Recent advances in the development of single-molecule approaches have made it possible to study the dynamics of biomolecular systems in great detail. More recently, such tools have been applied to study the dynamic nature of large multi-protein complexes that support multiple enzymatic activities. In this review, we will discuss single-molecule studies of the replisome, the protein complex responsible for the coordinated replication of double-stranded DNA. In particular, we will focus on new insights obtained into the dynamic nature of the composition of the DNA-replication machinery and how the dynamic replacement of components plays a role in the regulation of the DNA-replication process.

Hylkje J. Geertsema and Antoine M. van Oijen

The power of single-molecule experiments lies in their ability to reveal the dynamic behavior of individual molecules and thereby obtain information on unsynchronized, stochastic events that otherwise would be hidden by ensemble averaging. In particular, single-molecule tools greatly facilitate our ability to study complexes consisting of multiple proteins by allowing the direct observation of dynamics of association and dissociation as well as the stoichiometry of protein components within a complex. Besides providing quantitative information on previously established molecular processes, the possibility to follow in real time multi-protein complexes as they undergo multiple sequential transitions has led to a number of novel mechanistic insights (68,69,70). In this review, we describe recent advances in single-molecule studies of one such multi-protein complex, the DNA replication machinery, and how these studies have revealed dynamic properties that might be widely shared with other multi-protein systems.

An intensively studied model system for DNA replication is the bacteriophage T7 replisome, mainly because of its relatively simple composition (Figure 9a) while displaying a remarkable resemblance to more complex replisomes of higher organisms (26,27). Replication of DNA is initiated by the unwinding of parental double-stranded DNA (dsDNA) by the helicase domain of the T7 gene 4 protein (gp4) (71,72), yielding two single-stranded DNA (ssDNA) templates. The ssDNA is covered by single-stranded binding protein (gp2.5), which plays a critical role in coordinating the intermolecular interactions between the replication proteins. After unwinding, DNA polymerases incorporate complementary nucleotides to each of the ssDNA templates. The T7 DNA polymerases consist each of a T7-encoded protein (gp5) and a processivity factor thioredoxin (trx) (73), which is provided by the host organism Escherichia coli. As is the case for all DNA polymerases, they can only support DNA synthesis in the 5’ to 3’ direction. Consequently, one of the strands, the so-called leading strand, can be copied continuously but the other strand, the lagging strand, is replicated in a discontinuous fashion. To allow synthesis of the lagging strand in a direction opposite to that of the leading strand, the lagging strand is hypothesized to form loops that permit the lagging-strand DNA polymerase to remain associated with the rest of the replication complex (74). The discontinuous replication of the lagging strand results in the synthesis of short fragments, Okazaki fragments, with a length of ~1 kb. Okazaki-fragment synthesis is initiated from short RNA primers that are synthesized by the primase domain of gp4.

Single-molecule experiments that relied on a real-time readout of the length of a hydrodynamically stretched DNA molecule (Figure 9b) resulted in the first direct, real-time observation of the dynamic nature of enzymatic processes that underlie DNA replication. For example, such experiments demonstrated that primer synthesis temporarily stalls the replisome to prevent leading-strand synthesis from outpacing lagging-strand synthesis (52) and visualized the formation and release of replication loops during coordinated leading- and lagging-strand synthesis (51) (Figure 9b and c). Previous biochemical studies suggested two possible mechanisms...
for replication loops to be released after the production of an Okazaki fragment. The so-called collision model describes the release of the polymerase from the lagging-strand when it encounters the 5' end of the previously synthesized Okazaki fragment, whereas the signaling mechanism will cause the replication loop to be released when a new primer is synthesized, even before the nascent Okazaki fragment is finished (51,75,76). After finishing an Okazaki fragment, the lagging-strand polymerase can be recycled to synthesize the next Okazaki fragment or dissociate from the replisome, in which case a new polymerase has to be recruited to synthesize the next Okazaki fragment. The single-molecule observation of loop lengths and pauses between loop-formation events allowed for a characterization of these release mechanisms in the context of a fully active replication complex. These studies resulted in a model that the active replisome employs both collision and signaling mechanisms, likely as a fail-safe method to ensure successful loop release.

Figure 9: a) Schematic view of the T7 bacteriophage replication machinery. b) Experimental design to visualize replication-loop formation (blue arrow) and release (red arrow) by the length changes of hydrodynamically stretched DNA molecules. c) Length changes over time of a single DNA molecule during replication. Loop formation and release are visible as a transient shortening of the flow-stretched DNA. (9a adapted from ref 26; 9b,c adapted from ref 51)
With such a complicated choreography of proteins at the replication fork, it is important to understand how the composition of the complex evolves over time. Numerous biochemical and biophysical studies have contributed tremendously to the detailed knowledge of the structure of replication proteins and the interactions between them (reviewed in 26,27,77), but a clear understanding of how these individual proteins interact dynamically is still largely lacking. Recent single-molecule experiments have begun to suggest a picture of a highly dynamic replisome that is characterized by a continuous exchange of components (78,79). These observations are emphasizing that the replisome does not have the static composition often suggested by the textbook pictures, but instead is a continuously changing complex, dynamically exchanging components while replicating DNA. In this review, we will focus on the highly dynamic exchange of polymerases at the replication fork, and argue that similar exchange mechanisms may be important to support subunit exchange in many other processes.

The precise nature of the molecular interactions between the DNA polymerases and the rest of the replisome is one of the important determinants in processes such as loop release and polymerase recycling. Biochemical studies revealed that the T7 DNA polymerases are physically tethered to the T7 gp4 helicase by two different binding interactions (31,37). During DNA synthesis, an interaction between the helicase and the palm domain of the polymerase keeps the polymerase tethered to the replisome with high affinity. Alternatively, a weaker, electrostatic interaction can be formed between the acidic and unstructured C-terminal tail of gp4 and a basic patch on the outside of the T7 polymerase. The latter interaction does not require a primer-template for the polymerase to be present. The availability of two interactions with different binding stability is hypothesized to mediate a switching mechanism that provides, on the one hand, stable binding of polymerases while replicating and, on the other, allows the polymerase to be tethered loosely to the replisome. Earlier biochemical experiments showed that even though T7 replisomes are highly resistant to dilution (46), leading- and lagging-strand polymerases showed rapid exchange when challenged with excess polymerase in solution (80). Based on these experiments and similar ones on the T4 replisome (81), a switching mechanism was hypothesized that would both confer high processivity to the polymerase via a stable conformation supporting synthesis and allow polymerase exchange via a more loose secondary interaction with the replisome.

The dynamic nature of these interactions between the polymerases and the helicase in the T7 system raises an immediate paradox. How can the replisome processively replicate thousands of basepairs, while polymerases are able to readily exchange? A recent study by Loparo et al. (48) utilized a novel single-molecule approach to address this question. By combining the observation of the DNA replication rate of a single T7 replisome with the visualization of the recruitment of individual fluorescently labeled DNA polymerases from solution to the complex, the authors showed that DNA polymerases bind to the helicase tens of seconds before they are
utilized in actual DNA synthesis. The authors showed that a polymerase is initially recruited via the helicase’s acidic C-terminal tail, followed by an exchange of the synthesizing polymerase by the newly recruited one. This exchange event is initiated by a transient release of the DNA synthesizing polymerase from the DNA template, providing all polymerases bound to the helicase an opportunity to compete for binding to the exposed primer-template substrate. This two-step mechanism potentially allows for up to six polymerases, associated with the six acidic helicase C-terminal tails, to be readily available to replace the DNA-synthesizing polymerase. In the absence of polymerases in solution, and thus in the absence of additional competing polymerases bound to the helicase C-termini, the synthesizing polymerase while still tethered to the helicase will simply rebind the primer very rapidly after dissociation. Thus, the replisome ensures high processivity even in the absence of excess polymerase.

Over the past years, both recruitment of more polymerases to the replisome than minimally necessary for coordinated DNA replication and rapid exchange of polymerases have been observed in other organisms, suggesting that the aforementioned exchange mechanism may be a general one. First, the leading- and lagging-strand polymerases of the bacteriophage T4 have been shown to form a homodimer (82), but both polymerases exchange within a minute when they are challenged by a mutant polymerase (81). The polymerase exchange mechanism is found to be induced by the attachment of an extra polymerase to the clamp, which competes for DNA binding with the replicating polymerase. In addition, the T4 replisome has also been observed to be able to bind up to three polymerases (83). These observations suggest that polymerase exchange within the T4 replisome may follow a similar exchange mechanism as has been reported for the T7 replisome. Second, polymerase exchange has also been observed for the bacterial replication machinery of *E. coli*, both *in vivo* and *in vitro*. In *E. coli*, polymerases are tethered to the replisome by the τ subunits of the clamp loader, which in turn connect to the helicase (DnaB) (84,85). A clamp loader can contain up to three τ subunits, each of which can bind a polymerase during DNA replication (86). Notably, recent single-molecule *in vivo* experiments have confirmed the biological relevance of having three polymerases at the replisome (87) and of frequent and dynamic polymerase exchange within the replisome in living *E. coli* cells (50).

The biological advantage of the presence of a ‘backup’ polymerase may be most apparent when the replisome encounters a lesion in either the leading- or lagging-strand template, when the lagging-strand polymerase is left behind due to premature stalling of Okazaki-fragment synthesis, or generally when one of the polymerases loses functionality. The *E. coli* replication machinery overcomes lesions in the DNA template by either bypassing the lesion by generating a downstream primer on the leading-strand from which processive DNA replication can restart (88) or repairing the lesion by rapidly exchanging the high-fidelity polymerase III for the low-fidelity lesion bypass polymerase IV. The rapid polymerase exchange is
regulated by the β sliding clamp, which can bind both polymerases simultaneously (89). The presence of an extra polymerase at the replication fork may provide the opportunity to quickly load a polymerase on the downstream primer to continue leading-strand synthesis as well as the possibility to exchange a processive polymerase by an error-prone polymerase to fix lesions. Additionally, single-molecule measurements demonstrate that replisomes containing three polymerases are more efficient in nucleotide incorporation and generate shorter ssDNA gaps in the lagging-strand than dual-polymerase replisomes (49). Dohrmann et al. (90) recently showed that the dissociation rate of a single polymerase from the 3’ end of a DNA template is too slow to successfully recycle lagging-strand polymerases. Taken together, these studies strongly suggest a picture in which the third polymerase binds a newly formed primer to start synthesis of the next Okazaki fragment, such that the former lagging-strand polymerase can finish the Okazaki fragment, possibly by being released from the replisome, or has enough time to be recycled to the second-next Okazaki fragment.

In this review, we have focused on the molecular mechanisms underlying stable binding and high processivity of polymerases at the replisome while allowing rapid exchange. Interestingly, recent single-molecule experiments have revealed similar dynamic subunit exchange mechanisms in other multi-protein complexes. FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) experiments demonstrated that the MotB and FliM subunits of the bacterial flagellar motor are exchanged in a signal-dependent way (91,92). In addition, the structural maintenance of chromosome proteins of E.coli, MukBEF, was determined to form multimers that can exchange single MukBEF complexes with freely diffusing complexes in the cytosol (93,94). The multimeric form of MukBEF is proposed to allow the release of one DNA segment without releasing the whole multimeric complex from the chromosome. The observation of multiple examples of stable multi-protein complexes that can readily exchange subunits suggests a more general picture of multi-protein complex that are able to dynamically exchange components with spare parts available in solution while maintaining high stability in the absence thereof.

We hypothesize that the dynamic exchange of subunits within multi-protein complexes while maintaining its overall composition and structure is a widespread mechanism. Stable binding of subunits while allowing rapid exchange is made possible by the presence of multiple sites of interaction between the subunit and the rest of the complex. Such a stable binding would be mediated by binding a subunit at two sides simultaneously, while a transient disruption of one of the two interactions would not immediately result in dissociation of this subunit but would allow, however, a second subunit from solution to bind at the vacated binding site. The presence of two subunits would then be quickly resolved by one of them competitively displacing the other altogether. Such a mechanism could be compared to two monkeys reaching for the same hand of bananas while swinging from a tree.
branch. A monkey holding the branch can stably grab the bananas, but a transient release will allow another monkey, associated with a second binding site, to join in and potentially take over the desired bananas (Figure 10, left). However, the ability to stably hold on to the branch allows the monkey to keep its position, close to the bananas. In the absence of a competing monkey, the secondary binding site allows the monkey to quickly take hold again of the bananas after having released them (Figure 10, right). Single-molecule techniques are very well-suited to characterize such exchange mechanisms at the molecular scale. The technical advances in these methodologies and our increasing understanding of the molecular details of the interactions involved place us in an ideal situation to further study and understand such molecular gymnastics with unprecedented dynamic detail.

Figure 10: Schematic representation of entities competing for a single preferential binding site. On the left, competition of two monkeys for the same preferential binding site (a hand of bananas). Transient dissociation from the bananas by the monkey allows the left monkey to compete for the same bananas. On the right, with just one monkey present, temporary unbinding from the bananas still allows a rapid re-association by the same monkey.