Coupling of DNA unwinding to nucleotide hydrolysis in a ring-shaped helicase

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The ring-shaped T7 helicase uses the energy of dTTP hydrolysis to perform the mechanical work of translocation and base pair (bp) separation. We have shown that the unwinding rate of T7 helicase decreases with increasing DNA stability. Here, we show that the dTTPase rate also decreases with increasing DNA stability, which indicates close linkage between chemical transition steps and translocation steps of unwinding. We find that the force-producing step during unwinding is not associated with dTTP binding, but dTTP hydrolysis or P_i release. We determine that T7 helicase extracts ~ 3.7 kcal/mol energy from dTTPase to carry out the work of strand separation. Using this energy, T7 helicase unwinds ~ 4 bp of AT-rich DNA or 1–2 bp of GC-rich DNA. T7 helicase therefore adjusts both its speed and coupling ratio (bp/dTTP) to match the work of DNA unwinding. We discuss the mechanistic implications of the variable bp/dTTP that indicates T7 helicase either undergoes backward movements/futile hydrolysis or unwinds DNA with a variable bp-step size; ‘long and fast’ steps on AT-rich and ‘short and slow’ steps on GC-rich DNA.

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Introduction

Helicases are key components of the biological machinery that catalyse the numerous DNA and RNA metabolic processes in the cell (Matson *et al.*, 1994; Lohman and Bjornson, 1996; Patel and Picha, 2000; Singleton *et al.*, 2007). Helicases are classified as nucleic acid motor proteins because of their unique ability to convert the chemical energy of NTP hydrolysis into mechanical work of directional movement and nucleic acid strand separation. The unidirectional translocation activity of helicases drives processes such as DNA recombination, nucleosome remodelling, packaging of genomes, DNA restriction, and protein dissociation, whereas the nucleic acid base-pair (bp) separation activity of helicases is critical for genome replication and repair. Very little is known about the energetics and the chemo-mechanical coupling

during nucleic acid unwinding by helicases. We investigate here the mechanism of a hexameric ring-shaped helicase, the bacteriophage T7 helicase (T7 gp4A' protein), which has been a model protein to understand DNA replication as well as the mechanisms of DNA unwinding (Donmez and Patel, 2006).

Movement along nucleic acids is an important property of helicases. It is assumed that processive helicases move and unwind nucleic acids by taking discrete elementary steps; each step being fuelled by the energy from NTP hydrolysis. T7 helicase uses the chemical energy from dTTP hydrolysis to move along ssDNA in a highly processive manner and to unwind dsDNA (Matson and Richardson, 1983; Matson *et al.*, 1983). The cooperativity among the six subunits of T7 helicase and the topological association of the hexameric ring with the DNA are likely responsible for the high processivity of T7 helicase (Egelman *et al.*, 1995). The six subunits of T7 helicase exert an effect cooperatively and fire dTTPs in a sequential manner, which facilitates sequential hands-off of DNA to neighbouring subunits providing a mechanism for stepwise and processive DNA movement (Liao *et al.*, 2005; Crampton *et al.*, 2006).

T7 helicase unwinds dsDNA using the strand exclusion mechanism, whereby it threads and moves unidirectionally along one strand of the dsDNA while excluding the complementary strand from its central channel (Ahnert and Patel, 1997; Hacker and Johnson, 1997; Jezewska *et al.*, 1998; Patel and Picha, 2000; Kaplan and O'Donnell, 2002). Recent studies have shown that T7 helicase unwinds DNA by a partially active mechanism (Donmez *et al.*, 2007; Johnson *et al.*, 2007). That is, it destabilizes the bp at the junction, but the interaction energy of T7 helicase with the ss/ds junction is not sufficient to allow the helicase to move through duplex regions as fast as it does along ssDNA. Therefore, the dsDNA poses a barrier to helicase's movement and decreases its translocation rate during DNA unwinding (Donmez *et al.*, 2007; Johnson *et al.*, 2007).

The unidirectional translocation of T7 helicase has been measured by ensemble methods and more recently by single-molecule force measurements (Kim *et al.*, 2002; Johnson *et al.*, 2007). T7 helicase moves efficiently along ssDNA and hydrolyses one dTTP on an average to move ~ 3 nt of ssDNA (Kim *et al.*, 2002). When T7 helicase is stalled at a lesion on ssDNA, its dTTP hydrolysis is inhibited and the helicase–dTTP complex remains bound to the DNA at the site of modification (Brown and Romano, 1989, 1991). The data indicate that the dTTPase activity of T7 helicase is tightly coupled to movement along ssDNA, and therefore the experimentally measured ~ 3 nt/dTTP coupling ratio is likely to be close to the elementary nt step-size of T7 helicase translocating along ssDNA (nt step-size is the average distance moved per dTTP hydrolysed). As compared with non-ring-shaped helicases such as PcrA (Dillingham *et al.*, 2000) and UvrD (Tomko *et al.*, 2007) that move on an average 1 nt for every ATP hydrolysed, the ring-shaped T7 helicase is three times more fuel efficient.

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The hydrolysis of a single NTP molecule can provide enough energy to unwind 4–6 bp of dsDNA (Lohman and Bjornson, 1996). This information can be obtained from the determination of the bp/NTP coupling ratio, which is not known for any ring-shaped helicase. Similarly, it is not known how much energy is extracted from NTP hydrolysis by the helicase to carry out the work of strand separation (thermodynamic efficiency). As it takes more energy to separate GC-rich DNA regions than AT-rich DNA regions, it is of interest to determine how the thermodynamic efficiency changes as the helicase unwinds DNA regions of different stabilities. It also remains unknown whether the coupling ratio (bp unwound/NTP hydrolysed) remains constant or changes as the helicase faces an increasing opposing force from more stable DNA regions.

To address the questions raised above, we have measured the rates of DNA unwinding and dTTP hydrolysis using transient state kinetic methods for T7 helicase, and more importantly carried out these studies with a range of dsDNA substrates with defined DNA stability. The DNA stability exerts an effect as a load or an opposing force to the forward movements of the translocating helicase. Studying helicase parameters as a function of dsDNA stability provides not only the energetics of unwinding but can also provide the location of the mechanical step of movement within the dTTPase cycle. Through studies of unwinding and dTTPase, we explore the possibility that the rate-limiting step for DNA unwinding is one of the chemical transition steps. Our results show that the coupling ratio bp/dTTP of T7 helicase is not a constant, but decreases with increasing dsDNA stability. The results provide new mechanistic insights into the mechanisms of DNA unwinding and reveal possibilities of backward movements or variable bp-step sizes of T7 helicase during DNA unwinding.

Results

Real-time unwinding of DNA substrates

To create DNA substrates with predefined stabilities, we introduced different percentage of GC bps, from 5 to 80% uniformly distributed in a 40-bp duplex DNA region (sequences in Supplementary data). The average bp stability of the DNA substrates was calculated using the nearest neighbour analysis (Breslauer *et al*, 1986), and it ranged from 1.1 to 2.1 kcal/mol/bp. Each DNA substrate contained a 5'-dT₃₅ tail, which is of sufficient length to load a single T7 helicase hexamer on the DNA before starting the unwinding reaction, and a 3'-dT₁₅ tail for providing strand exclusion during unwinding (Ahnert and Patel, 1997). To measure the average DNA-unwinding rate, we used the all or none unwinding assay (Ali and Lohman, 1997), which is commonly carried out in a discontinuous manner whereby radioactively labelled DNA substrates and unwound products are resolved using gel electrophoresis after the reactions are stopped with EDTA and SDS. The discontinuous nature of the assay, however, creates an uncertainty in the actual length of the dsDNA unwound. This is because partially unwound duplexes, especially those intermediates that have a very short stretch of dsDNA remaining to be unwound, can spontaneously fall apart in the time period between reaction quenching and product analysis by gel electrophoresis. Thus, the actual length of dsDNA that is unwound by the

helicase could be shorter and needs to be defined. The length of the duplex region that spontaneously falls apart (L_m) has been measured experimentally to be around 10 bp (Galletto *et al*, 2004; Jeong *et al*, 2004; Eoff and Raney, 2006). This is not desirable because the 10 bp L_m is almost 25% of the 40-bp length of the dsDNA substrates used here. To circumvent the L_m problem, we have developed a fluorescence-based unwinding assay that measures dsDNA unwinding in real time.

In this fluorescence-based assay, a fluorescein dye was covalently attached to the 5'-end of the excluded strand, and four consecutive guanines (G) were placed at the 3'-end of the loading strand (three of the G's constituting an overhang) (Figure 1A). The G nucleotides being in close proximity to the fluorescein dye quench the dye fluorescence only when the DNA is duplexed (Supplementary Figure 1a) (Torimura *et al*, 2001; Noble *et al*, 2005). When the dsDNA gets completely unwound and the dye-labelled strand moves away from the G-containing complementary strand, the fluorescein fluorescence increases. Neither the binding nor the translocation of T7 helicase on the dye-labelled strand changed the fluorescein fluorescence (Supplementary Figures 1c and d). The fluorescence-based assay is an all or none assay (two signal

Figure 1 Determination of L_m using the real-time unwinding assay. (A) Fluorescent DNA-unwinding substrate used for the real-time unwinding assays. The DNA contained two ssDNA overhangs, -GGG overhang at the 3'-end of the loading strand, and a fluorescein dye (F) at the 5'-end of the displaced strand. The ring-shaped helicase (blue ring) travels along the 5'-ssDNA to unwind the dsDNA region. (B) T7 helicase hexamer (40 nM) was preincubated with the fluorescent DNA substrates (5 nM) with dTTP (1 mM) and EDTA at 18°C in one syringe of the stopped-flow apparatus and unwinding reaction was initiated by addition of Mg(II) and SSB. Four DNA substrates with the same GC content (5%), but varying lengths of duplex regions (40, 50, 60, and 70 bp in black, red, green, and orange, respectively) were assayed for unwinding. The signal results from the separation of the fluorescein label opposite to several guanines at the end of the duplex. The resulting time traces of unwinding (Supplementary Figure 3a) were normalized between the values of 1 and 2. The time traces were globally fit to Equation 1) in which stepping rate (k), step size $m = (L - L_m)/n$ (where L is the length of the dsDNA region, n is the number of steps required to unwind the dsDNA), and the length of the spontaneously unwound region at end (L_m) were globally constrained. The best fit results (solid lines) provided k of 42.03 (36.6, 51.5) bp steps/s, m of 1.34 (1.09, 1.56) bp, and L_m of 1.5 (0, 3.37) bp. The average unwinding rate calculated from these parameters ($k \times m$) is 56.2 bp/s. The upper and lower limits for the parameters given in parentheses were obtained as described (Straume and Johnson, 1992).

levels corresponding to ss and dsDNA), but it has the advantage of monitoring DNA unwinding in real time.

To test the effectiveness of this assay, we first determined the L_m value of our DNA substrates by measuring the unwinding kinetics as a function of dsDNA length for substrates with an average GC content of 5%. We used DNA substrates with the lowest GC content to minimize processivity complications and because it is expected to provide the greatest L_m being the least stable of all the DNAs used. Four duplexes with the same 5% GC content, but varying lengths of duplex regions (40, 50, 60, and 70 bp) were used as unwinding substrates. T7 helicase was preincubated with the dye-labelled DNA in the presence of dTTP and EDTA (which prevents dTTP hydrolysis and unwinding, but allows T7 helicase to form a stable hexamer on the DNA) (Hingorani and Patel, 1993, 1996; Picha and Patel, 1998). The unwinding reactions were initiated by the addition of Mg(II) and *Escherichia coli* single-stranded binding (SSB) in a stopped-flow apparatus. The SSB protein acts as a DNA trap and stops unwinding beyond a single turnover (Donmez *et al.*, 2007). The SSB was used as a trap instead of a ssDNA to keep the reaction conditions as similar as possible between measurement of DNA-unwinding and dTTP hydrolysis rates (see below). The unwinding reactions were carried out at 18°C in a stopped-flow instrument and monitored by recording the fluorescein fluorescence in real time. All the DNA substrates were unwound with lag kinetics consistent with the all-or-none type of assay used and the observed lag time increased progressively with increasing dsDNA lengths (Figure 1B and Supplementary Figure 3a). The time courses were fit globally to the stepping model (Equation (1)), where three parameters were shared: L_m , the step size, $m = (L - L_m)/n$, and the stepping rate, k (where L is the length of the dsDNA and n is the apparent number steps to unwind the dsDNA). The best global fit provided L_m of 1.5 bp, k of 42 bp step/s, and m of 1.4 bp. Note that the L_m of 1.5 bp obtained when using the real-time unwinding assay is much smaller than the ~12 bp reported for ~30% GC-rich DNA whose unwinding was measured using the gel assays (Jeong *et al.*, 2004). We expect the L_m for the DNA substrates with a higher GC content to be even smaller, and hence the unwinding rates measured by the real-time assay are accurate and not significantly affected by the L_m .

Mechanochemistry of the DNA-unwinding reaction

Very little is known about the interdependence of the chemical reaction of dTTP hydrolysis and DNA movement or the chemo-mechanical coupling. It is expected that one or more of the elementary steps in the dTTPase pathway are associated with the DNA movement step. By studying the kinetics of unwinding as a function of [dTTP] and dsDNA stability, we can gain insights into the location of the step associated with DNA movement (Bustamante *et al.*, 2004). This is because the stability of the dsDNA is effectively a load or an opposing force that the helicase experiences when it is unwinding the dsDNA; thus, the dependence of unwinding rate on dsDNA stability can provide insights into the mechanical step.

The DNA-unwinding kinetics were measured using the fluorescence-based assay for the 40 bp dsDNA substrates, 5–80% GC rich at 500 μ M dTTP. T7 helicase (40 nM) was preincubated with the DNA substrate (5 nM) in the presence of EDTA and dTTP, and reactions were initiated with Mg(II)

and SSB in a stopped-flow instrument. A typical set of unwinding time traces at [dTTP] of 500 μ M (Figure 2A and Supplementary Figure 3b) shows that the unwinding lag time increases as the dsDNA stability increases. The kinetic traces were fit to the stepping model and the average rate of DNA unwinding was determined from k/n parameters (Supplementary Table 1). The average unwinding rates were plotted against dsDNA stability (Figure 2B), which shows a linear decrease in rate from ~60 to 9 bp/s over the range of 5–80% GC content in the DNA. Thus, the decrease in unwinding rate of T7 helicase with increasing dsDNA stability is nearly seven-fold.

The unwinding kinetics were measured at various [dTTP] ranging from 20 μ M to 1.28 mM for each of the 40 bp DNA substrates (Supplementary Figures 3c and d), and the average unwinding rates derived from fitting to the stepping model were plotted as a function of dsDNA stability (Figure 3A). At all [dTTP], the unwinding rate decreases almost linearly with increasing dsDNA stability. At high [dTTP], the decrease in the unwinding rate with increasing dsDNA stability is steeper as compared to when the [dTTP] was low.

The data in Figure 3A were replotted as unwinding rates against [dTTP] (Figure 3B). The plots show that the unwinding rates increase in a hyperbolic manner with increasing [dTTP] and hence were fit to the Michaelis–Menten equation. The derived k_{cat} and K_m were then plotted against dsDNA

Figure 2 Dependence of DNA-unwinding rate on dsDNA stability. (A) The unwinding kinetics of the 40-bp DNA substrates with GC content from 5 to 80% were measured as described in Figure 1 at 500 μ M dTTP. Experimental data (normalized between 1 and 2 for visual purposes, see Supplementary Figure 3b for unnormalized traces) and the fits to the simplest model (Equation (1), Materials and methods) in solid lines are shown. The fitted parameters with their confidence intervals are shown in Supplementary Table 1 from which the average unwinding rates ($40 \times k/n$) were calculated. (B) The average unwinding rates are plotted against average bp stability. The error bars shown here represent standard deviations of 6–8 independent determinations.

Figure 3 Dependence of DNA-unwinding rate on dsDNA stability and [dTTP] (A). The average DNA-unwinding rates, determined as shown in Figure 2, at various [dTTP] when plotted against bp stability show linear decreases at all [dTTP]. Individual time traces for 5 and 80% GC DNAs at various [dTTP] are shown in Supplementary Figures 3c and d. (B) The same data in (A) are replotted as average DNA-unwinding rates against [dTTP] for each of the DNA substrates ranging in GC content from 5 to 80%. The solid lines are the fits of the rate versus [dTTP] to a hyperbola (observed unwinding rate = $k_{cat} \times [dTTP]/K_m + [dTTP]$). (C) The maximal rate of DNA unwinding (k_{cat}) and dTTP K_m are plotted against average bp stability. (D) The ratio k_{cat}/K_m shows a slight decrease with increasing bp stability. The error bars are standard errors from computer fits and from combined errors of measurements (Fersht, 1999).

stability (Figure 3C). Examination of the dependence of these kinetic parameters on dsDNA stability allowed us to investigate which step/s in the dTTPase cycle is associated with movement. As the GC content increased from 5 to 80% in the DNA substrates, the maximal unwinding rate or k_{cat} decreased 8.5-fold and the K_m decreased 6.6-fold. The ratio k_{cat}/K_m , however, showed a relatively small decrease of about 25% and thus remained essentially independent of dsDNA stability (Figure 3D). The observation that both k_{cat} and K_m decrease with increasing dsDNA stability, but the k_{cat}/K_m ratio, which is the apparent second order rate constant for dTTP binding, remains independent of dsDNA stability indicates that the dTTP-binding steps are not the force-producing steps for dsDNA unwinding (Bustamante *et al*, 2004).

dTTP hydrolysis is coupled to DNA unwinding

The above experiments indicate the presence of a mechanically sensitive rate constant in the cycle of dTTP hydrolysis. To explore this finding further and to determine the bp/dTTP coupling ratio of T7 helicase, we designed experiments to measure the dTTP hydrolysis rate during DNA unwinding. For the determination of the bp/dTTP coupling, we made sure that the dTTP hydrolysis rate was measured under solution conditions (temperature, buffer composition, [dTTP] and T7 helicase) similar to DNA unwinding (Martinez-Senac and Webb, 2005). The reactions were also carried out under conditions where T7 helicase is a stable hexamer (500 μM dTTP and 20 nM helicase hexamer) (Hingorani and Patel, 1993, 1996; Patel and Hingorani, 1993; Picha and Patel, 1998). To optimize the signal, the

dTTPase reaction was carried out at [DNA] > [helicase], and to make the reactions single turnover *E. coli* SSB was added, which acts as a DNA trap and stops both unwinding and DNA-stimulated dTTP hydrolysis beyond a single turnover (Donmez *et al*, 2007). The ssDNA is a commonly used protein trap that works well in the unwinding reaction, but it stimulates the helicase's dTTPase activity and hence cannot be used as a trap to measure the dTTPase kinetics. The dTTPase rate is also sensitive to the active [helicase-DNA] and processivity. The active [helicase-DNA] was experimentally determined from its K_d measured using a functional unwinding assay (Supplementary Figure 1e). The unwinding processivity of T7 helicase has been experimentally determined (Donmez *et al*, 2007) and was taken into account when determining the dTTPase rate from model fittings (Equations (2) and (3)).

The dTTP hydrolysis kinetics were measured in real time using the phosphate-binding protein (PBP) that acts as a sensitive sensor for fast detection of low concentrations of P_i (Brune *et al*, 1994). This assay enables an instantaneous monitoring of P_i concentration from the fluorescent signal produced when P_i binds to coumarin dye-labelled PBP. T7 helicase was preincubated with the 40-bp dsDNA substrates of different stabilities and the P_i release kinetics were measured at 500 μM dTTP in a stopped-flow instrument. Two prominent phases of P_i release kinetics were observed (Figure 4A). The fast phase of P_i production (burst phase) occurs in the same time scale as dsDNA unwinding (compare time traces in Figure 2A to Figure 4A) for each of the DNA substrates. The prominent burst phase of P_i production,

Figure 4 Dependence of dTTP hydrolysis rates on dsDNA stability. T7 helicase hexamer (40 nM) was preincubated with the unwinding substrate (200 nM) in the presence of dTTP (1 mM), EDTA, P_i -mop, and MDCC-labelled PBP (as described in the Materials and methods) at 18°C in one syringe of the stopped-flow apparatus and reaction was initiated by addition of Mg(II), P_i -mop, and SSB from a second syringe. **(A)** Time traces of P_i production (P_i /T7 helicase hexamer, M/M) during T7 helicase unwinding of DNA substrates with different stability. The solid lines are fit to the model for DNA unwinding and dTTP hydrolysis (Equations (2) and (3), Materials and methods). The following were the best fit parameters from model fitting for DNA substrates (5, 20, 35, 50, 65, and 80% GC): b (15.2, 13.4, 11.0, 8.4, 9.5, and 5.7 s⁻¹), c (2.1, 2.2, 2.1, 2.2, 2.7, and 1.6 s⁻¹), k (40.3, 14.85, 7.98, 2.27, 4.4, and 1.2 bp step/s), and n (48.6, 19.1, 15.1, 5.5, 12.9, and 7.0). **(B)** The rates of dTTP hydrolysis during dsDNA unwinding obtained from model fitting, b , or slopes of the burst phase are plotted against the dsDNA stability. The error bars are standard deviations of average data from 6–8 independent measurements.

therefore, provides information on the rate of dTTP hydrolysis during the time T7 helicase is unwinding the dsDNA. This was further supported by a set of P_i release kinetics carried out with DNA substrates of different lengths (40, 50, 60, and 70 bp 5% GC-rich DNAs). Consistent with the analysis above, the burst amplitude of P_i production increased with increasing length of dsDNA (Supplementary Figure 2a). The linear increase in P_i production after the initial burst phase occurs at the same rate as the intrinsic dTTPase rate of T7 helicase free from bound DNA (Jeong *et al.*, 2002). A brief phase at the beginning of the reactions with an even steeper slope was relatively invariant from DNA to DNA

(Supplementary Figure 2e). This phase likely results from the fast hydrolysis of pre-bound dTTPs.

Analysis of the P_i release burst amplitudes (Figure 4A) showed that about 15–20 dTTP molecules were hydrolysed during the unwinding time of the 40 bp dsDNAs. In principle, the amplitude can be used to determine the bp/dTTP coupling ratio. However, the amplitude needs to be corrected for the imperfect processivity of the helicase and for the active helicase–DNA complex in the reaction. The dTTP hydrolysis rate associated with DNA unwinding can also be determined from the slope of the burst phase. A more rigorous treatment of the dTTPase data requires correction

for the imperfect processivity of the helicase, the active [helicase–DNA], and the intrinsic hydrolysis rate (dTTP hydrolysis rate in the absence of DNA). The P_i release kinetics were fit to the model (Equations (2) and (3), Materials and methods) to obtain the dTTPase rate during DNA unwinding. The dTTP hydrolysis rates (b) derived from model fitting are plotted next to the rates obtained from the simpler treatment of slope determination (Figure 4B). The rates from both treatments are quite similar, and the data clearly show a decrease in the dTTPase activity with increasing dsDNA stability. The decrease in unwinding rate (Figure 2) and the corresponding decrease in the dTTPase rate with increasing dsDNA stability indicate that the chemical transition steps are closely linked with the translocation steps during DNA unwinding. When the helicase slows down due to greater opposing force from dsDNA stability, its dTTPase activity also slows down.

The bp/dTTP coupling ratio decreases with increasing DNA stability

Knowing the unwinding rate and the dTTP hydrolysis rate allowed us to calculate the bp/dTTP coupling ratio during DNA unwinding. The coupling ratio was calculated by dividing the unwinding rate with the dTTPase rate (from data in Figures 2B and 4B) for each of the DNA substrates at $[dTTP] = 500 \mu M$. Interestingly, we find that the coupling ratio is not a constant, but decreases with increasing dsDNA stability (Figure 5). The coupling ratio for the AT-rich DNAs is 3–4 bp/dTTP, which is very similar to the coupling ratio nt/dTTP of T7 helicase when it is translocating along ssDNA. The coupling ratio for the GC-rich DNAs, on the other hand, is lower and 1–2 bp/dTTP.

The thermodynamic efficiency of T7 helicase as a function of dsDNA stability

Knowing the bp/dTTP coupling ratio and the energetics of base pairing in dsDNA, we can determine the average work involved in unwinding AT-rich versus GC-rich DNA. We define the term work-step here as the average work carried out in separating the DNA strands per dTTP hydrolysed. Work-step (kcal/mol) = [(unwinding rate (bp/s)/dTTPase

rate (s^{-1})] $\times \Delta G/bp$ (kcal/mol/bp). Where $\Delta G/bp$ is the average free energy of the bp for a particular substrate calculated from the free energy of the dsDNA using the nearest neighbour analysis (Breslauer *et al.*, 1986). The work-step was calculated for each of the DNA substrates and plotted against dsDNA stability (Figure 5). The results show a relatively small decrease in work-step from 4 to 3 kcal/mol with increasing dsDNA stability. We calculated an average work-step of 3.7 kcal/mol for T7 helicase over the physiological range of DNA stability, which is the free energy that T7 helicase extracts from dTTP hydrolysis to carry out useful work of unwinding dsDNA.

Knowing the work carried out for dsDNA separation allowed us to calculate the thermodynamic efficiency of the helicase, which is the ratio of the work done by the helicase for bp separation to the energy input from dTTP hydrolysis. Assuming that dTTP hydrolysis can generate ~ 10 kcal/mol of free energy, the average thermodynamic efficiency of T7 helicase is $\sim 37\%$ (3.7 kcal/mol divided by 10 kcal/mol). The average thermodynamic efficiency of the T7 helicase motor is very similar to other motor proteins such as $\phi 29$ packaging motor, RNA and DNA polymerases, and cytoskeletal motors such as kinesin and myosin (Bustamante *et al.*, 2004). The thermodynamic efficiency of T7 helicase decreases slightly however from 40 to 30% within the physiological range of dsDNA stability.

Discussion

The studies in this paper show that the DNA-unwinding rate of T7 helicase as well as its presteady-state dTTPase rate during DNA unwinding are strongly influenced by dsDNA stability and both decrease as the GC content in the dsDNA increases. In principle, the decrease in the unwinding rate or the translocation speed of the helicase with increasing dsDNA stability can be due to slowed chemical transitions or due to a slower movement of the helicase. The observed decrease in the presteady-state rate of dTTP hydrolysis with increasing dsDNA stability indicates that the decrease in translocation speed is linked to a slowed chemical transition. To investigate which step in the dTTPase pathway is most sensitive to dsDNA stability, the unwinding rates were measured as a function of DNA stability at various $[dTTP]$. The kinetic parameters obtained from these experiments show that both k_{cat} and K_m decrease with increasing DNA stability, but the k_{cat}/K_m ratio remains nearly constant. This characteristic behaviour is similar to DNA stability acting as an uncompetitive inhibitor affecting equilibrium between states not associated with dTTP binding. The data indicate that movement is not associated with the dTTP-binding steps. A similar behaviour has been observed for the $\phi 29$ packaging motor (Chemla *et al.*, 2005) where ATP-binding step has been eliminated as the force-producing step.

To narrow down the step involved in force production during DNA unwinding by T7 helicase, we examined the kinetic pathway of dTTP hydrolysis that has been studied in detail (Hingorani *et al.*, 1997; Jeong *et al.*, 2002; Liao *et al.*, 2005). To perform an energetic unfavourable movement on DNA, the helicase needs to have a controllable hold on the DNA. The ssDNA does not bind tightly to the T7 helicase–dTDP state (Hingorani and Patel, 1993, 1996); therefore, the possibility of dTDP release being the power stroke step is

Figure 5 Dependence of bp/dTTP coupling ratio and work-step on dsDNA stability. The bp/dTTP coupling ratio was determined by dividing the unwinding rate (from Figure 2B) and the dTTP hydrolysis rate (from Figure 4B, model fitting) at $500 \mu M$ dTTP. The work-step was calculated by multiplying the bp/dTTP with the average bp stability of the DNA substrates. The error bars were determined from the combined errors of measurements (Fersht, 1999).

low. We are left therefore with P_i release or a conformational change associated with dTTP hydrolysis as the power stroke step. It has been argued that the bond cleavage reaction of nucleotide hydrolysis is unlikely to cause major conformational changes that can drive movement (Oster and Wang, 2000). Therefore, a more likely candidate for a power stroke step is P_i release. The P_i release is the rate-limiting step during translocation of T7 helicase along ssDNA (Jeong *et al.*, 2002; Liao *et al.*, 2005). Therefore, it is very likely that translocation is limited by the rate of P_i release during DNA unwinding. This possibility needs to be examined by further studies.

Knowing the rates of dTTP hydrolysis and DNA unwinding allowed us to calculate the bp/dTTP coupling ratio that provides information on how many bp of DNA are unwound on an average per molecule of dTTP hydrolysed. We observed that the average bp/dTTP ratio of T7 helicase is 3–4 when it is unwinding AT-rich DNA (<50% GC content) and 1–2 when it is unwinding GC-rich DNA. Thus, the coupling ratio of T7 helicase is not a constant and it decreases with increasing dsDNA stability. One reason for the decrease in the coupling ratio with an increase in dsDNA stability could be that T7 helicase undergoes futile dTTP hydrolysis cycles during DNA unwinding. The futile dTTP hydrolysis may arise from occasional backward movements of the helicase or continued dTTP hydrolysis during helicase pauses (Figure 6A). Backward movements have been detected in single molecule studies of recBCD helicase (Perkins *et al.*, 2004) and pauses have been observed in single-molecule studies of the HCV helicase (Dumont *et al.*, 2006; Cheng *et al.*, 2007; Myong *et al.*, 2007). Although such data on T7 helicase are not available, based on the observation that T7 helicase does not hydrolyse dTTP when it is stalled on ssDNA (Brown and Romano, 1989, 1991), we argue that futile dTTPase hydrolysis during pauses of the helicase is less likely. Therefore, one explanation for the decrease in coupling ratio with increasing dsDNA stability is that T7 helicase undergoes occasional backward movements that increases in frequency with increasing dsDNA stability. This possibility needs to be tested experimentally.

Figure 6 Two models of DNA unwinding. (A) Helicase unwinds DNA with a constant bp-step size, but has varying probability of achieving a productive step due to occasional backward movements during DNA unwinding. The backward movements shown here are not fuelled by dTTP hydrolysis and are not necessarily of the same size as the forward movements. The backward movements result in futile dTTP hydrolysis cycles because a segment of dsDNA occasionally is unwound by more than one attempt from the helicase. The higher probability of backward movements on GC-rich DNA can explain its lower coupling ratio (bp/dTTP) as compared with AT-rich DNA. (B) Helicase unwinds DNA with variable bp-step sizes. The helicase takes longer steps on AT-rich DNAs and shorter steps on GC-rich DNAs.

There is, however, an alternative explanation for the decrease in coupling ratio of T7 helicase. If T7 helicase does not undergo backward movements during DNA unwinding or futile dTTPase, then the coupling ratio should be equal to the elementary bp-step size, which is the average distance moved per dTTP hydrolysed. If this were the case, then our data would indicate that the bp-step size of T7 helicase is longer for AT-rich DNA and shorter for GC-rich DNA (Figure 6B). A possible rationale for this behaviour could be that by decreasing the bp-step size the motor is able to keep moving forward at a reasonable speed under increasing load from dsDNA stability (similar to changing gears). Such behaviour has been observed for the cytoplasmic dynein, which is a tightly coupled motor; that is, it takes a forward step for every ATP hydrolysed. The step size of dynein, however, depends on the load and decreases with increasing opposing load (Mallik *et al.*, 2004).

One way that T7 helicase can achieve variable bp-step size is if the helicase's stepping process was dictated by the availability of ssDNA at the fork junction. Such a molecular mechanism for variable step size is illustrated in Supplementary Figure 4. A variable bp-step size can be achieved if the ssDNA-binding loop of T7 helicase in the central channel is flexible and can move 1–3 nt of ssDNA. On AT-rich DNA, the size of the frayed DNA segment could be longer such that ~3 nt of ssDNA are available to the DNA-binding loop for interactions during the lifetime of a bound dTTP. Therefore, the bp-step size of T7 helicase on AT-rich DNA is ~3 bp. On GC-rich DNA, fewer bps are frayed, and thus the bp-step size is shorter (1–2 bp) (Supplementary Figure 4).

Further studies will be required to distinguish between the two models. Such studies could include direct step size measurements by single-molecule methods and investigation of backward movements. T7 helicase, as many helicases, works in concert with other proteins to catalyse DNA unwinding during DNA replication. T7 helicase–primase is a part of the replication machinery that includes proteins such as the DNA polymerase and SSB proteins. Our studies here were carried out with the *E. coli* SSB protein, but in the absence of the DNA polymerase. It has been shown that T7 DNA polymerase increases the speed of T7 helicase unwinding (Stano *et al.*, 2005). The DNA polymerase could accelerate the helicase by dampening helicase's backward motions or by destabilizing the junction and trapping the unwound bp through DNA synthesis. Studies of T7 helicase with the DNA polymerase will be very important in understanding not only the mechanism of DNA replication but also the possible pathways of DNA unwinding. It will be interesting to investigate how the DNA polymerase would modulate the mechanochemical coupling in T7 helicase during DNA unwinding.

Materials and methods

DNA-unwinding assays

All the reactions were performed at 18°C in a stopped-flow instrument (Kintek Corp). An equal volume of a mixture of fluorescein-labelled DNA-unwinding substrate, T7 helicase, and dTTP from one syringe was mixed with Mg(II) and *E. coli* SSB protein to initiate the reaction. Final reaction buffer was 50 mM Tris-Cl pH 7.6, 50 mM NaCl, 10% glycerol, 2 μM BSA. Final concentrations unless indicated otherwise were dTTP 500 μM,

DNA substrate 2.5 nM, T7 helicase hexamer 20 nM, MgCl_2 4 mM, and SSB 1 μM . The reaction solution was excited at a wavelength of 480 nm with a slit width of 2 mm (~ 8 nm spectral bandwidth) and fluorescence was detected by a photomultiplier tube (Hamamatsu) filtered through a long-pass filter with a cutoff of 515 nm.

dTTP hydrolysis kinetics

The dTTP hydrolysis kinetics was measured using the same buffer conditions and protein concentrations, except that the unwinding substrate was unlabelled and its final concentration was 100 nM. To eliminate contaminant inorganic phosphate, both syringes of the stopped-flow instrument were treated with the P_i -mop (PNPase 1 U/ml and 7 mM, 0.5 mM) for 10 min before starting the reactions. Syringe A contained the P_i -mop (PNPase 0.1 U/ml and 7 mM, 0.5 mM), PBP (4 μM), T7 helicase, DNA-unwinding substrate, and dTTP, and syringe B contained P_i -mop, $\text{Mg}(\text{II})$, and SSB. The mixed solution was excited with 425 nm with 1 mm slits (~ 4 nm spectral bandwidth) and the emission was detected by PMT with a 450 nm long-pass filter (Corion).

For each set of experiment, a P_i calibration curve was created as follows: standard P_i solutions (Sigma) were mixed with the PBP protein and 6–8 time traces were collected at each $[\text{P}_i]$. The time courses were corrected for the experimentally determined dead time of the stopped-flow instrument (4 ms) and fit to a first degree polynomial to determine the amplitudes, which were corrected for no P_i control, and plotted against $[\text{P}_i]$ to obtain the slope.

Data analysis

The unwinding kinetics were fit to Equation (1) (Lucius *et al.*, 2003; Jeong *et al.*, 2004; Donmez *et al.*, 2007).

$$F(t) = (F_{ss} - F_{ds}) \left(1 - \frac{\Gamma(n, k_0 t)}{\Gamma(n)} \right) + F_{ds} \quad (1)$$

where F_{ss} is the background fluorescence signal, F_{ds} is the maximum fluorescence signal after completion of reaction, n the apparent number of steps to unwind the dsDNA, and k_0 the apparent unwinding rate per step ($k + k_d$) (k being the stepping rate and k_d the dissociation rate). This was the simplest model that takes into account only two states (ds and ssDNA signal levels) and ignores the slight peaking due to fast SSB binding to the newly generated ssDNA. We used a number of more sophisticated models to take care of the third mode that is manifested just before the trace is completed; however, the final normalized unwinding rates were not significantly different, so we present the fits for the simplest model above.

Typically, 6–8 P_i release reaction time courses were averaged, corrected for the instrument dead time, and normalized. The fluorescence signal was converted to $[\text{P}_i]$ using the P_i standard curve. To use as initial values in model fitting, we estimated the P_i release rates during different phases by taking the derivative of the time courses with respect to time.

We used Mathematica to obtain the model equations for fitting the P_i release kinetics. We assume that the helicase traverses the

DNA by the n -step sequential model.

Equation (2) describes the time evolution of the free helicase concentration.

$$H(t) = 1 - \frac{H_0 e^{-k_d t} \Gamma(n, kt)}{\Gamma(n)} \quad (2)$$

where $H(t)$ is the fraction of free helicase at given time t , and H_0 is the bound helicase fraction at the start of the reaction. Inorganic phosphate will be generated from these two states of the helicase (DNA bound and free):

$$\frac{d[\text{P}_i]}{dt} = b \sum_{j=0}^{j=n-1} [HD_j] + c[H]$$

Equation (3) describes the time evolution of $[\text{P}_i]$.

$$\begin{aligned} P(t) = & P_0 + ct + \frac{H_0(b-c)(1-R^n)}{k_d} \\ & + \frac{H_0(b-c)}{k_d} \left[R^n \left(1 - \frac{\Gamma(n, k_0 t)}{\Gamma(n)} \right) - e^{k_d t} \left(1 - \frac{\Gamma(n, kt)}{\Gamma(n)} \right) \right] \end{aligned} \quad (3)$$

where, processivity $[k/(k + k_d)]$ is denoted by R , P is the P_i concentration, P_0 is P_i concentration at the start of the reaction, b is the dTTP hydrolysis rate during dsDNA unwinding, and c is the dTTP hydrolysis of the helicase free from DNA. To simplify regression analysis, we fixed as many parameters as possible: P_0 was 0, because we could estimate it by extrapolation, H_0 was 0.85 from the known K_d of the T7 helicase–DNA complex. We fixed k_d in an indirect way by fixing the processivity (k_d would be constrained dynamically) to the value from previous measurements (Donmez *et al.*, 2007). Processivities for 5–80% GC DNAs are 0.9982, 0.9972, 0.9957, 0.9934, 0.9889, and 0.9803. At each step of iteration, the value of $k_d = k \times R^{(-L/n)} - 1$, where k is the forward stepping rate, L is total length of dsDNA, and n is the number of steps.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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