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Single-Molecule Fluorescence Reveals the Unwinding Stepping Mechanism of Replicative Helicase

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SUMMARY

Bacteriophage T7 gp4 serves as a model protein for replicative helicases that couples deoxythymidine triphosphate (dTTP) hydrolysis to directional movement and DNA strand separation. We employed single-molecule fluorescence resonance energy transfer methods to resolve steps during DNA unwinding by T7 helicase. We confirm that the unwinding rate of T7 helicase decreases with increasing base pair stability. For duplexes containing >35% guanine-cytosine (GC) base pairs, we observed stochastic pauses every 2–3 bp during unwinding. The dwells on each pause were distributed nonexponentially, consistent with two or three rounds of dTTP hydrolysis before each unwinding step. Moreover, we observed backward movements of the enzyme on GC-rich DNAs at low dTTP concentrations. Our data suggest a coupling ratio of 1:1 between base pairs unwound and dTTP hydrolysis, and they further support the concept that nucleic acid motors can have a hierarchy of different-sized steps or can accumulate elastic energy before transitioning to a subsequent phase.

INTRODUCTION

DNA helicases are motor enzymes that convert the chemical energy of nucleotide triphosphate hydrolysis into mechanical energy for translocation on single-stranded DNA (ssDNA) and unwinding of double-stranded DNA (dsDNA) (Lohman and Bjornson, 1996; Patel and Picha, 2000). These enzymes encounter DNA sequences of different stabilities, and studying the effect of base pair stability can provide insights into the unwinding mechanisms (Betterton and Jülicher, 2005; von Hippel and Delagoutte, 2001). Previous studies have shown that the unwinding rates of T7 helicase, T4 helicase, and hepatitis virus C NS3 helicase depend on the stability of nucleic acid base pairs (Donmez et al., 2007; Johnson et al., 2007; Lionnet et al., 2007; Cheng et al., 2007).

T7 helicase (gp4A′ protein) has served as a good model system for hexameric helicases (Patel and Hingorani, 1993; Richardson, 1983). It assembles into a ring-shaped hexamer in the presence of deoxythymidine triphosphate (dTTP) and ssDNA (Donmez and Patel, 2006; Egelman et al., 1995), and translocates on ssDNA in the 5′ to 3′ direction. It unwinds dsDNA using a strand-exclusion mechanism whereby it binds and moves along one strand of the dsDNA in the 5′ to 3′ direction while excluding the complementary strand from its central channel (Ahnert and Patel, 1997; Hacker and Johnson, 1997; Jezewska et al., 1998; Kaplan and O'Donnell, 2002).

Using ensemble single-turnover kinetic analysis, Jeong et al. (2004) estimated the kinetic step size of unwinding by T7 helicase to be ~10 bp. As defined, the kinetic step size provides an estimate of how often a recurrent rate-limiting step takes place during processive unwinding. However, the kinetic step size estimated from ensemble measurements can be inflated if there exists significant heterogeneity in the reaction rate among individual molecules (Park et al., 2010). In addition, the crystal structures of hexameric helicases published thus far have not given detailed information on the unwinding mechanisms because either the nucleic acid substrates were not included in the structures (Bailey et al., 2007; Gai et al., 2004; Li et al., 2003; Singleton et al., 2000; Wang et al., 2008) or only the single-stranded substrates were cocrystallized (Enemark and Joshua-Tor, 2006; Itsathitphaisarn et al., 2012; Thomsen and Berger, 2009). Although single-molecule techniques have provided detailed insights into the mechanisms of various helicases (Bianco et al., 2001; Cheng et al., 2007, 2011; Dessinges et al., 2004; Dohoney and Gelles, 2001; Fili et al., 2010; Ha et al., 2002; Honda et al., 2009; Johnson et al., 2007; Karunatilaka et al., 2010; Klaue et al., 2013; Lee et al., 2006; Lionnet et al., 2007; Manosas et al., 2009; Myong et al., 2005, 2007, 2009; Park et al., 2010; Perkins et al., 2004; Qi et al., 2013; Spies et al., 2003; Sun et al., 2011), unwinding steps have not been detected for any hexameric helicase. In this report, we used single-molecule fluorescence resonance energy transfer (smFRET) (Ha et al., 1996) to measure real-time DNA unwinding by individual T7 helicase molecules. Taking advantage of the sequence-dependent unwinding rate, we used a designed DNA substrate to find a relation between FRET efficiency and the number of base pairs unwound. From substrates with a

high guanine-cytosine (GC) content, we could detect the individual steps of DNA unwinding and analyze their kinetics.

RESULTS

smFRET Unwinding Assay Shows the Duplex Stability Dependence of the Unwinding Rate

We probed the activity of T7 helicase using a single-molecule unwinding assay (Myong et al., 2007; Yodh et al., 2009) based on smFRET (Ha et al., 1996). We used forked substrates, where the Cy3 (donor) and Cy5 (acceptor) fluorophores were introduced at the ss/ds junction of the DNA with a 40 bp duplex (Figure 1A). The placement of fluorophores at the ss/ds junction does not alter the unwinding behavior (Pandey et al., 2009). The DNA was tethered to a polymer-treated quartz surface via biotin-neutravidin interaction (Figure 1A). After assembling T7 helicase on the DNA in the presence of 2 mM dTTP but no Mg(II), we initiated the unwinding reaction by flowing a solution containing 4 mM Mg(II) and 1 mM dTTP. This method of initiation served to remove the unbound protein in solution and enabled us to observe DNA unwinding catalyzed by prebound proteins only. Before unwinding starts, the donor and acceptor fluorophores are close together and therefore FRET is high (Figure 1B). As the unwinding reaction proceeds, the time-averaged distance between the fluorophores increases, resulting in a FRET decrease over time (Figure 1B; Ha et al., 2002; Myong et al., 2007; Yodh et al., 2009). When the donor-labeled strand departs from the surface after complete unwinding, the total fluorescence signal drops to the background level because the acceptor is not excited efficiently by the excitation laser at 532 nm.

To quantify the unwinding behavior, we measured the total unwinding time, which is the duration of the time interval from the moment FRET starts to decrease until the moment the total fluorescence signal disappears (Figure 1B, bottom panel, marked with arrows), and plotted the distributions (Figure 1C). A 10-fold difference in the unwinding time from 0% GC sequence to 80% GC sequence was observed (1 and 10 s, respectively; Figure 1C). After the unwinding reaction has progressed to a certain point, the remaining base pairs may separate spontaneously (Jeong et al., 2004). Therefore, we employed the following alternative method to estimate the absolute unwinding rate: First, we measured the time it takes to change FRET from 0.9 (the average value before unwinding starts) to 0.3. As we will show below, 0.3 FRET corresponds to ~10 bp unwound (Figure S1). Dividing 10 bp by the average time it takes to unwind 10 bp gives the unwinding rate. We then plotted the total unwinding rate for five different substrates and plotted it against ΔG/bp (dsDNA stability) calculated using the nearest-neighbor approach (Breslauer et al., 1986) and the HyTher web-based program (SantaLucia, 1998). When the unwinding rate is measured in this way, the difference between 0% GC and 80% GC becomes 13-fold. The results confirm that the unwinding rate decreases as the base pair stability of the duplex increases (Donmez et al., 2007). Moreover, the results further validate our single-molecule assay because the unwinding rates obtained are in agreement with previously reported rates obtained from ensemble measurements (Donmez and Patel, 2008; Donmez et al., 2007).

Calibration of the Number of Base Pairs Unwound to FRET Efficiency

In order to make an approximate assignment of FRET values to the number of base pairs unwound, N_{uw}, we performed the unwinding experiment using a substrate with ten contiguous adenine-thymine (AT) base pairs followed by 30 contiguous GC base pairs in the duplex region (Figure 2A; Table S1). After the unwinding reaction was initiated, FRET dropped rapidly to ~0.3 in ~0.9 s, likely due to unwinding of the AT base pair block, followed by a slow decrease to the lowest value (Figure 2B). To estimate the FRET value for $N_{uw} = 10$, we measured FRET values during the 1 s window after the initial, rapid FRET drop had ceased, and found that their histogram (>50 molecules) peaked at 0.3 (Figure 2C). Therefore, we will assume below that FRET is ~0.3 for $N_{uw} = 10$.

Unwinding Step Size of T7 Helicase

Although all AT sequence DNA was unwound rapidly without any evidence of steps or pauses, DNA sequences containing ≥35% GC base pairs showed clear evidence of steps (Figure 1B). Because the apparent steps may be caused by GC base pairs that are slower to unwind (i.e., the steps may be sequence-dependent pauses rather than elementary steps), we analyzed in detail three substrates with a stretch (2, 3, or 4 bp) of AT base pairs followed by a stretch of GC base pairs in a repeating pattern with 48%, 50%, and 80% GC content, respectively (Figure 3A). We observed that T7 helicase unwound all three substrates in a stepwise manner, indicated by the plateaus observed during the FRET decrease (Figure 3B). For a minor fraction of molecules, FRET decreased with no detectable pause or only a single pause. However, in the majority of molecules, regardless of the sequence, three or four pauses could be visually identified, corresponding to four or five steps, respectively, in reaching from the highest to the lowest FRET values. We observed two or three pauses (or three or four steps) by the time FRET reached a value of ~0.3, corresponding to $N_{uw} = 10$ (Figures 3B and 3C). Therefore, we can deduce that the pauses were separated by ~2–3 bp. However, FRET histograms during the FRET decrease did not show distinct peaks, suggesting that there are not well-defined FRET states that are visited during unwinding, as would be expected if pauses occur when the helicase encounters a boundary caused by GC base pairs (Supplemental Experimental Procedures; Figure 3D).

To further quantify the pausing behavior, we used an unbiased step-finding algorithm that generated the average FRET value for each pause and its dwell time (Kerssemakers et al., 2006; Myong et al., 2007). We then built transition density plots (McKinney et al., 2006) that represented the two-dimensional histogram for pairs of FRET values, determined using the step-finding algorithm, before and after each pause (Figure 3B). The transition density plots also illustrate that the pauses during unwinding did not occur at well-defined FRET states (Figure 3E). Furthermore, the histogram of pause durations determined using the step-finding algorithm showed a nonexponential distribution with an initial lag phase, and the average pause duration increased with the increase in GC content (Figure 3F). We fit the data into a gamma distribution $((\Delta t)^{N-1} \exp(-k\Delta t)$, where Δt = dwell time, k = rate of hidden stepping within a dwell, and