Bacteriophage T7 DNA Helicase Binds dTTP, Forms Hexamers, and Binds DNA in the Absence of Mg\(^{2+}\)

THE PRESENCE OF dTTP IS SUFFICIENT FOR HEXAMER FORMATION AND DNA BINDING*

(Received for publication, March 23, 1998, and in revised form, August 5, 1998)

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The role of Mg\(^{2+}\) in dTTP hydrolysis, dTTP binding, hexamer formation, and DNA binding was studied in bacteriophage T7 DNA helicase (4A protein). The steady state \(k_{cat}\) for the dTTPase activity was 200–300-fold lower in the absence of MgCl\(_2\) but the \(K_m\) was only slightly affected. Direct dTTP binding experiments showed that the \(K_m\) of dTTP was unaffected, but the stoichiometry of dTTP binding was different in the absence of Mg\(^{2+}\). Two dTTPs were found to bind tightly in the absence of Mg\(^{2+}\) in contrast to three to four in the presence of Mg\(^{2+}\). In the presence of DNA there was little difference in the stoichiometry of dTTP binding to 4A. These results indicate that Mg\(^{2+}\) is not necessary for dTTP binding, but Mg\(^{2+}\) is required for optimal hydrolysis of dTTP. Gel filtration of 4A in the presence of dTTP without Mg\(^{2+}\) showed that Mg\(^{2+}\) was not necessary, and dTTP was sufficient for hexamer formation. The hexamers formed in the presence of dTTP without Mg\(^{2+}\) were capable of binding single-stranded DNA. However, the 4A hexamers formed in the presence of dTTP with or without Mg\(^{2+}\) did not bind DNA, indicating that hexamer formation itself is not sufficient for DNA binding. The hexamers need to be in the correct conformation, in this case in the dTTP-bound state, to interact with the DNA. Thus, the \(\gamma\)-phosphate of dTTP plays an important role in causing a conformational change in the protein that leads to stable interactions of 4A with the DNA.

Helicases are motor proteins that translocate on DNA and catalyze separation of the double-stranded DNA into single-stranded DNAs. The ssDNA regions generated by the action of helicases serve as templates during DNA replication and repair and as intermediates in various processes of DNA metabolism (1–4). The strand separation activity of helicases requires hydrolysis of NTP, and the NTPase activity in helicases, as in other motor proteins, plays a central role in the enzymatic process that leads to translocation and DNA unwinding. Studies of T7 DNA helicase and other helicases such as *Escherichia coli* Rep and *E. coli* DnaB have shown that the type of nucleotide bound to the helicase, NTP versus NDP, influences its affinity for the DNA (5–7). Therefore, NTP binding and hydrolysis steps must act as switches that allow binding and release of DNA from the protein, and these steps are necessary for translocation. In the hexameric class of helicases, the MgNTP binding is also necessary for protein oligomerization (8–13). Bacteriophage T7 DNA helicase (4A protein) forms ring-shaped hexamers in the presence of Mg\(_2\)TmPPCP (14, 15). The T7 helicase preferentially uses dTTP as its substrate for DNA unwinding (16–19). The hexamers that are formed in the presence of Mg\(_2\)TmPPCP interact tightly with ssDNA, which binds in the central hole of the ring-shaped hexamer (5, 20). The MgNTP is therefore involved in multiple activities of the helicase, yet the roles of Mg\(^{2+}\) and dTTP have not been dissected. The studies in this report are directed toward understanding the roles of Mg\(^{2+}\) and NTP in hexamer formation and DNA binding.

To understand the role of Mg\(^{2+}\), we have studied dTTP hydrolysis, dTTP binding, hexamer formation, and DNA binding in 4A in the absence of added Mg\(^{2+}\) and in the presence of EDTA, which chelates contaminating metal ions. Our results showed that Mg\(^{2+}\) was required for efficient hydrolysis of dTTP into dTDP and P\(_i\). This was not surprising, since MgNTP is believed to be the substrate of most NTPases. Interestingly, Mg\(^{2+}\) was not necessary for dTTP binding, hexamer formation, or DNA binding. Stable 4A hexamers were formed in the absence of Mg\(^{2+}\) and in the presence of dTTP, and these hexamers were capable of binding ssDNA. The affinity of the hexamers for the DNA in the absence of Mg\(^{2+}\) was very similar to that observed in the presence of Mg\(_2\)TmPPCP. These studies provide novel insights into the roles of Mg\(^{2+}\) and dTTP in the helicase mechanism. At the same time, they have provided us with conditions to preassemble the helicase hexamer on the DNA. Such preassembly is desirable for detailed biochemical characterization of the dTTPase and unwinding kinetics, since it will allow us to measure the rate of unwinding and hydrolysis that is not limited by protein assembly and DNA binding (21).

EXPERIMENTAL PROCEDURES

Nucleotides and Other Reagents—\([\alpha\]\(^{32}\)P\]dTTP was purchased from Amersham Pharmacia Biotech, and its purity was checked and corrected for in all of the experiments. dTTP was purchased from Sigma. The nitrocellulose (BA-S) and DEAE (NA-45) membranes were purchased from Schleicher & Schuell. The Bio-Gel P-30 resin was purchased from Bio-Rad, and the polyethyleneimine cellulose TLC plates were purchased from EM Separations Technology.

Proteins and Enzymes—The 4A protein was purified and stored as described previously (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. T4 polynucleotide kinase (10 units/μl) used to \(\gamma\)-radio label the oligodeoxynucleotides was purchased from Boehringer Mannheim.

Synthetic Oligodeoxynucleotides—The sequence of the 30-mer DNA used in all of the experiments was as follows: AGCTT GCATC ATAGT TGCCAC CTGTG AGCTT. The 30-mer was purified on a 16% polyacryl-
amide gel with 3.2 m urea in Tris-borate-EDTA. The DNA was electrophoresed using an Elutrap apparatus from Schleicher & Schuell. The concentration of DNA was determined spectrophotometrically at 260 nm in Tris-EDTA buffer, 5 m urea, using an extinction coefficient of 305,010 m⁻¹ cm⁻¹.

dTTPase Assays—The dTTPase assays were carried out in an 18 °C room. 4A' protein was mixed with dTTP and [α-32P]dTTP with or without 30-mer DNA in the following reaction buffer: 50 mM Tris-Cl (pH 7.5), 40 mM NaCl, 0.1 mg/ml bovine serum albumin, with 10 mM MgCl₂ or 5 mM EDTA (the concentrations of the enzymes and substrate are given in the figure legends). After various times of incubation, the reactions were quenched with 1M HCl, treated with chloroform, and neutralized with 1 M NaOH, 0.25 M Tris base. The [α-32P]dTTP was separated from [α-32P]dTDP with polyethyleneimine cellulose TLC using 0.3 M potassium phosphate, pH 3.4, as the running buffer. The radioactive activity was quantitated using a PhosphorImager (Molecular Dynamics). The molar dTTP was plotted as a function of time, and the slope of the plot provided the reaction rate. The experiment was repeated at various concentrations of dTTP, and the rate of each reaction was plotted versus dTTP concentration. The curve was fit to Equation 1 to obtain the \( K_m \) and \( k_{cat} \) values.

\[
v = \frac{[E][S]k_{cat}}{K_m + [S]} \quad \text{(Eq. 1)}
\]

where \( v \) is the rate of the reaction, \([E][S]k_{cat}\) is the maximal rate, \([S]\) is the concentration of dTTP, and \( K_m \) is the Michaelis constant.

**dTTP Binding Assay**—The nitrocellulose (NC) filter binding assay was used to quantitate dTTP binding to 4A' protein as described previously (8, 22). The assays were performed at 18 °C using a constant amount of the protein and increasing dTTP. The NC membranes (25 mm) were washed with 0.5 M NaOH for 3 min, rinsed with double-distilled water, and equilibrated overnight in membrane wash buffer (50 mM Tris-Cl (pH 7.5), 5 mM NaCl, with either 10 mM MgCl₂ or 2 mM EDTA). 4A' (12 μM) was mixed with dTTP (0–100 μM) and [α-32P]dTTP with or without 30-mer DNA (5 μM) in 50 mM Tris-Cl (pH 7.5), 40 mM NaCl, 10% glycerol, in the presence of either 10 mM MgCl₂ or 0.5 mM EDTA in a volume of 15 μl. The reactions were incubated for 30 s, and 10-μl aliquots were filtered through the NC membrane assembly. The membranes were washed before and after filtration with 0.6 ml of membrane wash buffer. One-microliter aliquots were spotted onto a separate nitrocellulose membrane to measure the total radioactivity. The radioactivity on the membranes was quantitated using a PhosphorImager (Molecular Dynamics). The results of the nucleotide binding assays were fit to a hyperbola (Equation 2)

\[
[E][S] = \frac{[E][S]k_{cat}}{K_m + [S]} \quad \text{(Eq. 2)}
\]

where \([E][S]\) is the concentrations of 4A'-dTTP complex, \([E]\) is the concentration of 4A', \([S]\) is the concentration of dTTP, and \( K_m \) is the dissociation constant.

**HPLC Gel Filtration**—The assembly of 4A' into hexamers was analyzed in the presence of dTTP (in the absence of Mg²⁺) using HPLC gel filtration chromatography. The elution buffer consisted of 50 mM Tris-Cl (pH 7.5), 40 mM NaCl, 10% glycerol, 0.5 mM EDTA, and the appropriate concentration of dTTP (0–250 μM). Protein samples (10 μM) were prepared by mixing 4A' (3.6 μM) with the desired concentration of dTTP (0–250 μM) in the presence or absence of 30-mer DNA (3.6 μM). The 4A' hexamer was resolved from the monomer or dimer species at a flow rate of 0.8 ml/min using a 30-cm HPLC gel-filtration column (Bio-Sil SEC 400–5, Bio-Rad) that was pre-equilibrated in the elution buffer. Chromatography was performed on a Waters model 625 LC system, and proteins were detected using a Waters 470 scanning fluorescence detector (λex/emission = 280 nm and 340 nm). The fraction of hexamers was plotted against the concentration of free dTTP, and the data were fit to the Hill equation (Equation 3) to obtain the apparent equilibrium constant for hexamer formation.

\[
Y_L = \frac{[L]^n/K_m + [L]^n}{n}
\quad \text{(Eq. 3)}
\]

where \( Y_L \) is the fractional saturation, \([L]\) is the concentration of free dTTP, \( K_m \) is the apparent dissociation constant for the hexamer, and \( n \) is the Hill coefficient.

**Equilibrium DNA Binding Assays**—The DNA binding assay was carried out as described previously (5). The 30-mer DNA was radiolabeled with [32P]ATP and T4 polynucleotide kinase. Excess [γ-32P]ATP was removed by Bio-Gel P-30 spin gel filtration. The NC and DEAE membranes were washed with 0.5 M NaOH for 10 min, rinsed with double-distilled water, and equilibrated with the membrane wash buffer (50 mM Tris-Cl (pH 7.5), 5 mM NaCl, and either 10 mM MgCl₂ or 2 mM EDTA) for at least 1 h at room temperature. The assays were performed at constant DNA (1 μM) and increasing 4A' (0–20 μM) concentration in buffer containing 50 mM Tris-Cl (pH 7.5), 40 mM NaCl, 10% glycerol, and in the presence of 10 mM MgCl₂ or 0.5 mM EDTA. Experiments were carried out under the following conditions: (a) in the presence of dTMPCCP (1 mM) and Mg²⁺ (10 mM); (b) in the presence of dTOP (1 mM) and Mg²⁺ (10 mM); (c) in the presence of dOTP (1 mM) and EDTA (0.5 mM). The reactions were preincubated for 20 min at room temperature before the samples were filtered through the membrane assembly. The membranes were washed, before and after filtration of samples, with 60 μl of membrane wash buffer (above). After the samples were filtered, radioactivity on both NC and DEAE filters was quantitated using a PhosphorImager (Molecular Dynamics).

**RESULTS**

To investigate the role of Mg²⁺ and dTTP in hexamer formation, hydrolysis, and DNA binding by T7 4A' protein, we have characterized the activities of 4A' protein both in the presence and absence of Mg²⁺. The studies in the absence of Mg²⁺ were carried out in the presence of EDTA, which serves to chelate the contaminating metal ions in the reaction mixture. To determine the optimal amount of EDTA for use in the Mg²⁺-free experiments, the dTTP hydrolysis rate was measured at increasing concentrations of EDTA in the reaction. We found that, as the concentration of EDTA was increased from 0.5 mM to 20 mM, the rate of dTTP hydrolysis decreased and was saturated at 0.5–1 mM EDTA (data not shown). These results indicate that the reaction mixture without EDTA contained contaminating Mg²⁺ or other divalent cations that were able to partially activate the dTTPase activity of 4A'. To chelate the free Mg²⁺ ions in the reaction mixture, we included 0.5–5 mM EDTA in all the reactions.

**The Role of Mg²⁺ in dTTP Hydrolysis Reaction Catalyzed by 4A'**—To investigate the role of Mg²⁺ in the 4A'-catalyzed dTTP hydrolysis reaction, we measured the dTTPase activity in the absence of Mg²⁺. Our results showed that 4A' was able to hydrolyze dTTP at a reduced rate in the absence of Mg²⁺. To further investigate this dTTPase activity, we determined the steady-state \( K_m \) and \( k_{cat} \) values of dTTPase in the absence and presence of DNA, with and without Mg²⁺ (see Fig. 1A–D). The measured \( k_{cat} \) and \( K_m \) values are reported in Table I. In the absence of DNA and Mg²⁺, the \( k_{cat} \) for dTTP hydrolysis was reduced 300-fold relative to the rate in the presence of Mg²⁺. The difference in the \( K_m \) values of dTTPase in the absence and presence of Mg²⁺ was relatively small. The \( K_m \) increased by only 2-fold in the absence of Mg²⁺. In the presence of 30-mer ssDNA, we also observed a large change in the \( k_{cat} \) and a small change in the \( K_m \) value in reactions without Mg²⁺. The dTTPase \( k_{cat} \) decreased about 200-fold from the DNA-stimulated rate in the presence of Mg²⁺ and the \( K_m \) value decreased about 2-fold in the absence of Mg²⁺.

**Table I**

| Steady state dTTPase \( k_{cat} \) and \( K_m \) values |
|-----------------|-----------------|
| No DNA | 30-mer ssDNA |
| --- | --- | --- | --- |
| \( k_{cat} \) | \( K_m \) | \( k_{cat} \) | \( K_m \) |
| [mol/dTTP hydrolyzed s⁻¹] · [mol 4A' monomer] μM | [mol/dTTP hydrolyzed s⁻¹] · [mol 4A' monomer] μM | [mol/dTTP hydrolyzed s⁻¹] · [mol 4A' monomer] μM | [mol/dTTP hydrolyzed s⁻¹] · [mol 4A' monomer] μM |
| 10 mM Mg²⁺ | 0.053 ± 0.003 | 9.4 ± 1.6 | 0.28 ± 0.020 | 52.6 ± 10.0 |
| No Mg²⁺ | 1.7 ± 0.07 × 10⁻⁴ | 18.8 ± 1.9 | 1.2 ± 0.02 × 10⁻³ | 32.2 ± 10.2 |
The rate of dTTP hydrolysis was plotted in the presence of 5 mM EDTA and 1 mg/mL of protein. The gel filtration experiments were performed by incubating 4A protein with various concentrations of dTTP in the presence and absence of 30-mer ssDNA (1 mM) (Table 1). The rate of dTTP hydrolysis was plotted versus the concentration of dTTP, and the curve was fit to a hyperbola to obtain the $K_{cat}$ and $K_m$ values under various conditions. A shows the $K_m$ dependence of hydrolysis in the presence of Mg$^{2+}$. The $K_{cat}$ and $K_m$ values in the presence of 10 mM Mg$^{2+}$ were 0.053 ± 0.003 s$^{-1}$ and 9.4 ± 1.8 μM, respectively. B shows the $K_m$ dependence in the absence of Mg$^{2+}$. The $K_{cat}$ and $K_m$ values in the absence of Mg$^{2+}$ (in the presence of 5 mM EDTA) were 1.7 ± 0.7 × 10$^{-4}$ s$^{-1}$ and 18.8 ± 1.9 μM, respectively. C shows the $K_m$ dependence in the presence of Mg$^{2+}$ and DNA. The $K_{cat}$ and $K_m$ values in the presence of 10 mM Mg$^{2+}$ and 1 μM 30-mer DNA were 0.28 ± 0.020 s$^{-1}$ and 52.6 ± 10.0 μM, respectively. D shows the $K_m$ dependence in the absence of Mg$^{2+}$, and in the presence of DNA. The $K_{cat}$ and $K_m$ values in the absence of Mg$^{2+}$ (in the presence of 5 mM EDTA) and 1 μM 30-mer DNA were 1.2 ± 0.02 × 10$^{-3}$ s$^{-1}$ and 32.2 ± 10.2 μM, respectively.

dTTP Binding to 4A with and without Mg$^{2+}$—The equilibrium binding of dTTP to 4A protein was measured both to determine the number of dTTPs that bind 4A and their affinity in the absence of Mg$^{2+}$. Fig. 2A shows that in the presence of 10 mM Mg$^{2+}$, 4A protein bound 3–4 dTTPs per hexamer, consistent with our previous results (8, 22). In the absence of Mg$^{2+}$, 4A bound only 2 dTTPs per hexamer. The $K_d$ for the bound nucleotides was very close to that in the presence of Mg$^{2+}$ (11 ± 2 μM with Mg$^{2+}$ and 9 ± 1 μM without Mg$^{2+}$). The binding of dTTP was also investigated in the presence of 30-mer ssDNA. In the presence of 10 mM Mg$^{2+}$, 4A bound 3–4 dTTPs per hexamer (Fig. 2B), and the same number of dTTPs were bound in the presence of DNA without Mg$^{2+}$. The affinity of the dTTPs was about 2-fold weaker when Mg$^{2+}$ was removed (35 ± 6 μM without Mg$^{2+}$ and 13 ± 2 μM with Mg$^{2+}$ in the presence of DNA). Together, the above results indicate that Mg$^{2+}$ is necessary for dTTP hydrolysis, but the absence of Mg$^{2+}$ does not impair dTTP binding to 4A to a significant extent.

The Formation of 4A Hexamer in the Absence of Mg$^{2+}$—The 4A protein assembles into stable hexamers in the presence of Mg$^{2+}$. The gel filtration experiments were performed by incubating 4A protein with increasing concentrations of dTTP in the presence of EDTA. The samples were applied to the gel filtration column eluted with a buffer containing the same amount of dTTP and EDTA. Fig. 3A shows the gel filtration profiles of 4A protein in the presence of various concentrations of dTTP. The gel filtration profiles show that 4A protein is capable of forming stable hexamers in the absence of Mg$^{2+}$, and the formation of hexamers was dependent on the concentration of dTTP. Thus, 4A monomer or dimer species were converted to the hexamer species as the concentration of dTTP was increased. The dTTP dependence of the hexamer peak area was sigmoidal as expected for a ligand-linked oligomerization process (Fig. 3B). The data were fit to the Hill equation with an apparent $K_d$ of 38 μM for dTTP-dependent hexamer formation and a Hill coefficient of 1.5. The gel filtration experiments were also carried out in the presence of 30-mer ssDNA (Fig. 3C). The dTTP-dependent hexamer formation occurred with a tighter $K_d$ in the presence of DNA. The apparent $K_d$ for dTTP-dependent hexamer formation in the presence of DNA was 7 μM and the Hill coefficient was 2.5 (Fig. 3D). These results show that Mg$^{2+}$ is not necessary for hexamer formation in 4A helicase, and dTTP binding is sufficient for formation and stabilization of the hexamer.

4A Protein Binds DNA in the Absence of Mg$^{2+}$—We have shown earlier that 4A helicase binds DNA with a high affinity only in the presence of Mg$^{2+}$. The nonhydrolyzable analog of dTTP, and the interactions with the DNA are weak in the presence of Mg$^{2+}$ (5). These results suggested that the dTTP-bound form of 4A may interact more tightly with the DNA. However, dTTP hydrolysis in the presence of Mg$^{2+}$ is fast, thus we were unable to test this idea. Now we have conditions under which dTTP can be bound to 4A without extensive hydrolysis, and hence we are able to test DNA binding in the presence of dTTP instead of dTTPCP. The binding of DNA was measured in a quantitative manner using the NC-DEAE filter binding assay. The experiment was carried out using a constant concentration of radiolabeled 30-mer DNA, which was titrated with increasing concentrations of the 4A protein. Experiments were carried out in the presence of various ligands such as Mg$^{2+}$, Mg$^{2+}$, Mg$^{2+}$, and dTTP with EDTA. The positive control (dTTPCP and Mg$^{2+}$) showed tight binding of DNA with a stoichiometry of one 30-mer DNA per hexamer (Fig. 4). Very little DNA binding was observed in the presence of dTTP and Mg$^{2+}$. This is because the protein...
tography performed at a flow rate of 0.8 ml/min.

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alysis of experiments described in monomer or dimer 4A (21). We have shown that dTTP binding is not greatly affected by Mg²⁺.

Hecilases is not known, but it is likely to be similar to the F1-ATPase enzyme, which is an oligomer (27, 30–33). In the F1-ATPase, it is known that ATP binding is necessary for hexamer formation. This behavior of 4A₉ protein was found to assemble into stable hexamers in the absence of Mg²⁺ as well as Mg⁴⁺. That is, NTP binding is sufficient, for hexamer formation. Since no high resolution structural information of hexameric helicases is available at the present time, it is not known exactly where dTTP binds with respect to the hexamer subunits and how it promotes hexamer formation. There is, however, considerable evidence that the structure of hexameric helicases may be similar to the F1-ATPase enzyme, which is hexameric (27, 30–33). In the F1-ATPase, it is known that ATP may be bound at the subunit interface. The binding of dTTP at the interface provides a simple mechanism by which it can stabilize hexamer formation.

They are just as small as the nucleophile is either activated by protein active site residues (23, 24), or the γ-phosphate of the NTP may serve to activate the water molecule (25, 26). Thus, the role of Mg²⁺ is most likely in neutralizing the negative charges that develop during the reaction. Our results also show that DNA binding by itself can activate the dTTPase activity about 10-fold in the absence of Mg²⁺. The structure of E. coli Rep helicase and hepatitis C virus helicase show that the DNA binding site is close to the NTP binding site (27, 28). Therefore, it is possible that the DNA activates the dTTPase by a direct mechanism. Alternatively, the DNA may cause changes in the conformation of the active site residues that lead to stabilization of the dTTPase transition state.

dTTP binding in the absence of Mg²⁺ showed that only two dTTPs were bound to 4A. Since the nitrocellulose filter-binding assay detects sites that have high affinity, we cannot rule out the possibility that other sites are occupied in the absence of Mg²⁺. We can, however, conclude that only two sites are tightly bound in the absence of Mg²⁺ as compared with three to four tight binding sites in the presence of Mg²⁺. The presence of DNA restored the binding of one or two dTTPs to 4A. The dTTP binding in the presence of DNA was therefore the same with or without Mg²⁺. We have shown previously that 4A has two kinds of dTTP binding sites (29). Approximately half of the dTTP binding sites on the hexamer appear to be noncatalytic. The dTTPs bound at the noncatalytic sites do not turn over very rapidly and hence these dTTPs are most likely not involved in providing the energy for translocation and DNA unwinding. Future studies are directed toward determining whether the dTTPs that are bound in the absence of Mg²⁺ are at the catalytic sites or the noncatalytic sites.

The 4A protein was found to assemble into stable hexamers even in the absence of Mg²⁺. Thus, Mg²⁺ is not necessary, and dTTP or dTDP binding is sufficient, for hexamer formation. Since no high resolution structural information of hexameric helicases is available at the present time, it is not known exactly where dTTP binds with respect to the hexamer subunits and how it promotes hexamer formation. There is, however, considerable evidence that the structure of hexameric helicases may be similar to the F1-ATPase enzyme, which is hexameric (27, 30–33). In the F1-ATPase, it is known that ATP may be bound at the subunit interface. The binding of dTTP at the interface provides a simple mechanism by which it can stabilize hexamer formation. We show here that Mg²⁺ is not necessary for hexamer formation. This behavior of 4A is different from that of E. coli DnaB helicase that was shown to
form hexamers in the presence of Mg$^{2+}$ alone (34). Since we have not been able to detect stable hexamers of 4A protein in the presence of Mg$^{2+}$ alone (data not shown), we believe that nucleotide binding, either dTTP or dTDP, is necessary for stable hexamer formation.

Our studies show that the γ-phosphate plays a very important role in regulating the interactions of the helicase with the DNA. Previous studies have shown that 4A helicase interacts tightly with the DNA in the presence of Mg$^{2+}$TMPPCP, and very little DNA binding was observed in the presence of Mg$^{2+}$TDP (5). We were not sure if dTMPPCP was a good analog of dTTP because the bond lengths and the bond angle of P-C-P are different from those of P-O-P. It is possible that the structure of the dTMPPCP-bound 4A is different from the dTTP-bound form, and the dTMPPCP-bound form resembles an intermediate in the dTTPase reaction pathway that has strong interactions with the DNA. Part of the reason for believing this was because weak DNA binding was observed in the presence of Mg$^{2+}$TTP. Now we are able to observe tight binding of DNA in the presence of dTTP without Mg$^{2+}$, conditions under which dTTP hydrolysis is very slow and hence translocation and dissociation of 4A from the DNA is slow. These results indicate that the dTTP-bound form of 4A does interact tightly with the DNA. We can also conclude that hexamer formation is not sufficient for DNA binding, since no DNA binding was observed in the presence of dTDP, although stable hexamers were formed in the presence of Mg$^{2+}$TDP (8 and dTTP data not shown). It appears that the γ-phosphate of dTTP may be involved in causing a critical conformational change in the protein that “opens up” the DNA binding site on a 4A subunit. We can infer that the hydrolysis of dTTP or the dissociation of P leads to dissociation of the DNA. These steps provide the switch mechanism that allows the helicase subunit to switch from a tight DNA binding state to a weak one. Since the DNA predominately interacts with one or two subunits (20), the resulting DNA binding and release steps can occur in a sequential manner among the hexamer subunits, providing a mechanism for processive translocation of the helicase on the DNA.

Finally, the findings from this study are likely to be general for helicases that assemble into hexamers in the presence of Mg$^{2+}$ (8–13). This study has provided us with conditions where a complex of hexamer bound to the DNA can be performed without extensive dTTP hydrolysis occurring in the complex. This is the first time that we have been able to form such a complex. The ability to preform this complex will allow us to examine the presteady state kinetics of the DNA-stimulated dTTP hydrolysis reaction, and the kinetics of DNA unwinding at a replication fork, without complications from oligomerization and/or DNA binding processes.

Acknowledgments—We thank Mikhail Levin for preliminary observations that led to this study, Dr. M. Todd Washington for carefully reviewing the manuscript during preparation, and the members of the Patel laboratory for helpful discussions.

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