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Critical Reviews in Biochemistry and Molecular Biology

ISSN: 1040-9238 (Print) 1549-7798 (Online) Journal homepage: http://www.tandfonline.com/loi/ibmg20

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To cite this article: Bo Sun & Michelle D. Wang (2015): Single-molecule perspectives on helicase mechanisms and functions, Critical Reviews in Biochemistry and Molecular Biology, DOI: 10.3109/10409238.2015.1102195

To link to this article: http://dx.doi.org/10.3109/10409238.2015.1102195


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Critical Reviews in Biochemistry and Molecular Biology

http://informahealthcare.com/bmg
ISSN: 1040-9238 (print), 1549-7798 (electronic)
Editor: Michael M. Cox
Crit Rev Biochem Mol Biol, Early Online: 1–11
© 2015 Taylor & Francis. DOI: 10.3109/10409238.2015.1102195

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REVIEW ARTICLE

Single-molecule perspectives on helicase mechanisms and functions

Bo Sun[1,2,3] and Michelle D. Wang[1,2]

[1]Laboratory of Atomic and Solid State Physics, Department of Physics, Cornell University, Ithaca, NY, USA, [2]Howard Hughes Medical Institute, Cornell University, Ithaca, NY, USA, and [3]School of Life Science and Technology, ShanghaiTech University, Shanghai, P.R. China

Abstract

Helicases are a diverse group of molecular motors that utilize energy derived from the hydrolysis of nucleoside triphosphates (NTPs) to unwind and translocate along nucleic acids. These enzymes play critical roles in nearly all aspects of nucleic acid metabolism, and consequently, a detailed understanding of helicase mechanisms at the molecular level is essential. Over the past few decades, single-molecule techniques, such as optical tweezers, magnetic tweezers, laminar flow, fluorescence resonance energy transfer (FRET), and DNA curtains, have proved to be powerful tools to investigate the functional properties of both DNA and RNA helicases. These approaches allow researchers to manipulate single helicase molecules, perturb their free energy landscape to probe the chemo-mechanical activities of these motors, and to detect the conformational changes of helicases during unwinding. Furthermore, these techniques also provide the capability to distinguish helicase heterogeneity and monitor helicase motion at nanometer spatial and millisecond temporal resolutions, ultimately providing new insights into the mechanisms that could not be resolved by ensemble assays. This review outlines the single-molecule techniques that have been utilized for measurements of helicase activities and discusses helicase mechanisms with a focus on functional and mechanistic insights revealed through single-molecule investigations in the past five years.

Keywords

Helicase, replication, single-molecule studies, translocation, unwinding mechanisms

History

Received 7 June 2015
Revised 27 September 2015
Accepted 28 September 2015
Published online 4 November 2015

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Introduction

Nucleic acid metabolic processes, such as replication, repair, recombination and transcription, require the duplex form of DNA or RNA to be unwound transiently to single-stranded (ss) intermediates (Delagoutte & von Hippel, 2003). Helicases are motor enzymes that use the chemical energy from nucleoside triphosphate (NTP) binding and hydrolysis to catalyze this strand separation (Jankowsky *et al.*, 2010; Lohman & Bjornson, 1996; Spies, 2013). In addition, some helicases are also known to act in reverse by rewinding nucleic acids, and rewinding may play a role in fork regression during DNA repair, transcription and telomere metabolism (Wu, 2012). Furthermore, helicases, as nucleic acid translocases, have recently been found to function in other biological processes, such as protein displacement from nucleic acids and structural rearrangement of nucleic acids and protein complexes (Lohman *et al.*, 2008; Patel & Picha, 2000; Pyle, 2008; Singleton *et al.*, 2007). Therefore, it is not surprising that defects in helicases that have been reported to result in several human diseases, including Bloom, Werner and Rothmund-Thomson syndromes (van Brabant *et al.*, 2000). Thus, a complete understanding of helicase mechanisms and functions is essential for deciphering their roles in fundamental metabolic processes.

Helicases are ubiquitous and have been identified in various organisms, ranging from viruses to eukaryotes. On the basis of their primary structures, they have been classified into six superfamilies (SF1–SF6) (Patel & Picha, 2000; Singleton *et al.*, 2007). Those belonging to SF1 and SF2 generally act as monomers or dimers on DNA or RNA substrates, whereas most of the SF3–SF6 helicases form ring-shaped hexameric structures that encircle the nucleic acid and function mainly in DNA replication. This review will focus on discussion of DNA/RNA helicases.

Ensemble studies have contributed tremendously to elucidating helicase functions and mechanisms, such as helicase substrate specificity, translocation directionality and kinetic parameters. However, these studies characterize the average molecular population, and have limited ability in detecting intermediate states or distinguishing heterogeneities of motor behavior. Over the past few decades, single-molecule techniques have proven to be exceedingly powerful in addressing this knowledge gap (Ha *et al.*, 2012; Lionnet *et al.*, 2006; Yodh *et al.*, 2010). These approaches allow researchers to study molecular motors on a DNA or RNA substrate one at a time, providing detailed, and often surprising, views of these motors in action. Moreover, single-molecule methods are also capable of resolving helicase motion at nanometer spatial and millisecond

Address for correspondence: Michelle D. Wang, Laboratory of Atomic and Solid State Physics, Department of Physics, Cornell University, Ithaca, NY, USA and Howard Hughes Medical Institute, Cornell University, Ithaca, NY, USA E-mail: mwang@physics.cornell.edu

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temporal resolution and correlating it with its conformational states, thus generating a more comprehensive understanding of helicase mechanisms and their corresponding functions *in vivo*. In this review, we first detail the major single-molecule approaches, and then highlight examples of insights into helicase mechanisms and functions obtained using these methods in recent years.

Single-molecule techniques for helicase studies

Single-molecule techniques that have been employed to study helicases can be roughly divided into two broad categories: manipulation and visualization. Manipulation methods, such as optical tweezers and magnetic tweezers, manipulate and measure helicase motion under application of an external mechanical force on a substrate. Visualization methods, including fluorescence resonance energy transfer (FRET) and DNA curtains, use fluorescence to visualize motions of labeled molecules of interest. Recently, techniques from these two categories have also been successfully combined and these ''hybrid'' methods add a new capacity to investigate helicase mechanisms. In this section, we will summarize these method categories and discuss their principles and capabilities.

Manipulation techniques

Helicases uses chemical energies derived from NTP or dNTP hydrolysis to unwind double-stranded (ds) nucleic acids, as the double stranded structure represents a barrier for helicase forward translocation. The extent of the barrier may be estimated by the force required to mechanically unwind or unzip dsDNA, in the absence of a helicase, via separation of the two complementary strands beginning at one end of the dsDNA. This unzipping force is generally in the range of 10–18 pN under physiological conditions, though it is DNA sequence-dependent, with GC rich regions requiring a higher force to unzip over AT rich regions (Essevaz-Roulet *et al.*, 1997; Huguet *et al.*, 2010). Single-molecule manipulation techniques, such as optical trapping and magnetic tweezers, are capable of applying forces from 0.1 to 100 pN and have been employed to influence helicase activity, ultimately providing information on the kinetics and thermodynamics of helicase processes.

Here, we will briefly summarize these techniques with DNA as substrates, however they also apply to RNA substrates. Several experimental configurations have been adopted to apply a force on a DNA substrate to monitor helicase unwinding (Figure 1A): (1) A commonly employed configuration requires that the two strands from one end of the dsDNA be tethered to two separate surfaces, one single-stranded end to each surface, and the positions of these surfaces can, in turn, be controlled and measured (Johnson *et al.*, 2007; Lionnet *et al.*, 2007; Patel *et al.*, 2011; Ribeck & Saleh, 2013). For each base pair unwound by the helicase, two nucleotides are released, contributing to the DNA end-to-end extension. Thus, the helicase translocation at the fork may be monitored by the extension increase, typically carried out when the DNA tether is held under constant force. Under the force typically used (6–12 pN), this corresponds to $\sim$1 nm increase in extension for each base pair unwound. (2) In an alternate configuration, a template is double stranded at one end and single stranded at the other end, with each end anchored to a separate surface (Dessinges *et al.*, 2004). Helicase unwinding starts at the junction of dsDNA to ssDNA, and will convert one base pair of dsDNA to one nucleotide of ssDNA in the DNA extension. In this case, helicase unwinding may still result in a DNA extension change, but the direction of the change depends on the force on the DNA, with a decrease in extension at $<$6 pN and an increase at $>$6 pN (Bustamante *et al.*, 2003; Lionnet *et al.*, 2006). The sensitivity of the extension to helicase unwinding is typically $<$0.2 nm of extension change for each base pair unwound. (3) A third configuration uses dsDNA tethered between a surface at one end and the helicase itself anchored to another surface at the other end. Helicase translocation may be monitored by the length of the dsDNA unwound (Perkins *et al.*, 2004). Helicase unwinding will yield $\sim$0.3 nm of extension change for each base pair unwound.

Figure 1(B) shows an example of the use of an optical trap, a versatile and commonly used manipulation technique, to investigate helicase unwinding (Johnson *et al.*, 2007). An optical trap is generated by using a high-numerical aperture microscope objective to tightly focus a laser beam to a diffraction limited beam waist. The trap center is located near the beam waist and the gradient of the intensity provides the trapping force. An optical trap can be used to manipulate a trapped dielectric microsphere, whose position in the trap, as well as the force exerted by the trap, are both monitored in real time. During a helicase unwinding experiment, the force on the ssDNA is often held constant, by modulation of the coverslip position to feedback on the force, and the position of the helicase on the DNA template may be obtained from the length of the ssDNA.

An optical trap also offers flexible control of both force and extension of the substrate, enabling rapid switching between different modes of operation. Data acquisition rates in the tens of kilohertz range can be achieved by monitoring the transmitted laser beam with a photodiode, allowing detection of fast dynamics of the biological systems. In addition, dual optical traps can be utilized, reducing the instrument noise by isolating the measurements from laser beam and sample chamber drifts and thus achieving base pair (bp) spatial resolution that is well suited for fine measurements such as step sizes (discussed below).

Figure 1(C) shows another commonly employed manipulation technique, the magnetic tweezers, to investigate helicase unwinding (Dessinges *et al.*, 2004; Lionnet *et al.*, 2007; Sun *et al.*, 2008). In this example, one of the ssDNA strands is attached to a magnetic bead that experiences a force due to the magnetic field gradient from external magnets. DNA unwinding is monitored by the displacement of the magnetic bead, whose position may be determined to nanometer precision via the diffraction pattern of the bead image. Unlike an optical trap, magnetic tweezers naturally maintain a constant force on the DNA and can potentially be used to monitor multiple molecules simultaneously (De Vlaminck & Dekker, 2012).

DNA may also be stretched by fluid flow to monitor helicase unwinding, as shown in Figure 1(D) (Hamdan *et al.*, 2009; Lee *et al.*, 2006). For efficient stretching, a microsphere

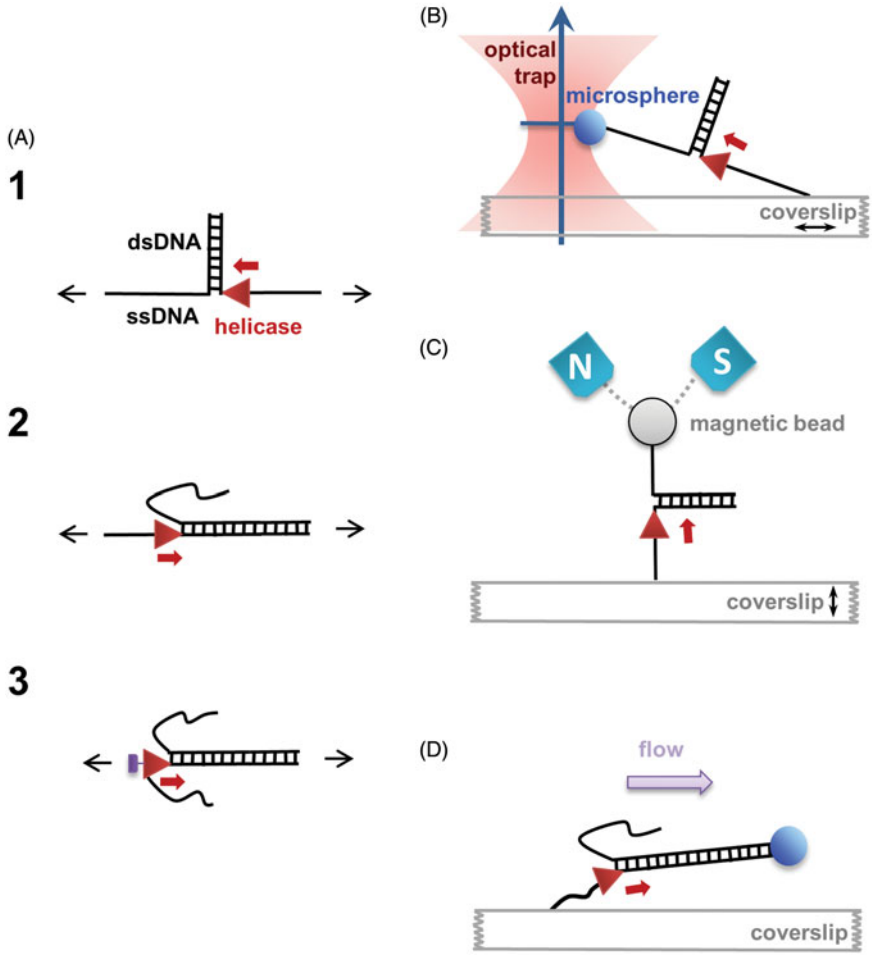


Figure 1. Single molecule mechanical manipulation methods. (A) DNA template configurations. A DNA template is held under tension between (1) both of the single strand ends (top panel), (2) a single strand end and the double strand end (middle panel) or (3) a helicase and DNA (bottom). Helicase unwinding results in a change in DNA held under tension. (B) Optical tweezers. This cartoon illustrates an example of the experimental configuration. One strand of a dsDNA molecule is attached to a μm-sized microsphere held in the optical trap for manipulation and measurement, while the other strand is anchored to a microscope coverslip surface. Helicase unwinding increases the number of single stranded nucleotides held under tension. (C) Magnetic tweezers. This cartoon illustrates an example of the experimental configuration, which is similar to that of the optical tweezers, except that the force on the magnetic bead is generated by a magnetic field. (D) Flow stretch. Laminar flow may also be used to apply a low force on a microsphere attached to a dsDNA that is unwound by a helicase, while one strand of the DNA is attached to the coverslip. Helicase unwinding of the fork converts dsDNA to ssDNA held under tension. (see colour version of this figure at www.informahealthcare.com/bmg).

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is attached to the end of the DNA. This increases the viscous drag force and the position of the microsphere is monitored via video-tracking. Although the resolution of the DNA extension is limited to a few hundred nanometers, the strengths of this method are the ease of implementation and its capability to take parallel measurements of multiple single molecules (Hamdan *et al.*, 2009; Lee *et al.*, 2006).

Atomic force microscopy (AFM) is another valuable single-molecule manipulation technique, though less commonly employed for helicase studies. AFM uses a cantilever to apply a force to the molecule of interest with the force detected by the deflection of the cantilever. Because of the high stiffness of cantilever, AFM is more suited for applications of larger forces, typically in the range of 10–10 000 pN. To detect helicase unwinding with AFM, a DNA or RNA molecule may be tethered between a surface and a cantilever (Fisher *et al.*, 2000). This approach has been employed to measure the force that helicase generates (Marsden *et al.*, 2006), but has limited applications to kinetic studies for helicases due to its force range and resolution.

Visualization techniques

Several techniques have also been developed to visualize helicase activities by fluorescently labeling either the helicase or the DNA substrate. These techniques complement the manipulation techniques and offer unique insights to helicase mechanisms.

Single-molecule FRET is a powerful technique that can monitor the nanometer scale motions of a helicase. In the example shown in Figure 2(A) (left), the two strands of dsDNA are separately labeled, by either a donor or acceptor dye, and the FRET signal provides a sensitive indicator of the distance between the donor and the acceptor in the range of 1–5 nm (Ha *et al.*, 2002). Helicase unwinding of the dsDNA induces a change in the distance between the donor and acceptor dyes, and thus a FRET signal change. Instead of labeling the DNA, different domains of a helicase may also be labeled with a donor and acceptor dye, allowing the investigation of helicase conformational changes (Figure 2A, right) (Myong *et al.*, 2005). Alternatively, helicase and DNA substrate may both be labeled so that the helicase's position on the template may be determined (Honda *et al.*, 2009; Wickersham *et al.*, 2010).

Another approach, termed protein-induced fluorescence enhancement (PIFE), allows for circumvention of helicase labeling. In PIFE, the intensity of a single fluorophore attached to the substrate is enhanced in the vicinity of a bound helicase (Hwang *et al.*, 2011). Unlike FRET, PIFE employs only a single dye as a reporter of the protein binding and its movement. Similarly, distance-dependent fluorescence quenching from a single dye mediated by an iron–sulfur cluster of helicases has also been employed in monitoring helicase activities (Ghoneim & Spies, 2014; Honda *et al.*, 2009). These high-resolution, high-throughput techniques inherently endure environmental noise as they report on the

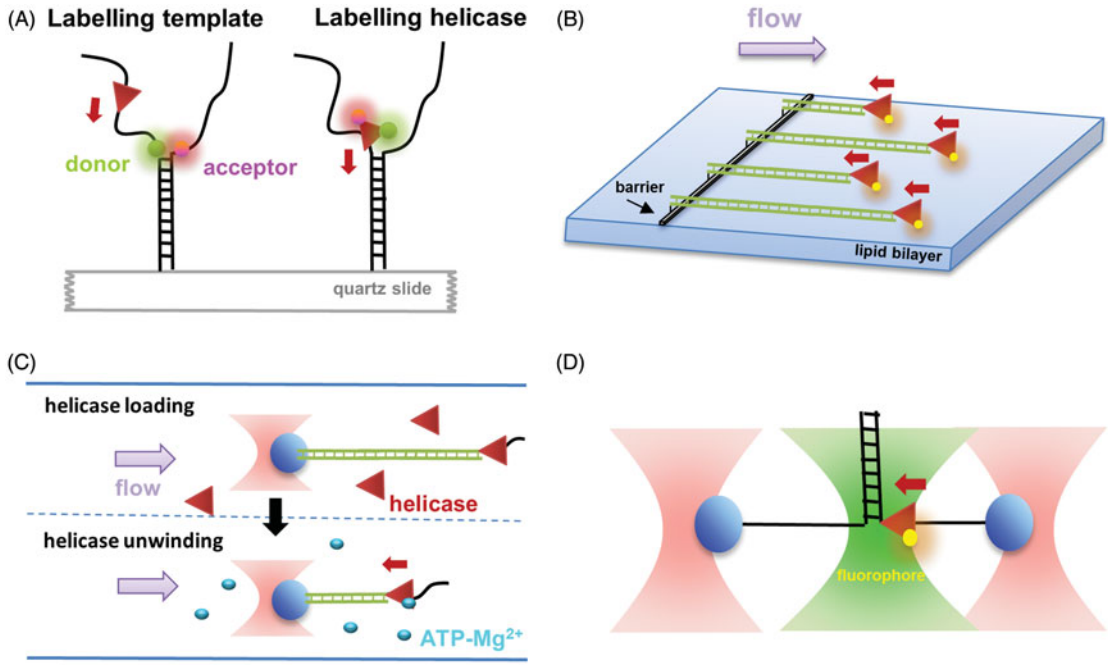


Figure 2. Single molecule visualization methods. (A) FRET. This cartoon shows an example of the use of FRET to detect helicase unwinding. Donor and acceptor fluorophores are attached to the two strands of dsDNA to be unwound (left). Helicase unwinding leads to a distance change between the donor and acceptor and thus a change in the FRET efficiency. Alternatively, different domains of a helicase may be labeled with a donor and an acceptor (right) to monitor helicase conformational changes during unwinding. (B) DNA curtains. An array of DNA molecules is aligned by a barrier in a lipid bilayer, while laminar flow stretches the molecules away from the barrier. DNA unwinding can be monitored as shortening in the dsDNA or as the movement of labeled helicase at the forks. (C) ''Visual biochemistry'' setup. A fluorescently labeled dsDNA to be unwound is attached to a microsphere held in an optical trap and stretched via a laminar flow force. Helicase can be loaded onto the DNA in one laminar flow channel and moved to another channel to start unwinding. (D) Dual optical trap with fluorescence. A DNA molecule is suspended between two microspheres, each of which is held in an optical trap. A confocal laser (green) allows visualization of fluorophore-labeled helicase at the fork (see color version of this figure at www.informahealthcare.com/bmg).

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relative distances between two fluorophores and are able to directly identify short-lived states of helicases.

High throughput visualization of helicase activities may also be attained with DNA curtains (Collins *et al.*, 2014; Finkelstein *et al.*, 2010; Greene *et al.*, 2010). Figure 2(B) shows that fluorescently labeled DNA substrates anchored on the lipid bilayer, driven by flow, will align with each other on the leading edges of a nanofabricated surface pattern. It is possible to observe hundreds of DNA molecules, in real time, under total internal reflection fluorescence. With this method, helicase unwinding is revealed by the disappearance of dsDNA.

Visualization techniques may be greatly enhanced when complemented with optical trapping and a multi-channel laminar flow cell, allowing for rapid exchange of chemical environments (Bianco *et al.*, 2001). Figure 2(C) shows an example of the use of such an approach to visualize the progression of helicase unwinding. Here an optical trap is used to manipulate fluorescently labeled DNA substrates through flow channels containing different chemical reagents or proteins, and the solution flow extends the DNA molecule and allows for visualization by fluorescent microscopy. Helicase unwinding may be directly visualized in real time as a decrease in the length of dsDNA. These combined features make this approach a powerful single molecule tool, which has been coined as ''visual biochemistry''.

When both ends of a DNA substrate are held in optical traps (dual trap) as shown in Figure 2(D), the fork dynamics can be monitored at high resolution while still permitting visualization of helicase on DNA (Comstock *et al.*, 2015). This approach allows for concurrent measurements of subnanometer-scale mechanical motions at the fork and detection of helicase conformational dynamics.

Both manipulation and visualization techniques offer advantages and challenges for experiments. Ultimately, the need to address specific helicase questions, and the capability of a specific method to do so, should determine the best suited technique.

Novel insights on helicase mechanisms and functions through single-molecule approaches

Single molecule techniques have offered new experimental approaches to investigate controversial and complex questions that are difficult or impossible to address using ensemble biochemical approaches. Below, we will highlight a few recent examples where single molecule approaches have brought unique and important insights into the mechanisms of helicases.

Helicase loading

The very first step in helicase activity is properly loading onto a nucleic acid template. This step is essential for helicase function and frequently regulates subsequent metabolic processes. Accessory loading proteins are often required and loading intermediates are often short-lived (Bell & Kaguni, 2013; O'Shea & Berger, 2014). For example, loading of eukaryotic replicative DNA helicase not only licenses origins

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of replication, but also directs the recruitment of the rest of the replication machinery (Yeeles *et al.*, 2015). Single-molecule imaging techniques are capable of detecting helicase loading intermediates and also allow stoichiometric determination of the protein composition, making significant contributions in characterizing helicase-loading mechanisms (Graham *et al.*, 2011; Phelps *et al.*, 2013).

As an example, in eukaryotes, MCM2–7 (an AAA + family of ATPases) serves as the principal replicative helicase and its loading requires three loading factors: the origin recognition complex (ORC), Cdc6 and Cdt1 (Yardimci & Walter, 2014). These factors assemble two MCM2–7 complexes into a head-to-head double hexamer before replication initiation. Although previous studies determined the structure of the double-hexamer, fundamental questions remained: (1) Do two MCM2–7 hexamers load simultaneously or sequentially? (2) How many copies of ORC and Cdc6 proteins are required to load two MCM2–7 hexamers? (3) Do the two MCM hexamers employ similar mechanisms to load?

Recently, Ticau *et al.* developed single-molecule loading assays with recombinant yeast proteins to address these questions (Ticau *et al.*, 2015). In this approach, both proteins and DNA were fluorescently labeled and protein loading and unloading on DNA were monitored in real time by co-localizing the fluorescence signals in which the helicase-loading intermediates were captured. They ultimately showed that distinct Cdc6 and Cdt1 molecules direct the loading of the two MCM2–7 hexamers in a sequential manner, in agreement with previous models (Fernandez-Cid *et al.*, 2013; Sun *et al.*, 2013, 2014). However, they also found that only one ORC molecule is required in the loading process. By combining their single molecule technique with FRET, they also demonstrated that two MCM2–7 hexamers load via distinct mechanisms, and the recruitment of the second hexamer requires interactions with the first MCM2–7, instead of ORC. This loading mechanism ensures that the two MCM2–7 hexamers assemble in a head-to-head orientation, which facilitates bidirectional replication initiation. A separate study by Duzdevich *et al.* using single-molecule DNA curtain confirmed these results and further investigated the regulation of Cdc6 on ORC binding, as well as the replisome firing in real time (Duzdevich *et al.*, 2015). These studies illustrate the power of single-molecule imaging techniques in addressing questions of stoichiometry and dynamics during helicase loading and have led to important insights into the eukaryotic helicase-loading mechanisms.

Step size

During translocation and unwinding, helicase motors consume chemical energy to translocate in a stepwise fashion along a DNA or RNA lattice. Thus, to have a complete understanding of the unwinding mechanism, it is essential to determine basic parameters, such as step sizes, of this stepping process. Step sizes can be defined in several ways, based on the type of measurement employed. A ''mechanical or physical step size'' is typically measured under a limited NTP concentration such that the forward translocation of a motor is limited by the binding of the next incoming NTP and the motor pauses between two binding events. The step size is then defined as the average number of base pairs/nucleotides that a motor unwinds/translocates on the nucleic acid lattice between two adjacent pause sites. Conversely, a ''kinetic step size'' is defined as the average base pairs/nucleotides unwound/translocated between two successive rate-limiting kinetic steps measured typically under normal NTP concentrations (Lohman *et al.*, 2008). The physical and kinetic step sizes may be different, as the rate-limiting kinetic step could occur several times in one NTP hydrolysis cycle or one time in several NTP hydrolysis cycles. Both physical and kinetic step sizes that a helicase motor takes are critical parameters, but their determination has been well recognized as a challenging technical endeavor. The minute nature of step sizes, likely on the order of one or a few nucleotides, requires an ultra-stable, exceedingly high resolution instrument for detection. Single-molecule methods, such as optical tweezers and FRET, allow for measurements of both kinetic and physical step sizes.

Take the hepatitis C virus helicase NS3, a representative SF-2 helicase essential for viral replication (Lam & Frick, 2006), as an example. Earlier single-molecule studies suggested a physical step size of 11 bp, each consisting of substeps of 3–4 bp (Dumont *et al.*, 2006). Discernment of finer steps was limited, at the time, by the resolution of the instrument, although single molecule FRET studies suggested that the 3-bp steps were, in fact, composed of even smaller steps of 1 bp (Myong *et al.*, 2007). Cheng *et al.* then took on the challenge to directly measure the physical step size of NS3 helicase using ultra-stable dual optical traps with angstrom-level resolution to follow NS3 unwinding of a single RNA hairpin (Cheng *et al.*, 2011). Single-base pair steps, independent of the mechanical force applied to the end of the hairpin were observed (Figure 3A). Surprisingly, additional step sizes of 0.5 bp increments (i.e. 1.5, 2.0, 2.5, etc.) were also reported. Cheng *et al.* proposed a model in which, with each ATP hydrolyzed, NS3 opens a single base pair at a time, but may release one nucleotide from one strand of the RNA while holding onto the other nucleotide from the other strand within itself. This asynchronous release of the two strands of RNA from the fork results in a 0.5-bp increment in step size. This proposed explanation is intriguing as crystal structural studies of NS3 suggest a 1-bp step size, and also show the possible sequestration of ssRNA within NS3.

Another SF2 helicase, XPD, was also found to take 1-bp physical steps. Qi *et al.* examined XPD unwinding and found that it took uniform 1-bp forward steps (Qi *et al.*, 2013), however, in reverse, XPD took 1- and 5-bp steps. They attributed the 1-bp reverse steps to helicase back stepping with the helicase remaining partially associated with the ssDNA, and the large 5-bp reverse steps to rearrangements of the translocating DNA strand within XPD.

Several SF1 helicases have also been reported to take 1-nt step sizes. The kinetic step size of SF1 helicase PcrA was first inferred from the crystal structure study to be 1 nt and was subsequently unambiguously demonstrated in both biochemical and single-molecule FRET studies (Dillingham *et al.*, 2000; Park *et al.*, 2010; Velankar *et al.*, 1999). Similarly, Pif1, another SF1 helicase, was also reported to translocate on ssDNA in a way consistent with 1-nt kinetic step size in FRET studies (Zhou *et al.*, 2014).

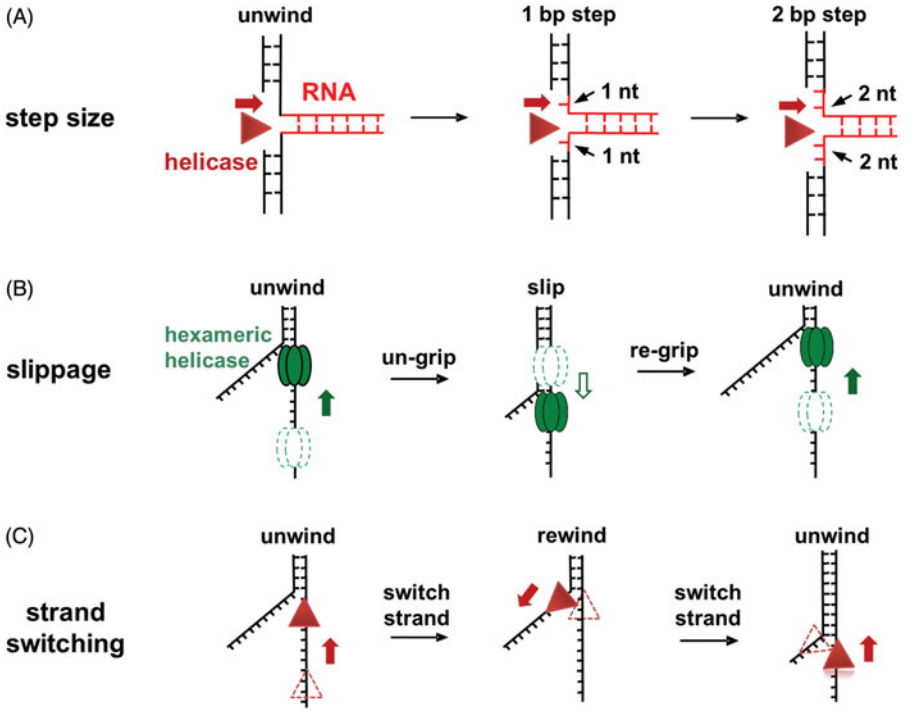


Figure 3. Helicase unwinding behaviors revealed by single-molecule techniques. (A) Step size. Helicase unwinds dsRNA one base pair at a time. The opening of each base pair generates two single-strand nucleotides. (B) Slippage. This cartoon illustrates helicase slippage behavior during unwinding. The helicase (green) unwinds, loses grip, slips, re-grips and resumes unwinding. Dotted helicase indicates a previous location of the helicase (see color version of this figure at www.informahealthcare.com/bmg). [Adapted from Sun *et al.* (2011) with permission.] (C) Strand switching. During unwinding, helicase may switch to the displaced strand, and its translocation on that strand results in helicase moving away from a re-annealing fork. Helicase may also switch back and resume unwinding. Dotted helicase indicates a previous location of the helicase. (see colour version of this figure at www.informahealthcare.com/bmg).

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Although 1-bp/nt step size appears to be characteristic of several non-hexameric helicases, this may not be the case for hexameric helicases. Single-molecule studies of T7 helicase suggest that the helicase unwinds dsDNA at discrete physical unwinding steps of 2–3 bp (Syed *et al.*, 2014).

These examples demonstrate that techniques with high spatial and temporal resolution, such as optical trapping and FRET, have enabled the resolution of elementary steps, and even substeps, of helicase translocation. This information, which had previously been difficult to access experimentally, combined with crystal structure data is essential to our understanding of helicase mechanisms.

Subunit coordination

Hexameric helicases are ubiquitous in organisms from phages to humans. The hexameric helicase of bacteriophage T7 is a model helicase for understanding how these types of helicases work to translocate along DNA and separate the strands of duplex DNA. Sun *et al.* investigated the unwinding mechanism of T7 helicase using a single-molecule optical trapping assay to monitor unwinding in real time (Sun *et al.*, 2011) (Figure 1B). Bulk studies had previously concluded that T7 helicase cannot unwind in the presence of ATP, preferring dTTP as a fuel source (Matson & Richardson, 1983). Surprisingly, they found that ATP, in fact, supported faster unwinding than dTTP, but unwinding was frequently interrupted by helicase slippage. During slippage the helicase loses grip of the ssDNA, without detachment from the ssDNA, slides in reverse direction along the ssDNA under the re-annealing stress of the DNA fork, and then regains its grip to the ssDNA and resumes unwinding (Figure 3B). These findings resolved the apparent lack of unwinding activity seen in ensemble studies with ATP – the frequent slippage prevents the helicase from traveling sufficient distance to be detected in a strand separation assay.

This discovery provided Sun *et al.* a window of opportunity to investigate how the hexamer's subunits coordinated their catalysis to maintain high unwinding processivity. Inspired by structural studies of hexameric ring-shaped helicases E1 (Enemark & Joshua-Tor, 2006) and Rho (Thomsen & Berger, 2009), they proposed a model for the T7 helicase that requires all, or nearly all, subunits of the T7 helicase coordinate their chemo-mechanical activities and DNA binding (Figure 4A). Although only one subunit at a time can accept a nucleotide, other subunits are nucleotide ligated and interact with DNA to ensure processivity. Combining both experimentation and theoretical modeling, they found that all, or nearly all, helicase subunits coordinate in both DNA binding and catalysis during unwinding. They speculated that slippage may provide an evolutionary advantage for replication: when dNTP concentrations are low, slippage can slow down helicase to allow its synchronization with a slow-moving DNA polymerase.

Thus far, T7 helicase is the only motor protein that has reported nucleotide-specific slippage behavior. Helicase slippage was also observed with other ring-shaped replicative helicases (Klaue *et al.*, 2013; Lee *et al.*, 2014; Manosas *et al.*, 2012b). However, these slippages appeared to be of different natures. The slippage behavior exhibited by both the T4 and XPD helicases occurred more often in GC-rich regions of DNA sequence (Manosas *et al.*, 2012b; Qi *et al.*, 2013). E1 unwinding occurs in a repetitive pattern in which E1 unwinds and backslips or rewinds, and then the helicase unwinds again (Lee *et al.*, 2014). It is unclear if E1 slippage depends on the type of nucleotides. NS3 unwound dsDNA in a repetitive shuttling manner where dsDNA was partially unwound and then re-annealed instantaneously, a process that was repeated many times before helicase dissociation (Myong *et al.*, 2007). This repetitive unwinding pattern was thought to arise from helicase maintaining contact with the end of one strand of

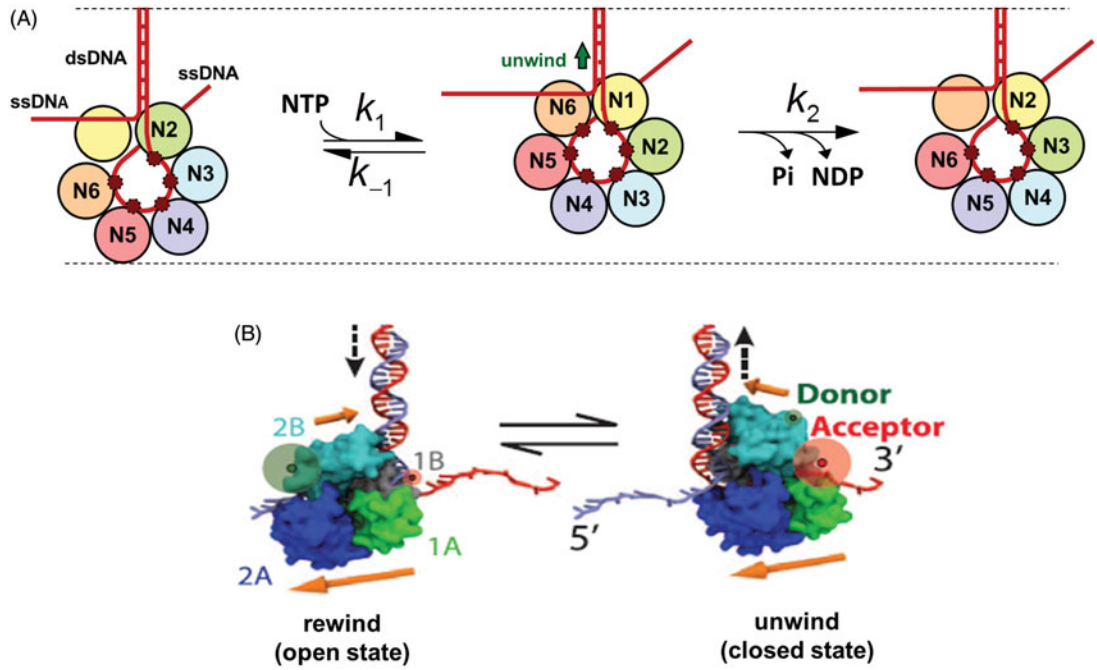


Figure 4. Conformational changes during unwinding. (A) Hexameric helicase subunit coordination. Each subunit is uniquely labeled with a different color and has a potential ssDNA-binding site (small dots). Nucleotide binding and subsequent hydrolysis occur sequentially around the ring. If a subunit is nucleotide-ligated (the state of hydrolysis indicated by N*i*), it has a non-zero probability of being bound to ssDNA. During unwinding, the leading subunit can bind to a nucleotide (N) and thus acquire affinity for the downstream ssDNA. This stimulates the last nucleotide-bound subunit to release its nucleotide and ssDNA. Then, the cycle proceeds again around the ring. Slippage occurs when all subunits simultaneously release ssDNA. [Adapted from Sun *et al.* (2011) with permission.] (B) Correlation between conformation states and unwinding activities. Non-ring-shaped UvrD helicase can exhibit two conformation states: ''open'' and ''closed'', as investigated by FRET using donor and acceptor labeled UvrD helicase. Monomeric UvrD unwinds dsDNA in the closed conformation, yet rewinds in the open conformation. [Adapted from Comstock *et al.* (2015) with permission.]. (see colour version of this figure at www.informahealthcare.com/bmg).

DNA during unwinding, and then snapping or slipping backwards rapidly before restarting the unwinding. Helicases, such as UvrD, RecQ, BLM, T4 and T7 helicases (Dessinges *et al.*, 2004; Johnson *et al.*, 2007; Klaue *et al.*, 2013; Lionnet *et al.*, 2007; Sun *et al.*, 2008; Wang *et al.*, 2015), have also been reported to switch strand during unwinding to translocate in a reverse direction, away from the DNA fork instead of moving toward it, such that a re-annealing fork follows behind the helicase (Figure 3C). The re-annealing process differs from helicase slippage in that helicase translocates on ssDNA instead of slipping along it.

Conformational dynamics

In contrast to ring-shaped hexameric helicases that function by coordination of six monomeric subunits, there have been conflicting *in vitro* reports about the oligomeric state of the non-ring-shaped SF1 and SF2 helicases. Several studies suggest that SF1 and SF2 helicases, such as Rep, PcrA and UvrD, translocate along ssDNA in a monomeric state, but unwind dsDNA only if two or more monomers are present (Lohman *et al.*, 2008; Sun *et al.*, 2008; Yang *et al.*, 2008). However, other studies propose that a helicase monomer can function as a processive unwinding motor on its own (Lee & Yang, 2006; Mechanic *et al.*, 1999). Take UvrD helicase as an example. It belongs to the SF1 family and consists of four domains: two RecA-like domains (1A and 2A) and two accessory domains (1B and 2B). A series of crystal structures of the *Escherichia coli* UvrD helicase complexed with DNA and ATP hydrolysis intermediates have been produced (Lee & Yang, 2006). These crystal structures suggest that monomeric UvrD-mediated DNA unwinding is achieved via directional rotation and translation of the DNA duplex in a combined wrench-and-inchworm mechanism (Lee & Yang, 2006). Furthermore, the 2B subdomain of UvrD can orientate in ''open'' and ''closed'' states, which is believed to be coupled to its function (Jia *et al.*, 2011).

Recently, Comstock *et al.* combined dual optical traps with single molecule FRET to simultaneously monitor unwinding and UvrD's conformational changes (Comstock *et al.*, 2015) (Figure 2D). They found that processive unwinding required two monomers which are referred to as a dimer, but a monomeric UvrD was only able to unwind 20 bp of dsDNA although it was a processive translocase on ssDNA. These results are consistent with earlier single-molecule studies (Lee *et al.*, 2013; Sun *et al.*, 2008; Yokota *et al.*, 2013). The remaining question is whether the second monomer simply pushes the first one or the monomers interact with each other and form a stable dimer during unwinding. Interestingly, they also uncovered two conformational states of a monomeric UvrD helicase, which correspond to ''open'' and ''closed'' states of the UvrD structure. The closed state supported helicase unwinding of dsDNA, whereas the open state supported rewinding (Figure 4B). These findings served as inspiration for the design of super-helicases of Rep and PcrA. By cross-linking 1B and 2B subunits of each helicase to lock it in the closed form, the helicase became more processive while working against opposing force (Arslan *et al.*, 2015). Taken together, these results underscore that helicase oligomeric and conformational states correlate with

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unwinding activities and functional mechanisms, and regulation of helicase-involved nucleic acid metabolism may be achieved via conformational control.

Unwinding heterogeneity

Traditional ensemble assays average molecular populations and may cancel out unsynchronized actions of individual helicases, obscuring potential behavioral heterogeneity. Single-molecule techniques have proven to be especially powerful for detecting helicase heterogeneity by examining the behavior of individual helicases.

It is well known that enzymatic activities of many motor proteins exhibit variability; however, it was unclear whether there are intrinsic differences from motor to motor, or whether each motor has the potential to sample the entire activity distribution given enough time. The answer to this question requires one to examine the ergodic hypothesis. That is, if all molecules are truly identical in their behaviors, then the behavior distribution of a single molecule observed over infinite amount of time should be identical to the behavior distribution from an infinite numbers of molecules taken at a snap shot. Experimentally, the behavior distribution from multiple molecules is attainable. However, it is challenging to monitor a single molecule over a sufficiently long time for it to reach an equilibrium condition.

A recent work by Liu *et al.* took on this challenge and brought the distinct advantages of single molecule studies to the forefront (Liu *et al.*, 2013). They addressed this question by examining unwinding of the *E. coli* RecBCD helicase. Helicase unwinding was detected by the shortening of a dsDNA template, which was extended using a combination of optical trap and laminar flow (Figure 2C). They measured unwinding rates from multiple helicase motors, and during the short period of measurement time, each motor assumed a well-defined speed. Together, these motor speeds formed a distribution that could be well described by two Gaussians representing a fast and slow species, and thus there appeared to be two distinct species of the motor. However, this was ultimately not true. They found a clever way to sample the speed of a single helicase motor, without the need to track its behavior over an infinite amount of time. They discovered that, by briefly depleting the ligand of the motor (Mg^{2+}-ATP), each motor could switch its speed to any other speed within the distribution. A single motor measured in this way yielded the same speed distribution as that from multiple motors. This provides strong evidence that these motors do not have intrinsic differences in speed and each motor has the ability to sample the entire distribution.

This discovery encourages us to revisit the causes of molecular heterogeneity that were often observed in single molecule studies. Heterogeneities might arise from thermal annealing or chemical refolding, but RecBCD's reported unwinding heterogeneities seem to originate from multiple conformations of different free energy states of the motor. Although Liu *et al.* switched the motor speed by the depletion of its ligand (Liu *et al.*, 2013), speed regulation is also known to occur via the recombination hotspot sequence χ, a recognition sequence at which RecBCD initiates homologous recombination and generates ssDNA (Dillingham & Kowalczykowski, 2008). Previous single molecule studies revealed that RecBCD moved processively at a very fast rate (Bianco *et al.*, 2001), and interactions with χ paused its translocation followed by translocation at approximately half of the initial rate (Handa *et al.*, 2005; Spies *et al.*, 2003). The ability of RecBCD to switch speeds upon demand suggests its intrinsic molecular plasticity, allowing it to adapt to cellular needs.

Dealing with obstacles

Although the helicases were initially thought to only catalyze strand separation of nucleic acids, there is increasing evidence for significantly broader functional roles of this class of enzymes. Recent single molecule studies are helping to uncover many of these additional functions and provide mechanistic understandings of these roles.

Under physiological environments, helicases that translocate along ssDNA and unwind dsDNA inevitably encounter protein obstacles that are bound to either substrate (Finkelstein & Greene, 2013). For example, the *E. coli* genome is occupied by nucleoid-associated proteins and DNA binding proteins, and most of the eukaryotic genome is coated in nucleosomes. How does a helicase overcome an obstacle? Does the helicase dissociate the obstacle, bypass it, or take it along the way (Figure 5)?

Finkelstein *et al.* challenged RecBCD helicase with a range of dsDNA binding proteins – RNA polymerase, EcoRI, lac repressor and even nucleosomes, and observed the outcome using DNA curtains (Finkelstein *et al.*, 2010) (Figure 2B). They found that after encountering these DNA binding proteins during unwinding, RecBCD helicase was able to push these proteins (or protein–DNA complexes) thousands of base pairs before displacing them from DNA (Figure 5).

Besides the removal of dsDNA binding proteins, helicases can also play a crucial role in evicting ssDNA binding proteins (Figure 5). Park *et al.* used a single-molecule FRET method to examine the activity of monomeric PcrA helicase on ssDNA coated with RecA (Park *et al.*, 2010), which is known to form a rigid nucleoprotein filament on ssDNA in the presence of ATP (Bell, 2005). They concluded that PcrA could efficiently displace RecA from the ssDNA. A subsequent study by Fagerburg *et al.* showed that RecA displacement by PcrA required the ATPase activity of RecA (Fagerburg *et al.*, 2012). Qiu *et al.* investigated the eukaryotic counterparts of these proteins and showed that Srs2 helicase could also efficiently dismantle Rad51 filaments (Qiu *et al.*, 2013).

Although helicases are typically known to unwind dsDNA, some seem to work in an opposite fashion by rewinding dsDNA. The thermodynamically favorable process of helicase-mediated DNA rewinding may be coupled with other thermodynamically unfavorable processes, such as displacement of bound proteins (Wu & Hickson, 2006). Using both magnetic tweezers and an optical trap, Manosas *et al.* demonstrated that both *E. coli* RecG helicase and the T4 bacteriophage UvsW helicase, involved in DNA repair and the rescue of stalled replication forks, display a strong preference for rewinding over unwinding, and are able to work against an opposing force as strong as 35 pN

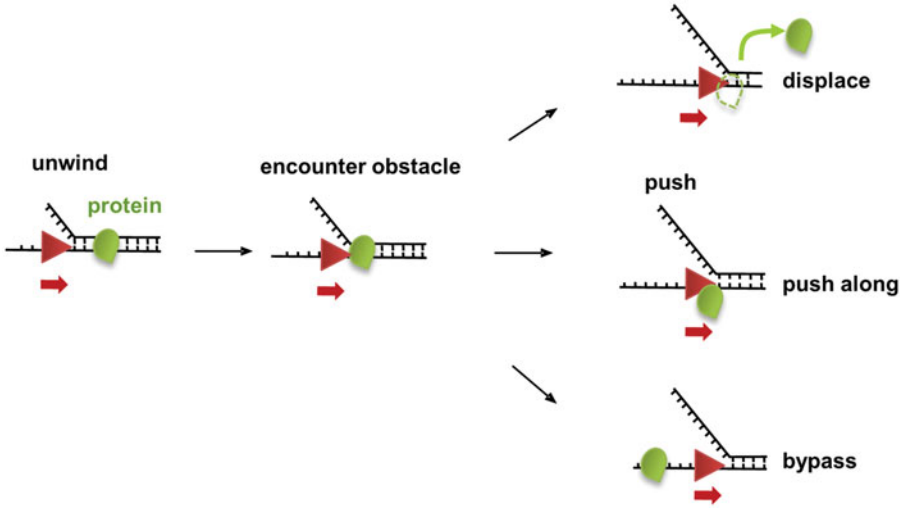


Figure 5. Collision of helicase with obstacles. Collisions between a helicase and a DNA binding protein may lead to bound protein dissociation, helicase pushing the bound protein forward, or helicase bypassing the bound protein. (see colour version of this figure at www.informahealthcare.com/bmg).

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(Manosas *et al.*, 2012a, 2013). They also found that, when the single-stranded DNA binding protein (SSB) is present, both RecG and UvsW can efficiently displace SSB while rewinding dsDNA at a rate close to that in the absence of SSB (Manosas *et al.*, 2013). This efficiency might be achieved via specific interactions between SSB and RecG such that the RecG's binding at the replication fork is remodeled (Buss *et al.*, 2008; Sun *et al.*, 2015). Such rewinding capability might facilitate the rescue of stalled replication forks by displacing bound proteins on ssDNA.

Upon encountering a DNA-binding protein, helicase might also circumvent the protein instead of displacing it. This might be particularly advantageous for DNA replicative helicase when replication needs to be completed in a timely manner for cell division. Several recent studies examined this possibility using single molecule fluorescence. Fu *et al.* showed that CMG, a eukaryotic replicative DNA helicase that translocates on DNA in the 3′–5′ direction, bypassed roadblocks on the lagging strand template much more readily than those on the leading strand (Figure 5), indicating that CMG helicase may unwind dsDNA via a steric exclusion model (Fu *et al.*, 2011). In contrast, Yardimci *et al.* found that the large T antigen replicative helicase of the simian virus 40 (SV40) was able to unwind dsDNA through bulky adducts on the leading strand (Figure 5), suggesting transient helicase ring opening for obstacle bypass (Yardimci *et al.*, 2012). Honda *et al.* also found that the XPD helicase can share the lattice with bound ssDNA-binding proteins and bypass them without promoting dissociation (Honda *et al.*, 2009).

Besides protein road blocks, helicase will also encounter obstacles comprised of nucleic acid structures. G-quadruplexes (G4s) (Hershman *et al.*, 2008) are formed by Hoogsteen hydrogen bonding in guanine-rich DNA sequences (Sen & Gilbert, 1988) and are known to impede replication and regulate transcription, and thus must be resolved by helicases (Fry & Loeb, 1999; Huber *et al.*, 2002; Paeschke *et al.*, 2011). Using single molecule FRET, Zhou *et al.* and Hou *et al.* showed that a monomeric Pif1 helicase can unfold a G4 structure in 2–3 discrete steps (Hou *et al.*, 2015; Zhou *et al.*, 2014). Another example is the BLM helicase that is associated with Bloom Syndrome. Chatterjee *et al.* found that G4 unfolding by BLM is highly dependent on 5′ ssDNA tail (Chatterjee *et al.*, 2014) and Budhathoki *et al.* showed that this unfolding requires only the RecQ-core of the BLM and may even occur in the absence of ATP (Budhathoki *et al.*, 2014).

Conclusions and future perspectives

As evident from this review, mechanisms and functions of a variety of helicases have been directly measured and characterized using single-molecule techniques. High-resolution methods, such as optical trapping, magnetic tweezers and FRET, are providing a rich toolbox for precision measurements of individual helicase motors. Techniques, such as DNA curtains and flow stretch, have the capability to monitor multiple molecules at the same time. Future enhancements of these approaches hold the promise of combining the accuracy of these single molecule measurements with high throughput (De Vlaminck *et al.*, 2011; Soltani *et al.*, 2014). In addition, recent demonstration of torque measurements of a DNA-based motor suggests that investigation of helicase unwinding under DNA supercoiling and well defined torque may also be possible (Bryant *et al.*, 2012; Ma *et al.*, 2013). Finally, an important future direction would be extending these approaches to investigate multi-component, dynamic machineries of replisomes. Indeed, efforts toward this direction have already begun (Manosas *et al.*, 2012a,b; Ticau *et al.*, 2015; van Oijen & Loparo, 2010). We anticipate that single molecule approaches will offer unprecedented opportunities to understand the mechanisms of helicases and replisomes.

Acknowledgements

We thank Drs. S. L. Moore, R. M. Fulbright and J. T. Inman from the Wang laboratory at Cornell University for critical reading of the manuscript.

Declaration of interest

The authors declare no conflict of interest.

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