuous nature of lagging-strand synthesis. In addition, the rate of decomposition was independent of the presence of the 44/62 protein, consistent with its catalytic nature. Equally important, these measurements further demonstrated that the clamp and polymerase dissociate simultaneously.

Figure 3



Proposed mechanisms for bacteriophage T4 DNA polymerase holoenzyme dissociation. **(a)** Upon encountering a previously completed Okazaki fragment (wavy line), the lagging-strand holoenzyme and the clamp protein are both triggered for rapid dissociation. **(b)** The more stable leading-strand holoenzyme appears to dissociate via a subunit-exchange mechanism, whereby the loss of a subunit from the 45 protein leads to holoenzyme dissociation. Holoenzyme components are represented by the same symbols as in Figure 2.

Incorporation of the 45 protein into the leading-strand holoenzyme complex increases the stability of the 45 protein on DNA at least 100-fold (K_{off} decreases from $1\ s^{-1}$ to $<0.01\ s^{-1}$). One likely disassembly mechanism involves the loss of a subunit from the 45 protein within the holoenzyme complex (Figure 3b). Subunit exchange within the 45 protein has been measured using a variant of the 45 protein that facilitates observation of inter-subunit FRET (PS Soumillion, DJ Sexton, SJ Benkovic, unpublished data). Upon mixing this protein with a nonfluorescent form of the 45 protein that contains 4-fluorotryptophan, subunit exchange was observed to occur with a first order rate constant of about $0.01\ s^{-1}$, which is very similar to the rate of holoenzyme dissociation [31••,43]. The latter rate, which would apply to leading-strand synthesis, is sufficiently slow, given a rate of polymerization of approximately 500 nucleotides s^{-1}, to accomplish processive replication of the T4 genome during a single pass of the holoenzyme.

In contrast to the T4 system, the *E. coli* holoenzyme is able to quickly dissociate (k_{off} <1 s^{-1}) upon completion of DNA synthesis, during which only the polymerase dissociates, leaving the β subunit behind [47]. The greater stability of the β clamp on DNA has apparently necessitated a catalytic clamp-unloading activity that is accommodated by the *E. coli* γ complex [48]. Even more intriguing is the observation that dissociation of the β-clamp requires ATP binding to the γ complex but not ATP hydrolysis. It is likewise suggested that the dissociation rate of the eukaryotic holoenzyme is dictated by the release of the polymerase and not by PCNA [49]. The difference in decomposition of the holoenzymes suggests an inherent difference in recycling of the clamp, a difference that may impact on the coordination of leading- and lagging-strand DNA synthesis. It is unclear how ATP binding and hydrolysis by the clamp loaders are regulated with regards to assembly versus disassembly of the holoenzyme.

Conclusions

The mechanisms of bacteriophage T4 DNA polymerase holoenzyme assembly and disassembly have been well elucidated using a combination of kinetic and biophysical methodologies. The multitude of individual protein–protein and protein–DNA interactions, as well as internal protein conformational changes, confirms that DNA replication is indeed mediated by an elegant protein machine. Now that the replication components from many eukaryotes and various pathogens have been identified, further information on these replication systems requires detailed mechanistic analysis. The techniques used to study T4 assembly and disassembly mechanisms will prove useful when applied to other replication systems. Future challenges include dissection of the assembly/disassembly mechanisms of higher-ordered structures, such as the coordination of leading- and lagging-strand DNA synthesis, and the interactions that couple the holoenzyme to the helicase/primase [50,51•,52–54].

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