Single-stranded DNA Enhances the Rate of Product Release During Nucleotide Hydrolysis Reaction by T7 DNA Helicase

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Received August 7, 2006

Bacteriophage T7 gp4A' is a ring-shaped hexameric DNA helicase that catalyzes duplex DNA unwinding using dTTP hydrolysis as an energy source. To investigate the effect of single-stranded DNA (ssDNA) on the kinetic pathway of dTTP hydrolysis by the T7 DNA helicase complexed with ssDNA, we have first determined optimal concentration of long circular M13 single-stranded DNA and pre-incubation time in the absence of Mg^{2+}, which is necessary for the helicase-ssDNA complex formation. Steady state dTTP hydrolysis in the absence of Mg^{2+} by the helicase-ssDNA complex provided k_{cat} of 8.5×10^{-3} sec^{-1}. Pre-steady state kinetics of the dTTP hydrolysis by the pre-assembled hexameric helicase was monitored by using the rapid chemical quench-flow technique both in the presence and absence of M13 ssDNA. Pre-steady state dTTP hydrolysis showed distinct burst kinetics in both cases, indicating that product release step is slower than dTTP hydrolysis step. Pre-steady state burst rates were similar both in the presence and absence of ssDNA, while steady state dTTP hydrolysis rate in the presence of ssDNA was much faster than in the absence of ssDNA. These results suggest that single-stranded DNA stimulates dTTP hydrolysis reaction by T7 helicase by enhancing the rate of product release step.

Key Words : DNA helicase, M13 single-stranded DNA, Pre-steady state kinetics, Chemical quench-flow technique

Introduction

Helicases are motor proteins that translocate along nucleic acids using the energy of nucleoside triphosphate (NTP) hydrolysis. This activity enables them to carry out nucleic acid metabolic processes including those that require the separation of duplex nucleic acids into their component single strands.[1-3] They are also involved in critical biological processes such as DNA replication, repair, and recombination. Therefore defects in helicases lead to many human diseases including xeroderma pigmentosum, Bloom's syndrome,[4,5] and Werner's syndrome.[6-8]

Bacteriophage T7 gp4A' is a hexameric helicase that translocates along DNA during DNA replication and unwinds double-stranded DNA (dsDNA).[2,9] The assembly of T7 helicase subunits into hexamer is driven by NTP binding, and the hexamer is also stabilized by DNA.[10-12] The energy source for the T7 helicase is dTTP and the chemical energy of dTTP hydrolysis is converted into mechanical work to translocate along the DNA[13]. T7 hexameric helicase has a high affinity for single-stranded DNA (ssDNA) that binds within the central channel of the hexameric ring in the presence of dTTP or dTMPPCP but not in the presence of dTDP.[14] Previous studies showed that T7 helicase passes only 5' strand of double-stranded DNA through central channel and excludes 3' strand.[15,16] While translocation of T7 helicase proceeds at a rate of ~130 nt/s along ssDNA, hydrolyzing one dTTP per three-base movement,[17] dsDNA separation catalyzed by T7 helicase proceeds at a rate of ~15 bp/s with forked DNA.[18]

To investigate the effect of ssDNA on the kinetic pathway of dTTP hydrolysis by the T7 DNA helicase complexed with ssDNA, we have determined optimal concentration of M13 ssDNA and pre-incubation time in the absence of Mg^{2+}, which is necessary for the helicase-ssDNA complex formation. Because M13 is a long circular single-stranded DNA (7250 bases), T7 hexameric helicase bound to M13 ssDNA does not fall off from the M13 ssDNA until available dTTP is consumed up. Based on this, we were able to compare the kinetics of dTTP hydrolysis reaction both in the presence and absence of M13 ssDNA. Subsequent pre-steady state analysis provided insights into the step at which ssDNA stimulates dTTP turnovers by the hexameric T7 helicase.

Materials and Methods

Protein, Nucleotides, and Buffer. T7 gp4A' protein (referred to as the T7 helicase) is a M64L mutant of T7 helicase-primase protein that was over-expressed and purified as described previously.[13,19] The protein concentration was determined both by absorbance measurements at 280 nm in 8 M urea (the extinction coefficient is 76,100 M^{-1} cm^{-1}) and by the Bradford assay using bovine serum albumin as a standard. Both methods provided similar concentrations. The M13 ssDNA (M13mp18) was purified as described.[20] dTTP and dTDP were purchased from Sigma Chemicals, and [α-^{32}P]dTTP was obtained from Amersham Pharmacia

Biotech. Tris buffer was used throughout the experiments unless specified otherwise, which contained 50 mM Tris/Cl (pH 7.6), 40 mM NaCl, and 10 % (v/v) glycerol.

Titration of T7 helicase with M13 ssDNA. T7 helicase (2 μM hexamer) was incubated with M13 ssDNA (0-50 nM) in the Tris buffer containing 5 mM EDTA and 1 mM dTTP. After 15 minutes, radio-labeled 76mer ssDNA (final 6 μM) was added and the samples were filtered through the NC (nitrocellulose)-DEAE (diethylaminoethyl) double filter membrane assembly.[21] The membranes were washed before and after filtration of samples with the membrane wash buffer (50 mM Tris/Cl (pH 7.5), 5 mM NaCl). After the samples were filtered, radioactivity on both NC and DEAE filters was quantified using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). The molar amount of T7 helicase-76mer complex was calculated from the ratio of radioactivity retained on the NC membrane over the sum of radioactivity on both membranes, as described.[14]

Steady state dTTP hydrolysis in the absence of Mg^{2+}. 2 μM hexamer T7 helicase was mixed with 1 mM dTTP plus [α-^{32}P]dTTP, 5 mM EDTA, and 30 nM M13 ssDNA at 25 °C. After various times of incubation, the reactions were quenched with 4 M formic acid. The [α-^{32}P]dTDP was separated from [α-^{32}P]dTTP by polyethyleneimine (PEI)-cellulose TLC using 0.4 M potassium phosphate (pH 3.4) as the running buffer. The radioactivity was quantitated using a PhosphorImager. The molar dTDP was plotted as a function of time, and the slope of the plot provided the reaction rate.

Determination of pre-incubation time to form the T7 helicase-M13 ssDNA complex in the absence of Mg^{2+}. T7 helicase (4 μM hexamer) was mixed with an equal volume of EDTA (10 mM), dTTP (840 μM) plus [α-^{32}P]dTTP, and M13 ssDNA (60 nM). After various incubation times (2-30 minutes), the mixture was mixed with an equal volume of $MgCl_2$ (14.42 mM) and dTTP (5 mM) in a chemical quench-flow instrument. The reactions were quenched with 4 M formic acid after 0.02 sec. An aliquot of the quenched reactions was spotted on PEI-cellulose TLC and analyzed as above.

Pre-steady state acid-quench kinetics of dTTP hydrolysis. The kinetic experiments were conducted on a rapid chemical quench-flow instrument at 18 °C. First, T7 helicase (4 μM hexamer) in Tris buffer was mixed with an equal volume of [α-^{32}P]dTTP + dTTP (840 μM) and EDTA (10 mM) in the presence (60 nM) and absence of M13 ssDNA. The mixture was immediately loaded into the quench-flow instrument, and in exactly 3 minutes 24 μL was mixed with an equal volume of $MgCl_2$ (9.42 mM in the same buffer). After various times, the reactions were quenched with 4 M formic acid. An aliquot of the quenched reactions was spotted on PEI-cellulose TLC and analyzed as above. The molar concentration dTDP was plotted versus the time of reaction, and the data were fit to the burst equation (Eq. 1).

$$D(t) = A^*(1 - \exp(-k_1^*t)) + m^*t \quad (1)$$

Where $D(t)$ is [dTDP] at time t, A is the amplitude of the burst phase, k_1 is the observed rate constant of the burst phase, and m is slope of the linear phase (steady state rate).

Results and Discussion

Determination of the optimal concentration of M13 ssDNA. Previous studies have shown that ssDNA binding within the central channel of T7 helicase is a slow process.[22] To measure the steady state kinetics of dTTP hydrolysis in the presence of ssDNA, ssDNA binding or hexamer assembly should not limit the dTTP hydrolysis rate. Therefore it is essential to form ssDNA-T7 helicase complex before pre-steady state dTTP hydrolysis reactions start. Since T7 helicase is known to form a hexamer in the presence of dTTP, it was necessary to determine the condition under which T7 helicase form helicase-dTTP-M13 ssDNA complex.

The hexamer was assembled by incubating T7 helicase with dTTP and various amount of M13 ssDNA. After 15

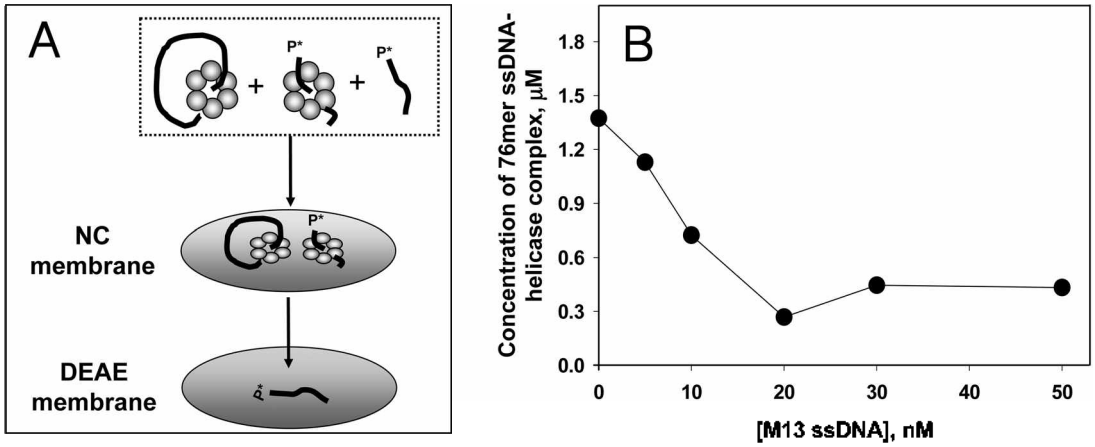

Figure 1. Determination of the optimal concentration of M13 ssDNA. (A) The schematic shows the design of NC/DEAE double filter binding assay. T7 helicase (2 μM hexamer), EDTA (5 mM), dTTP (1 mM), and various amount of M13 ssDNA were pre-incubated for 15 minutes prior to 76mer radio-labeled ssDNA (6 μM) trap addition. After 15 minutes, the mixtures were filtered through NC-DEAE membrane assembly. (B) Radioactivity on both NC and DEAE filters was quantified and molar amount of helicase-76mer ssDNA complex was calculated. Complex formation of helicase-76mer ssDNA reached bottom beyond 20 nM M13 ssDNA.

minutes, radio-labeled 76mer ssDNA trap was added and analyzed by NC/DEAE filter binding assay (Fig. 1A). NC/DEAE double filter binding assay is used to investigate equilibrium binding properties of protein-nucleic acid complexes.[21] While NC membrane retains protein and protein-DNA complexes, DEAE membrane is used as a trap for DNA that is passed through the NC membrane. In the absence of M13 ssDNA, a large amount of radio-labeled 76mer- helicase complex was observed (Fig. 1B). As the concentration of M13 ssDNA molecules was increased in the reaction, the amount of helicase-76mer complex decreased, and beyond 20 nM of M13 ssDNA, no more helicase-76mer complex was observed, indicating that helicase-M13 ssDNA complex formation was saturated. These experiments indicate that for each μmol of T7 helicase hexamer, we need at least 10 nmol of M13 ssDNA molecule to saturate the binding of protein to DNA. We therefore used a ratio of 15 nM M13 ssDNA per μM T7 helicase hexamer in all our experiments.

dTTP hydrolysis and T7 helicase-ssDNA complex formation in the absence of Mg^{2+}. The NTP hydrolysis activity of helicases converts NTP to NDP and P_i, and the produced energy is used for nucleic acid unwinding. Helicases are able to hydrolyze NTP in the absence of nucleic acid, but the rates are very slow. Previous studies have shown that Mg^{2+} is required for efficient hydrolysis of dTTP by T7 helicase, but not for dTTP binding, hexamer formation, or DNA binding.[12]

To measure the intrinsic dTTP hydrolysis ability of T7 helicase in the presence of M13 ssDNA, steady state dTTP hydrolysis reactions were performed in the absence of Mg^{2+} and steady state rate was obtained from the slope (Fig. 2). In the absence of Mg^{2+}, dTTP hydrolysis rate is insignificant (8.5×10^{-3} per second at 25 °C). This is about 5,000-fold slower than the dTTP hydrolysis rate in the presence of Mg^{2+}.[12] The slow dTTP hydrolysis enabled us to incubate T7 helicase with M13 ssDNA in the presence of dTTP (without Mg^{2+}) for three minutes without significant loss of dTTP bound to T7 helicase-M13 ssDNA complex.

T7 helicase-M13 ssDNA complex formation in the absence of Mg^{2+}. In a separate experiment, we varied incubation time of the helicase with M13 ssDNA and dTTP in the absence of Mg^{2+}. After various times of incubation, dTTP hydrolysis reactions were initiated by addition of Mg^{2+} and excess amount of non-radiolabeled dTTP (Fig. 3A). Fig. 3B shows that pre-incubation time does not influence on the amount of dTTP hydrolyzed even in a short (two minutes) period of incubation. This indicates that two minutes is sufficient for T7 helicase to form a complex with

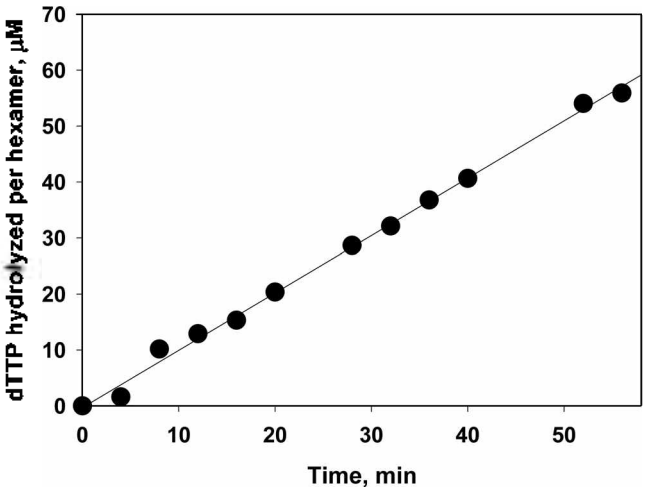

Figure 2. Intrinsic dTTP hydrolysis assay in the presence of M13 ssDNA without Mg^{2+}. T7 helicase (2 μM hexamer), EDTA (5 mM), dTTP (1 mM) + [α-^{32}P]dTTP, and 30 nM M13 ssDNA were incubated for various times at 25 °C and dTTP hydrolysis assay was performed. The radioactivity was quantitated using a PhosphorImager and the molar amount of dTDP was plotted as a function of time. The slope of the plot provided the reaction rate of 8.5×10^{-3} s^{-1}.

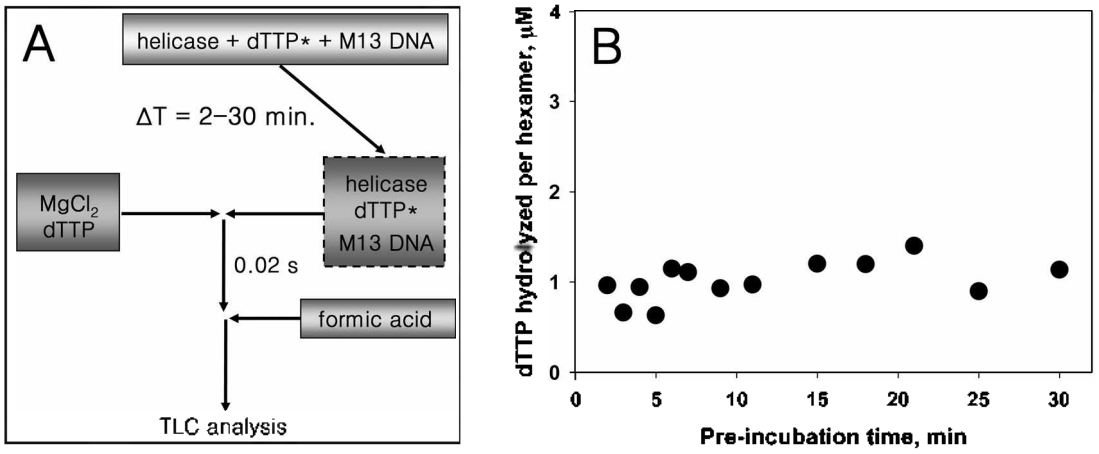

Figure 3. Dependence of pre-incubation time to form T7 helicase-M13 ssDNA complex in the absence of Mg^{2+}. (A) The schematic shows the design of determination of pre-incubation time to form T7 helicase-M13 ssDNA complex. Equal volumes of T7 helicase (4 μM hexamer) and a mixture of EDTA (10 mM), dTTP (840 μM) + [α-^{32}P]dTTP, and M13 ssDNA (60 nM) were incubated. After various incubation times (2-30 minutes), the mixture was mixed with an equal volume of $MgCl_2$ (14.42 mM) and excess amount of dTTP (5 mM), and the reactions were acid-quenched after 0.02 s. (B) shows the dTTP hydrolyzed by helicase-M13 ssDNA complex for 0.02 sec after various pre-incubation times.

M13 ssDNA. Based on this experiment, we chose a three-minutes pre-incubation period for the subsequent experiments, which also gives us enough time to load the pre-incubated sample in the quench-flow instrument.

Pre-steady state kinetics of dTTP hydrolysis in the presence and absence of M13 ssDNA. Previous studies show that gp4A' assembles into a hexamer in the presence of dTTP without Mg^{2+}.[12] The hexamer was assembled by incubating gp4A' with radio-labeled dTTP in the presence and absence of M13 ssDNA (without Mg^{2+}). We have already shown that 3-minutes pre-incubation time of gp4A' with dTTP was sufficient for dTTP binding and hexamer formation (Fig. 3B). After exactly 3 minutes, dTTP hydrolysis reaction was initiated by rapidly mixing the hexamer with $MgCl_2$ in a quench-flow instrument, and the reactions were quenched with formic acid after varying incubation time (3 msec to 1.0 sec) (Fig. 4A). The acid quench experiments exhibit biphasic kinetics, showing a burst of dTTP hydrolysis followed by a linear increase at the steady state rate both in the presence and absence of M13 ssDNA (Fig. 4B). The burst kinetics indicates that a step after dTTP hydrolysis is rate-limiting, because initial pre-incubation conditions eliminate the dTTP binding step. The observed burst rate and steady state rate at final 110 μM dTTP in the presence of M13 ssDNA were 11.1 ± 1.9 s^{-1} and 5.5 ± 0.3 s^{-1}, respectively. The burst rate and steady state rate at final 110 μM dTTP in the absence of M13 ssDNA were also measured to be 11.7 ± 4.4 s^{-1} and 1.1 ± 0.1 s^{-1}, respectively. Acid-quenched pre-steady state dTTP hydrolysis kinetics indicates that steady state dTTP hydrolysis rate in the presence of M13 ssDNA is faster than the one in the absence of M13 ssDNA, although pre-steady state dTTP hydrolysis rates both in the presence and absence of M13 ssDNA are similar (Fig. 5). Pre-steady state dTTP hydrolysis is involved in the hydrolysis of bound dTTP to hexamer. Therefore similar pre-steady state dTTP hydrolysis rates indicate that dTTP hydrolysis is not influenced by presence of the ssDNA during the first turnover. Importantly, the steady state dTTP hydrolysis rate in the presence of M13 ssDNA was five times faster than the steady state dTTP hydrolysis rate in the absence of M13 ssDNA. dTTP hydrolysis by the helicase consists of following minimal pathways; 1) dTTP binding to the helicase, 2) dTTP hydrolysis to products, dTDP and P_i, and 3) products release from the helicase. Previous studies show that dTTP binding occurs at a rate > 7×10^5 $M^{-1}s^{-1}$,[23] and the rate-limiting step would be the product release step. Although steady state reflects multiple turnover condition, the observed steady state rates are mainly governed by the rate of product release, which is the rate-limiting step. Therefore, in terms of minimal kinetic pathway of nucleotide

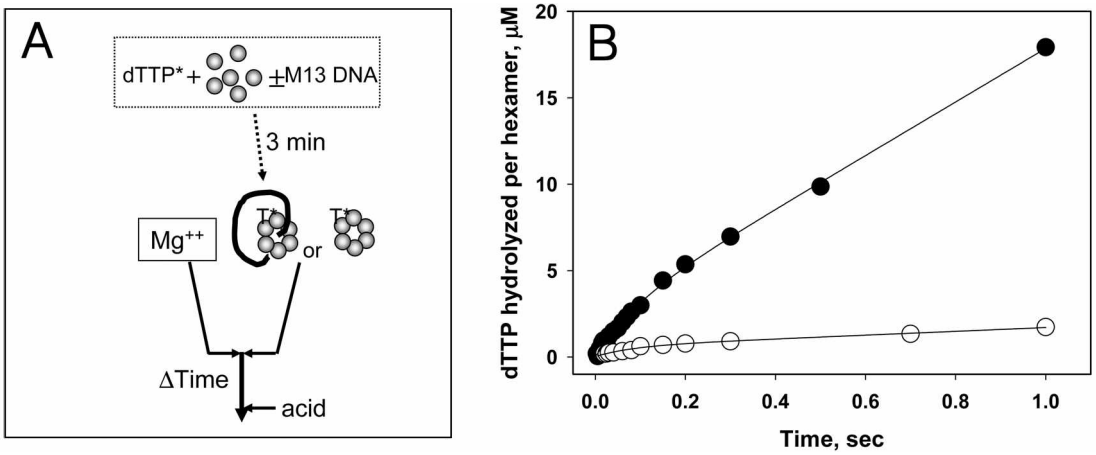

Figure 4. Pre-steady state acid-quenched kinetics of dTTP hydrolysis in the presence and absence of M13 ssDNA. (A) The schematic shows the design of the acid-quenched experiment. Equal volumes of T7 helicase (4 μM hexamer) and a mixture of EDTA (10 mM), dTTP (840 μM) + [α-^{32}P]dTTP in the presence (60 nM) and absence of M13 ssDNA were incubated for three minutes at 18 °C. The pre-formed hexamer was then mixed with an equal volume of $MgCl_2$ (9.42 mM) to initiate dTTP hydrolysis, and after various times, the reactions were acid-quenched. (B) shows the kinetic traces of dTTP hydrolysis in the presence (●) and absence (○) of M13 ssDNA. The dTTP hydrolysis kinetics fit best to the burst equation (Equation 1), as shown by the continuous line. The observed burst rate and steady state rate in the presence of M13 ssDNA were 11.1 ± 1.9 s^{-1} and 5.5 ± 0.3 s^{-1}, respectively. The burst rate and steady state rate in the absence of M13 ssDNA were 11.7 ± 4.4 s^{-1} and 1.1 ± 0.1 s^{-1}, respectively.

$$E + dTTP + DNA \xrightarrow{Mg^{2+}} E{\cdot}dTTP{\cdot}DNA \xrightarrow{Mg^{2+}} E{\cdot}dTDP{\cdot}P_i{\cdot}DNA \xrightarrow{fast} E{\cdot}DNA + dTDP + P_i$$

$$E + dTTP \xrightarrow{Mg^{2+}} E{\cdot}dTTP \xrightarrow{Mg^{2+}} E{\cdot}dTDP{\cdot}P_i \xrightarrow{slow} E + dTDP + P_i$$

Figure 5. Pathways of dTTP hydrolysis in the presence and absence of M13 ssDNA. E represents T7 helicase.

turnovers, M13 ssDNA stimulates products release step rather than the dTTP hydrolysis.

Acknowledgment. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) KRF-2005-041-C00270.

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