

Kinetic Measurement of the Step Size of DNA Unwinding by Bacteriophage T7 DNA Helicase gp4

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T7 bacteriophage gp4 is the replicative DNA helicase that unwinds double-stranded DNA by utilizing dTTP hydrolysis energy. The quaternary structure of the active form of T7 helicase is a hexameric ring with a central channel. Single-stranded DNA passes through the central channel of the hexameric ring as the helicase translocates (5'→3') along the single-stranded DNA. The DNA unwinding was measured by rapid kinetic methods and showed a lag before the single-stranded DNA started to accumulate exponentially. This behavior was analyzed by a kinetic stepping model for the unwinding process. The observed lag phase increased as predicted by the model with increasing double-stranded DNA length. Trap DNA added in the reaction had no effect on the amplitudes of double-stranded DNA unwound, indicating that the T7 helicase is a highly processive helicase. Global fitting of the kinetic data to the stepping model provided a kinetic step size of 10 - 11 bp/step with a rate of 3.7 s⁻¹ per step. Both the mechanism of DNA unwinding and dTTP hydrolysis and the coupling between the two are unaffected by temperature from 4 - 37°C. Thus, the kinetic stepping for dsDNA unwinding is an inherent property of the replicative DNA helicase.

Key words – DNA helicase/ Hexameric ring/ Rapid kinetics/ Global fitting/ Stepping model

DNA helicases are motor proteins that unwind double-stranded (ds) DNA during DNA replication, recombination, and repair and are also components of eukaryotic transcription complex [5,18,19]. These enzymes use the chemical energy obtained from nucleoside triphosphate binding and hydrolysis to perform the mechanical work of unwinding dsDNA, which also requires translocation of the helicase along DNA for processive unwinding. Bacteriophage T7 gp4A' (hereafter 4A') is a hexameric ring-shaped helicase that uses dTTP hydrolysis to separate the strands of duplex DNA. 4A' assembled into rings requires two noncomplementary single-stranded (ss) DNA tails at one end of the dsDNA to initiate dsDNA unwinding [1,9,27]. These studies of T7 4A' helicase indicate that one strand of the duplex DNA binds within the central channel of the 4A' ring and the complementary strand may interact with the outer parts of the hexamer.

A simple way for the helicase to unwind DNA is envisaged that 4A' hexamer couples the energy released during enzymatic dTTP hydrolysis cycles to unidirectional translocation along ssDNA, displacing the complementary DNA strand. Thus, unidirectional translocation is an intrinsic property of DNA helicase during unwinding. Kinetics of

unidirectional movement by PcrA helicase has been investigated using the stopped-flow kinetics method [8]. Recently, rate of the unidirectional translocation along the ssDNA by T7 gp4A' hexameric helicase and its accompanying dTTP hydrolysis rate has been measured precisely using the rapid kinetics technique [14]. To date, the rate and processivity of the dsDNA unwinding by the replicative hexameric helicase have not been however quantitatively measured.

The goal of this study was to better understand the dsDNA unwinding process by the hexameric DNA helicase. Reaction conditions have been previously established, in which DNA unwinding was not limited by processes such as protein oligomerization, protein-DNA binding or dissociation [1,21]. This allowed me to design single turnover kinetic experiments to directly measure DNA unwinding. It was observed that the accumulation of ssDNA follows lag kinetics, which depended on the length of the dsDNA.

A stepping model was developed to analyze the unwinding kinetics and extract the step size and rate of unwinding. A similar stepping model has been applied to the DNA unwinding process of the *E. coli* UvrD helicase [3,4]. UvrD unwinds short duplex DNA at a step size of 4 - 5 bp with a low processivity. It was observed that the processivity of 4A' helicase is very high. Global analysis of the kinetic data with forked DNA substrates of varying duplex length using the stepping mechanism revealed a stepping size of

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10 - 11 bp for dsDNA unwinding by 4A'. This stepping size was not affected by temperature. This study presents a first step toward the understanding of DNA unwinding by a hexameric replicative DNA helicase.

Materials and Methods

Buffers, Nucleotides, and Other Reagents

The reaction buffer without magnesium (Buffer T) contains 20 mM TrisHCl (pH 7.5), 50 mM NaCl, and 10% (v/v) glycerol. dTTP were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and radioactive nucleotides ($[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$) were purchased from Amersham Pharmacia Biotech. Quench buffer for stopping the helicase reaction of dsDNA unwinding is 50 mM TrisHCl (pH 7.5), 100 mM EDTA, 0.4% (v/v) SDS, and 20% (v/v) glycerol.

T7 Helicase gp4A' Protein and DNA Substrates

The 4A' protein was purified and stored as described previously [12,20]. The protein concentration was determined by two methods which provided similar concentrations. The first method was the Bradford assay using bovine serum albumin as a standard, and the second method was the absorbance measurements at 280 nm in 8 M urea (the extinction coefficient is $76,100\text{ M}^{-1}\text{cm}^{-1}$). The oligodeoxynucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). They were purified by Urea-PAGE, and their concentrations were determined spectroscopically in TBE buffer containing 8 M urea by taking absorbance

measurements at $\lambda_{\text{ex}} = 260\text{ nm}$ in a Hewlett & Packard 8452A diode array spectrophotometer. The extinction coefficients for each DNA species were calculated from the DNA sequences using extinction coefficients of individual bases ($\text{M}^{-1}\text{cm}^{-1}$): $\epsilon_{\text{A}} = 15,200$, $\epsilon_{\text{C}} = 7,050$, $\epsilon_{\text{G}} = 12,010$, and $\epsilon_{\text{T}} = 8,400$.

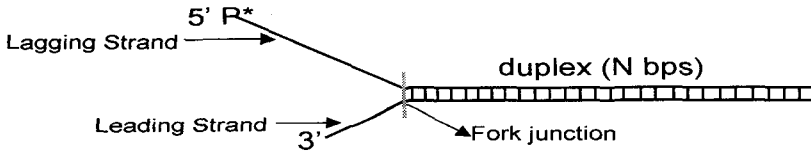
Forked DNA substrates used in this study are depicted in Table 1. The duplex regions of the DNA substrates were 30, 45, and 60 bp in length. The ssDNA regions, two single-stranded tails, were designed and prepared as illustrated in Table 1. The lengths of the single-stranded tail region for the lagging strand and the leading strand of the forked DNA substrates were 36 bases and 15 bases, respectively. The 5'-end of the longer DNA strand (lagging strand) was end-labeled with ^{32}P using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 nucleotide kinase.

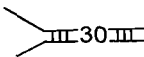
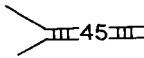
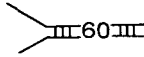


The DNA "trap" used in the dsDNA unwinding assay performed at high protein concentration was unlabeled single-stranded lagging strand of each forked DNA substrate. A $0.6\text{ }\mu\text{M}$ stock solution of dsDNA was prepared by mixing $0.6\text{ }\mu\text{M}$ of labeled DNA (lagging strand) with its complementary oligodeoxynucleotide ($0.9\text{ }\mu\text{M}$) in annealing buffer (50 mM NaCl, 10 mM Tris (pH 7.5) at $25\text{ }^{\circ}\text{C}$ and 1 mM MgCl_2) followed by heating for 5 minutes $90\text{ }^{\circ}\text{C}$ and slowly cooling to room temperature.

dsDNA Unwinding Assay - Rapid Chemical Quenched-Flow Kinetics Experiments

Rapid chemical quench experiments were carried out using a quench-flow apparatus (KinTek RQF-3). All reactions were performed in buffer T at specified temperature.

Table 1. Forked DNA substrates



Substrate	Structure	Sequence of top strand of duplex
ds30		5' - GAG CGG ATT ACT ATA CTA CAT TAG AAT TCA - 3'
ds45		5' - GAG CGG ATT ACT ATA CTA CAT TAG AAT TCA GAG TGT AGA GAT TCG - 3'
ds60		5' - GAG CGG ATT ACT ATA CTA CAT TAG AAT TCA GAG TGT AGA GAT TCG GTA AGT AGG ATC ATG - 3'
5' tail	 ssDNA	5' - TAC GAT CTA GCC TCA ATA CCA GGG TCA GGT TCG TTA
3' tail	 ssDNA	ATT GCT TGT ATG GTG - 3'

4A' protein (1.0 μM hexamer) was pre-mixed with the ^{32}P -labeled DNA substrate (4 nM) in buffer T containing 1.5 mM EDTA and 4 mM dTTP, and incubated at room temperature for 20 minutes and then loaded in one syringe of the quenched-flow machine. The other syringe contained 2.0 mM dTTP in buffer T plus 10 mM MgCl_2 and an appropriate trap DNA (unlabeled leading strand DNA, 3.0 μM). Reactions were initiated by rapidly mixing the two solutions, and quenched by the quench buffer added from the third syringe at varying time points (20 ms to 200 s). The fraction of ssDNA in the sample at time zero was determined by mixing the quench solution containing 10 mM MgCl_2 and trap DNA (3.0 μM) with the solution containing the 4A' protein and DNA substrates. The zero time control consisted of complete mixtures, but the quench buffer was added prior to addition of 4A' protein and DNA substrates. The quenched samples were analyzed by non-denaturing polyacrylamide gel electrophoresis (4 - 20% gradient PAGE) to separate the duplex from the ssDNA. The radioactivity in each band was quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and the fraction of DNA duplexes unwound at t, F(t) was calculated from the following equation as described previously [1];

$$F(t) = \frac{[\text{ssDNA}(t)]}{[\text{dsDNA}(t)]} = \left(\frac{R_s(t)}{R_s(t) + R_d(t)} - \frac{R_s(0)}{R_s(0) + R_d(0)} \right) \bigg/ \left(\frac{R_{sh}}{R_{sh} + R_{dh}} - \frac{R_s(0)}{R_s(0) + R_d(0)} \right) \quad \text{Eq.1}$$

Where $R_s(t)$ and $R_d(t)$ are radioactivity within each band corresponding to single-stranded and duplex DNA, respectively at time t. $R_s(0)$ and $R_d(0)$ are the corresponding quantities at $t=0$. R_{sh} and R_{dh} are the corresponding radioactivities obtained by heat denaturing the DNA for 5 min at 95°C and loading on the gel immediately, which is the control to measure the extent of maximum unwinding.

Analysis of dsDNA Unwinding Kinetics Containing a Lag Phase

The fraction of ssDNA was plotted against reaction time, and the data were fit numerically to an equation that relates the dsDNA unwinding rate constant to step size and duplex length as described in the following equation (Eq. 2);

$$\text{ssDNA}(t) = A_1 \left[1 - \sum_{r=1}^n \frac{(ku * t)^{r-1}}{(r-1)!} * \exp(-ku * t) \right] + A_2 [1 - \exp(-ks * t)] \quad \text{Eq.2}$$

In equation 2, A_1 is the amplitude of the lag phase, ku is the unwinding rate constant for each step, n is the number of steps required to unwind duplex DNA completely, and

A_2 and ks are the amplitude and rate constant of the second slower exponential phase. The first term in equation 2 represents the time-course of DNA unwinding, which occurs with a lag phase, whereas the second exponential phase reflects the slower rate of unwinding. Therefore, the unwinding time-course is a superposition of a rapid lag phase and a slower exponential phase.

Simulation and Global Fitting of the Stepping Mechanism

The stepping mechanism (Fig. 2A) was simulated using the stepping equation in the software package "Scientist" (MicroMath Software, SLC, Utah, USA). For global fitting of the mechanism all the data with the different lengths of duplex DNA were combined into one data set. To determine the step size, the data were fit globally to the stepping equation (Eq. 2). A separate equation, containing the appropriate number of steps, was assigned to each data set for a particular length of dsDNA. The quality of the fit was determined using the model selection criterion (MSC) provided by "Scientist" software. A larger value for the MSC indicates a better fit to the model. The MSC value was determined by a least squares method, and defined as a criterion to evaluate the suitability of the model used for fitting.

Steady State dTTPase Assay

The steady state dTTPase activity stimulated by ssM13 DNA was measured at various temperatures (4 - 38°C). ssM13 DNA was prepared and purified as described [25]. 4A' protein (0.10 μM hexamer) was mixed with dTTP (3.0 mM) and [α - ^{32}P]dTTP with ssM13 DNA (11 nM) in the following reaction buffer: 50 mM TrisHCl (pH 7.5), 40 mM NaCl, 10% (v/v) glycerol, and MgCl_2 (5.0 mM). After various times of incubation, the reactions were quenched with 2.0 M formic acid. The [α - ^{32}P]dTTP was separated from [α - ^{32}P]dTTP by polyethyleneimine (PEI)-cellulose TLC using 0.3 M potassium phosphate (pH 3.4) as the developing buffer. The radioactivity was quantitated using a phosphorImager. The molar dTTP was plotted as a function of time, and the slope of the plot provided the steady state dTTPase rate at the specified temperature.

Results

DNA Substrates for dsDNA Unwinding Catalysis by 4A' Helicase

T7 helicase gp4A' (4A') requires two noncomplementary

ssDNA tails and a DNA substrate resembling a fork to unwind DNA [1]. The 4A' hexamer interacts with the two strands of the fork asymmetrically. 4A' requires 35 bases of 5' ssDNA tail and 15 bases of 3' ssDNA tail to unwind DNA at maximal rates [1]. The asymmetric interactions of 4A' hexamer with the fork DNA suggests that only one strand of the duplex DNA is bound in the central channel. The 5' strand, corresponding to the lagging strand of the fork, would be bound in the central channel, and the 3' strand corresponding to the leading strand of the fork, would be excluded [1,19]. The 4A' helicase is proposed to move unidirectionally on the 5' strand during DNA unwinding. The DNA unwinding activity of 4A' was inhibited when a biotin-streptavidin block was placed in the 5' strand, but no inhibition was observed when the block was placed in the 3' strand [11]. Therefore, the forked DNA substrates used in this study were designed to possess two noncomplementary tails of 5' tail and 3' tail, flanking the duplex DNA region, with a length of 36 bases and 15 bases, respectively (See Table 1 for sequences).

Single Turnover Kinetics of dsDNA Unwinding by 4A'

Kinetic studies of 4A'-catalyzed DNA unwinding were performed with DNA substrates containing different lengths of duplex region, shown schematically in Table 1, using a rapid chemical quenched-flow apparatus. Experiments were carried out under conditions where other processes, such as oligomerization of 4A' and binding to DNA, were not limiting [2,21]. This was accomplished by incubating 4A' (1 μ M hexamer) with the DNA substrate (4 nM) and dTTP (4 mM) in the absence of Mg^{2+} (1.5 mM EDTA present), where unwinding does not occur, yet 4A' hexamer assembles at the junction of the forked DNA substrate. Without preincubation, the slow binding of 4A' binding to the forked DNA governs the overall unwinding kinetics. As indicated in the figure 1A, the DNA unwinding reaction was initiated by rapid mixing of the complex with $MgCl_2$ (10 mM) and a large excess (3 μ M) of a trap DNA (unlabeled lagging strand). The trap serves to prevent re-initiation of unwinding by trapping free 4A' and any 4A' that dissociates during unwinding. The trap DNA also prevents reannealing of the displaced leading strand ssDNA. These conditions therefore measure the single-turnover kinetics of DNA unwinding.

The amount of unwound DNA was determined at various times by non-denaturing PAGE, as shown in the figure 1. The 45 bp forked DNA was unwound rapidly with ~60% of

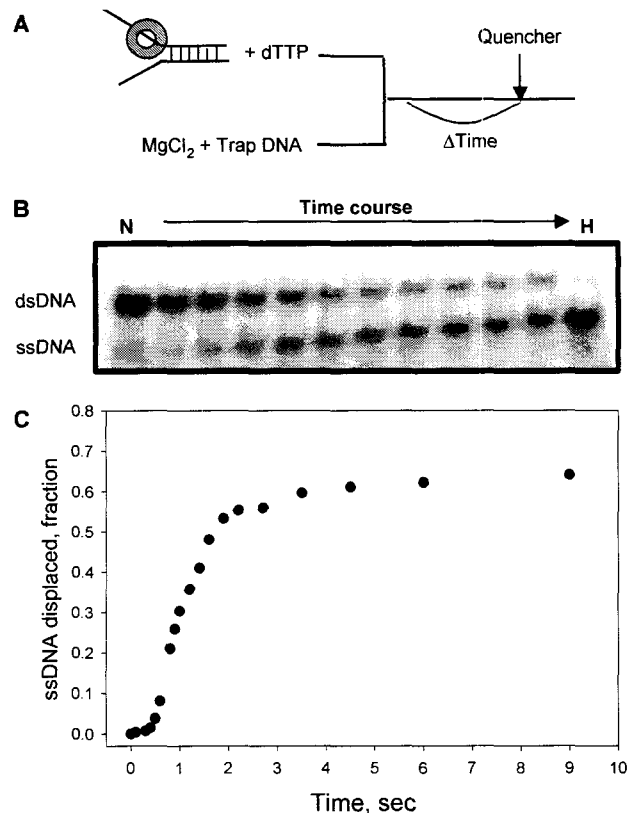


Fig. 1. Single-turnover kinetic time course for 4A'-catalyzed unwinding of a forked DNA substrate, ds45. (A) The design of the experiment using the quenched-flow method is shown. 4A' (1.0 μ M hexamer) was pre-mixed in one syringe with ^{32}P i end-labeled forked DNA (4 nM), dTTP (4 mM), and EDTA (5 mM) at 18°C in buffer T. An excess of trap DNA (unlabeled lagging strand ssDNA, 3.0 μ M) and $MgCl_2$ (10 mM) were added from a separate syringe to initiate the dsDNA unwinding reaction. The reaction mixture was incubated for varying time and quenched. The given concentrations are the concentrations in the syringes. (B) Image of a non-denaturing polyacrylamide gel (4-20% gradient gel) after electrophoresis of the ^{32}P i end-labeled DNA obtained from the rapid quench experiments. Lane N represents the zero time control, in which 4A' was added last to the solution containing both quench buffer and forked DNA substrate; lane H represents the heat-denatured control obtained by heating the entire reaction mixture at 95°C for 5 min. (C) Unwinding time course plotted as the fraction of duplex DNA unwound. Fraction of ssDNA displaced from the forked DNA substrate was calculated using the Eq. 1.

the DNA molecules unwound within 2 sec. The unwinding occurred with a distinct lag phase (Fig. 1C), which in a single-turnover experiment indicates the presence of at least one intermediate along the pathway to fully unwound DNA. This lag phase is not due to reannealing of the fully unwound

DNA because the trap DNA would prevent reannealing of the displaced ssDNA product to the isotope-labeled lagging strand ssDNA. The slow DNA-protein binding step was eliminated under the reaction condition, where 4A' was pre-incubated with forked DNA substrates. The lag phase suggests that DNA unwinding occurs by a multi-step process with highly populated intermediate states along the pathway. The rate constants for the formation and decay of these intermediates must be comparable in magnitude, otherwise a lag phase would not be observed [10].

The Step Size and the Rate of DNA Unwinding

One likely explanation for the lag phase is that partially unwound DNA molecules are formed as intermediates during unwinding [3]. This process was described by a stepping model (Fig. 2A). In the stepping model the helicase carries out DNA unwinding in kinetically discrete steps, which results in the transient accumulation of each intermediate (Fig. 2B). To illustrate this process, the kinetics of 60 bp duplex DNA unwinding was simulated. The transient formation of partially unwound intermediates is shown in figure 2B. In this kinetic simulation, step size of 15 bp and unwinding rate of 2.0 s^{-1} were assumed. With these parameters, unwinding proceeds with three intermediates and the final ssDNAs are formed with a lag. The stepping model predicts that the observed lag will depend on the dsDNA length (Fig. 2B). As the duplex length (L) increases, the number of steps required for unwinding dsDNA completely (n) and the lag phase increases (Fig. 2B).

To determine the 4A' helicase step size and the rate of DNA unwinding, the unwinding kinetics were measured with DNA substrates varying in duplex length, L, ranging from 30, 45, to 60 bp (Table 1). A lag phase was observed with each DNA substrate, and the lag increased with duplex length (Fig. 3A). The amount of unwound DNA remained constant with dsDNA length. This indicates that at least for the dsDNA lengths used, the helicase unwinds in a highly processive manner without dissociating from the DNA. The time courses of DNA unwinding were biphasic; a rapid lag phase (~50%) is completed within 1-2 seconds, followed by a significantly slower phase (~20%). The slower phase was not eliminated, when an excess of ssDNA trap (3 μM over 4 nM dsDNA substrate) was added. This slower exponential phase is likely due to a population of the 4A'-DNA complex that unwinds slowly. As a result, the time courses were analyzed using equation 2, including a term

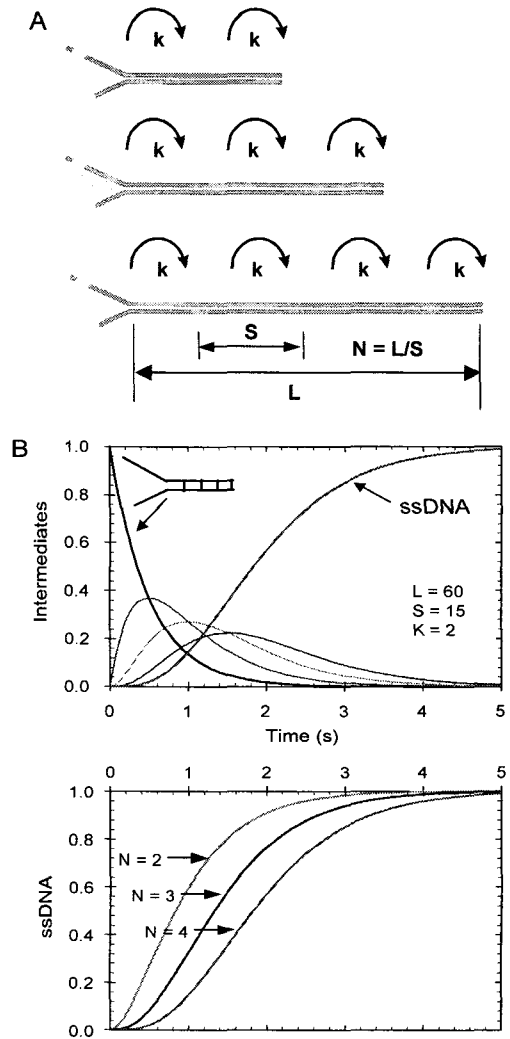


Fig. 2. The stepping model of DNA unwinding. In the stepping model, the helicase carries out DNA unwinding in kinetically discrete steps. (A) The implications of the stepping model are shown for the unwinding of three DNA molecules, with increasing length, by the same mechanism. The number of steps necessary for unwinding (n) depends on the length of the dsDNA (L) and the step size (S) as shown (see Materials and Methods). (B) Top panel; simulated kinetic profile for unwinding of an 60 bp duplex (ds60) showing the transient formation of partially unwound intermediates. Simulations were performed with the Scientist program, to the scheme shown in (A), with the parameters (S = 15 bp, n = 4, and $k_u = 2.0 \text{ s}^{-1}$). Unwinding proceeds with three partially unwound DNA intermediates, resulting in lag kinetics for the production of fully unwound ssDNA. It is assumed that upon quenching of 4A' helicase, partially unwound intermediates reform fully dsDNA. Bottom panel; Accumulation of ssDNA was simulated using mechanisms with the same step size (S = 15 bp). As L increases (L = 30, 45, and 60 bp), both n and the lag observed in the accumulation of ssDNA increase (from left to right L = 30, 45, and 60 bp duplex DNA).

describing the slow complexes (Fig. 3A).

The data shown in figure 3A were globally fitted to the stepping equation (Eq. 2) using the software "Scientist" to determine the step-size and rate of unwinding. Since it was not possible to include the step size (or the number of steps) as a floating parameter into the modeling procedure, the number of steps was specified in the stepping equation (Eq. 2) for each duplex length. The data were fit best with a step size of 10 bp and stepping rate of 3.7 s^{-1} . The quality of the fit with step sizes of 15 and 8 bp ranked second and third, respectively (Fig. 3B). The data could not be described well with a step size of 6. Thus, it was concluded that T7 helicase unwinds DNA with a kinetic step size of 10 bp. As expected, the number of steps, n , increases with duplex length, L , with the best integer values of n determined to be 3, 4, and 6 for $L = 30$ -, 45-, and 60-bp duplexes, respectively. For all three DNA substrates, the unwinding step size, S , determined from the ratio L/n varied from 10 to 11 bp, with an average value of 10.4 bp. Therefore, 4A' unwinds duplex about one-turn of a B-form DNA complex in each step. The fact that all time courses in figure 3A are well described by the same value of $k_u = 3.66 \pm 0.05 \text{ s}^{-1}$ for the observed unwinding rate per step, independent of duplex length, provides further support for the proposed model.

Temperature Dependence of dsDNA Unwinding and dTTPase Activities

The experiments described so far were performed at 18°C . The temperature dependence of the kinetics of dsDNA unwinding was examined and compared to the kinetics of dTTP hydrolysis stimulated by ssM13 DNA to investigate if the temperature would affect the step size of dsDNA unwinding. dsDNA unwinding assays were performed with ds30 forked DNA substrate at three different temperatures (4, 18, and 31°C). Time courses of dsDNA unwinding obtained at various temperatures were plotted in figure 4A, and the stepping equation (Eq. 2) was applied to each unwinding kinetics data. With the step size of 10 bp, the stepping equation described well all the dsDNA unwinding kinetics, as shown by the superimposed continuous lines in figure 4A. The rate constants for the dsDNA unwinding (k_u) increased with increasing temperature (see legends to Fig. 4A). Thus, the step size for dsDNA unwinding by 4A' does not appear to be affected by temperature, suggesting that the stepping mechanism is an inherent property not affected by the speed of enzyme catalysis. In other words, 4A'

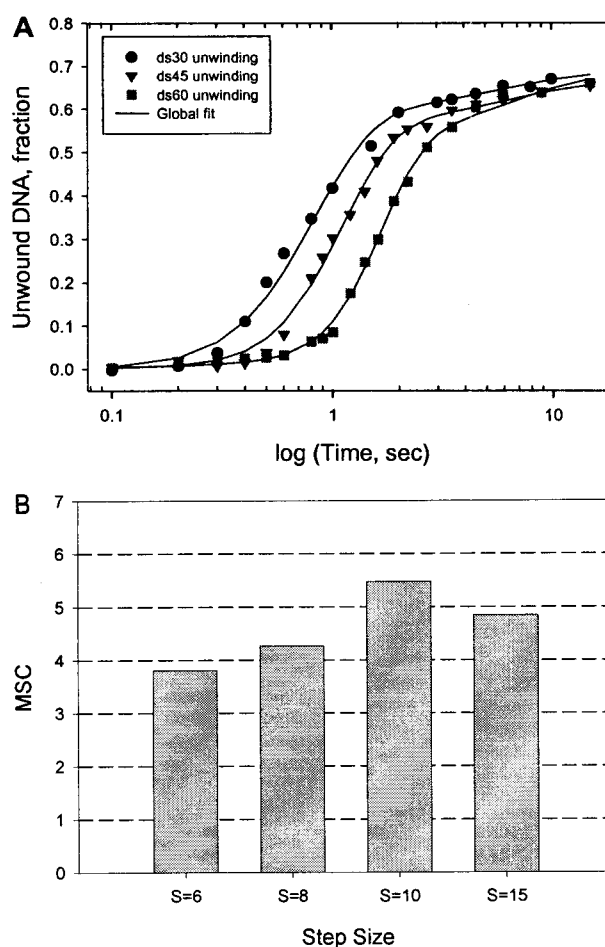


Fig. 3. Global fitting of the single-turnover kinetics of 4A'-catalyzed dsDNA unwinding. 4A' ($1.0 \mu\text{M}$ hexamer) was incubated with 4 nM DNA: ds30 (circles, $L = 30$), ds45 (triangles, $L = 45$), and ds60 (squares, $L = 60$), and unwinding was initiated with 10 mM MgCl_2 and $3.0 \mu\text{M}$ trap DNA. (A) Data from all three time courses were globally fitted to the stepping mechanism with Eq. 2 by fixing $n = 3, 4,$ and 6 for the $L = 30, 45,$ and 60 bp substrates, respectively. The fitted values of k_u and k_s were constrained to be the same for each time course, whereas the amplitudes for each time course (A_1 and A_2) were floated. The best fit parameters are as follows: $L = 30 \text{ bp}$ ($n = 3, A_1 = 0.569 \pm 0.015, A_2 = 0.12 \pm 0.028$); $L = 45 \text{ bp}$ ($n = 4, A_1 = 0.534 \pm 0.016, A_2 = 0.13 \pm 0.025$); $L = 60 \text{ bp}$ ($n = 6, A_1 = 0.472 \pm 0.025, A_2 = 0.214 \pm 0.029$), with $k_u = 3.66 \pm 0.049 \text{ s}^{-1}$, and $k_s = 0.17 \pm 0.04 \text{ s}^{-1}$ (average step size, $S = L/n = 10.4 \text{ bp}$). (B) shows the statistical results of global fitting, goodness of fit for global fitting. The bar graph shows the Model Selection Criterion (MSC) for several global fits performed with different step sizes. A larger value for the MSC indicates a better fit. The fit with the step size of 10 bp gives the best result with a highest MSC value of 5.47 . The MSC is based upon the quality of the fit of the model to the data, as predicted by a least squares method, and evaluates the suitability of the model used for fitting.

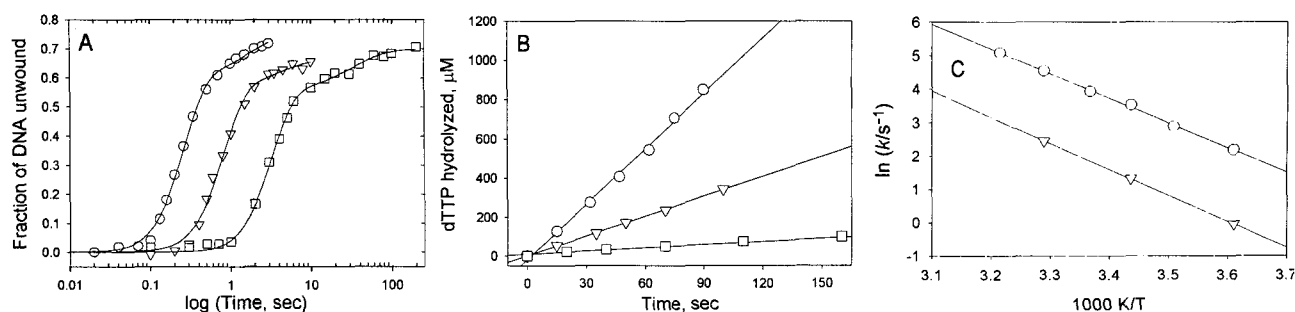


Fig. 4. Temperature dependence of dsDNA unwinding and steady state dTTPase activities by 4A'. (A) shows the single turnover kinetic time courses for 4A'-catalyzed unwinding of a forked DNA substrate, ds30, at various temperatures. Time courses of ds30 unwinding were obtained by rapid quench dsDNA unwinding experiment as performed in Fig. 3A. Each kinetic time course of ds30 DNA unwinding was fitted to Eq. 2 with a step size of 10 bp, as shown by continuous curves. The best fitting parameters are as follows: (\circ) unwinding at 31°C ($A_1 = 0.565 \pm 0.037$, $A_2 = 0.194 \pm 0.039$, $k_u = 11.66 \pm 0.32 \text{ s}^{-1}$, and $k_s = 0.55 \pm 0.41 \text{ s}^{-1}$); (∇) unwinding at 18°C ($A_1 = 0.532 \pm 0.051$, $A_2 = 0.134 \pm 0.039$, $k_u = 3.75 \pm 0.14 \text{ s}^{-1}$, and $k_s = 0.25 \pm 0.22 \text{ s}^{-1}$); (\square) unwinding at 4°C ($A_1 = 0.528 \pm 0.016$, $A_2 = 0.17 \pm 0.02$, $k_u = 0.94 \pm 0.027 \text{ s}^{-1}$, and $k_s = 0.03 \pm 0.007 \text{ s}^{-1}$). (B) shows temperature dependence of steady state dTTPase activities stimulated by ssM13 DNA. 4A' (0.10 μM hexamer) was mixed with dTTP (3.0 mM) and [α - ^{32}P]dTTP with ssM13 DNA (11 nM) in buffer T plus 5 mM MgCl_2 at various temperatures. dTTP hydrolysis kinetic time courses are fitted to linear equation to get k_{cat} values at each temperature. k_{cat} values for dTTP hydrolysis in the presence of ssM13 DNA are 94.5 ± 2.54 , 33.9 ± 0.35 , and $8.49 \pm 0.31 \text{ s}^{-1}$ per hexamer at 31 (\circ), 18 (∇), and 4°C (\square), respectively. (C) Arrhenius plots ($\ln k = \ln A - E_a/RT$) of the dsDNA unwinding rate constant (\circ) and of the steady state dTTPase rate constant, k_{cat} (∇), provide slopes of -7.80 and -7.41 K, respectively. The slope is equivalent to $-E_a/R$ in the Arrhenius equation (Eq. 3), where R is gas constant (1.98 kcal/mol K), and E_a is the activation energy (kcal/mol).

helicase unwinds duplex DNA in discrete steps, with a step size of one-turn of the DNA helix regardless of its speed of unwinding. Steady state dTTPase rates in the presence of ssM13 DNA were also measured at various temperatures (4 - 38°C). Initial velocities of dTTP hydrolysis increased with increasing temperature (Fig. 4B). Figure 4C shows Arrhenius plots of the rate constants of dsDNA unwinding and steady state dTTPase activities. The Arrhenius equation shown below (Eq. 3) provides activation energy for each catalytic process;

$$\ln k = \ln A - E_a/RT \quad \text{Eq.3}$$

where, A represents the pre-exponential factor, R is gas constant (1.98 kcal/mol K), and E_a represents the activation energy. The Arrhenius plots (Fig. 4C) give straight lines for dsDNA unwinding and dTTPase activities, indicating that the two catalysis occur with a mechanism, which is not changed over the range of temperatures (4 - 38°C). The apparent activation energies for dsDNA unwinding and dTTP hydrolysis turnovers with ssM13 DNA are 15.5 and 14.7 kcal/mol, respectively (Fig. 4C). The fact that the activation energies of these two catalysis processes are very close to each other indicates that these two activities are tightly coupled during the catalysis process.

Discussion

An understanding of the mechanism by which helicases

unwind DNA requires quantitative studies of the kinetics of duplex DNA unwinding. Most studies of helicase-catalyzed DNA unwinding have used steady state measurements, which generally do not provide mechanistic information on the elementary steps in the reaction. This is because such experiments measure only the slowest step in the unwinding reaction. In fact, the slowest step is often unrelated to the unwinding reaction and represents assembly of the initiation complex or dissociation of the products, ssDNA or the helicase itself. Therefore, single turnover kinetic experiments are necessary to probe the mechanistic details of DNA unwinding.

The 4A'-catalyzed dsDNA Unwinding Occurs in a Step-wise Manner

Helicase reaction condition under which the true single turnover rate can be measured was established. When 4A' protein is preincubated with the forked DNA substrate at high [dTTP] in the absence of Mg^{2+} a 4A'-forked DNA complex is formed [1,21]. This complex is preformed hence it overcomes the slow DNA binding process [2]. The DNA unwinding is then initiated by adding MgCl_2 when rapid unwinding occurs within 1~2 seconds. The rapid unwinding shows a lag in the production of fully unwound ssDNA, suggesting that 4A'-catalyzed DNA unwinding occurs in a step-wise manner producing partially unwound DNA

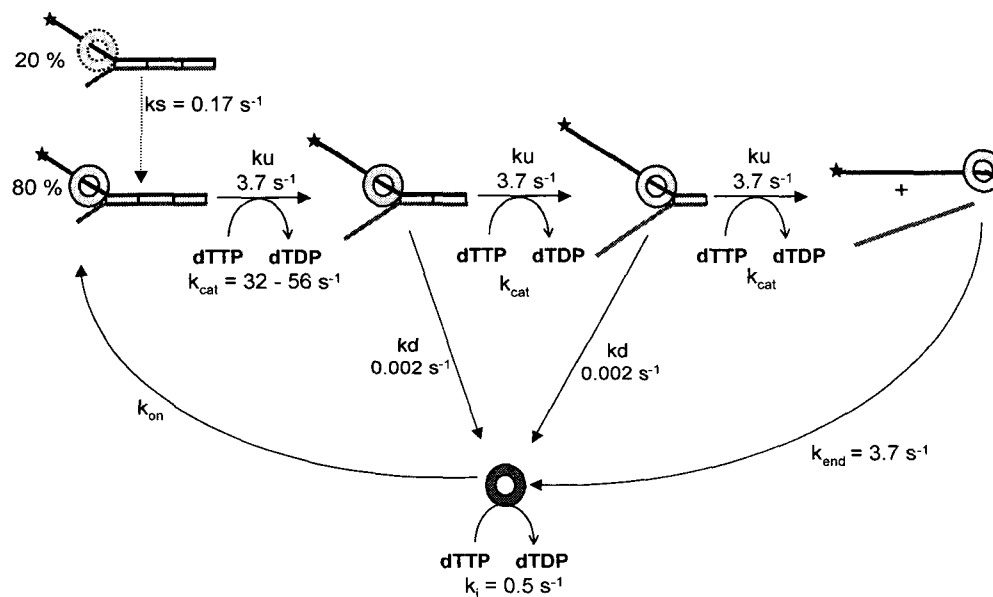


Fig. 5. Mechanism of dsDNA unwinding coupled with dTTP hydrolysis by 4A' helicase. The model shows a schematic of all the processes involved in dsDNA unwinding. Forked DNA of 30 bp duplex length is depicted. It is assumed that 20% of pre-bound 4A'-forked DNA complex undergoes a slow isomerization step, and ~80% of the complexes are competent that perform dsDNA unwinding in a step-wise manner. 4A' unwinds the forked DNA with a step size of 10 bp (each segment in dsDNA represents 10 bp) and a stepping rate of 3.7 s^{-1} , hydrolyzing dTTP at a rate of $32 \sim 56 \text{ s}^{-1}$. While unwinding, 4A' may fall off from the DNA at a rate of $k_d = 0.002 \text{ s}^{-1}$. After reaching the 3'-end of the lagging strand DNA (star-labeled strand), 4A' dissociates from the end at a dissociation rate of $k_{\text{end}} = 3.7 \text{ s}^{-1}$. Free 4A' hexamer recycles by binding to the junction of the forked DNA at a rate of k_{on} , meanwhile free 4A' hydrolyzes dTTP at an intrinsic rate, k_i .

intermediates. The lag period increases with increasing DNA duplex length, and quantitative analysis of the kinetics using the stepping mechanism (Fig. 2A) and Eq. 2 shows that the 4A'-catalyzed DNA unwinding occurs in discrete steps of 10-11 bp. The observed step rate constant, k_u , was determined from global analysis of the lag phase ($k_u = 3.66 \text{ s}^{-1}$ per step).

An additional slow phase following the rapid phase of DNA unwinding was also observed. The slower phase of unwinding was not affected by the presence and concentration of the ssDNA trap. I propose that the slower phase represents a portion of 4A'-DNA complex that is incompetent, must undergo a slow isomerization, with a macroscopic rate constant, $k_s = 0.17 \text{ s}^{-1}$, to form a productive complex before unwinding. This slow isomerization of pre-bound helicase-DNA complexes was reported for other helicases [3,13]. Because of this slow phase, the analysis of a single time course of unwinding provides only the lower limit of the number of steps, n [3]. However, simultaneous analysis of the time courses of 3 different DNA lengths shown in figure 3A provided additional constraints on the upper limit of n for each duplex, yielding a step size, $S = L/n$, equal to 10.4 bp, while the global fits with $S = 6$ or 8 gave poorer fits.

The Replicative Helicase T7 4A' Unwinds dsDNA with a High Processivity.

The processivity of DNA unwinding is the probability that the helicase will perform the next unwinding step rather than dissociate from the substrate. A helicase with a low processivity of DNA unwinding will show a smaller amplitude of dsDNA unwound with longer duplex DNA, under single turnover reaction conditions. The amplitude of DNA unwinding was analyzed as the fraction of decrease in the initially present amount of 4A'-forked DNA complex. Figure 3 shows that the amplitudes of dsDNA unwinding barely decreased with increasing duplex length. This indicates that 4A' does not dissociate during unwinding of dsDNA of 30-60 bp duplex length. Therefore, DNA unwinding by 4A' is highly processive. The other highly processive helicase, RecBCD, has been reported to unwind an average of 30 kbp before dissociating at 25°C [22], and the RecBC (without D subunit) DNA helicase was known to unwind up to 6.2 kbp per binding event [15,16]. Recently, a class of RNA helicase, the DEXH protein NPH-II has been reported to have a relatively high processivity at saturating concentration of ATP [13]. Other hexameric helicase, the *E. coli* DnaB also unwinds DNA with relatively high processivity, although

it has not been examined quantitatively [6,17]. In comparison, the DNA repair helicase UvrD of *E. coli* has been observed to be not highly processive, dissociating from the DNA every 40 - 50 bp [3]. Therefore, the hexameric, replicative T7 helicase gp4A' is designed to be highly processive as compared to the monomeric helicase, such as UvrD helicase.

Temperature Effect and Thermodynamic Efficiency of DNA Unwinding

Studies of the effect of temperature on the step size and the rate of DNA unwinding indicate that the discrete step size of 10~11 bp does not change with temperature, although the stepping rate decreases with decreasing temperature. This supports the conclusion that the step size reflects a mechanistic constraint of the 4A' helicase, which is not affected by the speed of catalysis process. The Arrhenius plot provides a linear relationship between rates and $1/T$, which can be extrapolated to obtain the unwinding rate at 37°C. The linear equation provides a rate of 190 bp/s for dsDNA unwinding at 37°C, which is faster than the rate at 18°C (37 bp/s). Studies in the literature show unwinding rates of other helicases; *E. coli* DnaB unwinds DNA at 30 - 60 bp/s at 30°C [17], RecBCD helicase unwinds duplex DNA at rates of 470 ± 30 bp/s at 25°C [23,24], *E. coli* Rep unwinds short duplex DNA at a rate of 23 ± 3 bp/s at 25°C [7], and *E. coli* Rho helicase unwinds RNA-DNA duplex at ~ 20 bp/s at 37°C [26].

Implications for the Model of dsDNA Unwinding by 4A' Helicase

The kinetic data of unwinding are summarized in a mechanistic model shown in figure 5. Under single turnover reaction conditions, it is proposed that some fraction ($\sim 20\%$) of 4A' helicase-DNA complex undergo a slow isomerization step at a rate of $k_s = 0.17 \text{ s}^{-1}$ prior to unwinding DNA. Since the ratio of A_1/A_2 appears to be constant (Fig. 3A), this suggests that two forms of pre-bound complex of 4A'-forked DNA are not in rapid equilibrium, otherwise a single unwinding phase would be observed. The competent complexes of 4A'-forked DNA unwind in a step-wise manner at a stepping rate (k_u) of 3.66 s^{-1} per step of 10 bp, displacing ssDNA products. During the dsDNA unwinding, dTTP hydrolysis occurs at a rate of k_{cat} ($32 - 56 \text{ s}^{-1}$ per hexamer, unpublished data). Eventually, the 4A' helicase reaches the 3'-end of lagging strand DNA, completing displacement of the complementary DNA strand. The slow steady state phase of dTTP hydrolysis is likely due to hydrolysis by free 4A'

at an intrinsic dTTPase rate ($k_i = 0.5 \text{ s}^{-1}$) and recycling of the helicase limited by the dissociation from the end of dsDNA at a rate of $k_{end} = 3.7 \text{ s}^{-1}$. These dissociation rates from ssDNA both at the 3'-terminal and during the translocation have been measured separately [14]. Taken together, I propose a mechanistic model as shown in figure 5, in which the quantitative evidences for the kinetic pathway of dsDNA unwinding are described.

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초록 : T7 박테리오파지 gp4 DNA helicase에 의한 DNA unwinding에서 step size의 반응속도론적 측정

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T7 박테리오파지 gp4는 dTTP 가수분해에너지를 이용하여 DNA 복제시 이중 나선 DNA를 단일가닥 DNA로 풀어내는 나선효소(helicase)이다. T7 나선효소의 활성형의 4차구조는 한가운데 구멍을 지닌 육량체 고리모양이다. 단일가닥 DNA는 나선효소가 5' → 3'방향으로 이동할 때 육량체 고리의 구멍으로 빠져나간다. 이러한 DNA의 이중나선 풀어헤침을 빠른 효소반응속도 측정법을 이용하여 정량적으로 측정하였으며, 그 결과 단일가닥 DNA 산물들이 생성되기 전에 지연상태(lag phase)가 존재함을 관찰하였다. 이러한 지연상태를 나선효소에 의한 이중나선 DNA의 풀어헤침이 속도론적 단계과정(kinetic stepping)을 거친다는 모델로써 분석하였다. 예상대로 이중나선의 길이가 클수록 지연상태의 지속시간이 늘어났다. T7 나선효소가 이중나선 DNA를 풀어내는 과정에서 놓여준 trap DNA는 풀어내는 이중나선 DNA의 양을 변화시키지 못하여서, T7 나선효소가 매우 큰 공정성을 지닌 효소임을 알 수 있었다. 이러한 속도론적 data를 global fitting법을 써서 kinetic stepping 모델에 적용한 결과 매 단계(step)마다 10~11개의 염기쌍이 풀려지고 1초당 3.7번의 step이 일어난다는 것을 알 수 있었다. DNA 풀어헤침과 dTTP가수분해의 메커니즘과 이들의 연계성은 4~37°C사이의 온도범위에서 영향을 받지 않았다. 이상을 종합할 때, T7 나선효소의 이중나선 DNA의 풀어헤침 시 나타나는 속도론적 단계과정은 DNA 복제시 이용되는 나선효소의 내재적 속성임을 알 수 있다.