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Nucleotide Binding Studies of Bacteriophage T7 DNA Helicase-Primase Protein

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ABSTRACT Bacteriophage T7 DNA helicase protein is a hexameric protein that contains identical subunits arranged in a ring-like structure. Single-stranded DNA binds through the hole of the ring, and the helicase protein translocates and unwinds duplex DNA using nucleoside triphosphate (NTP) hydrolysis. In our efforts to understand how NTP hydrolysis may be coupled to movement of the helicase on the DNA, we have quantitated the equilibrium binding of deoxythymidine triphosphate and thymidine 5′-(β,γ-methylenetriphosphate) using nitrocellulose binding assays. Even though the helicase consists of six identical subunits, each hexamer was found to bind only three NTP molecules. These results indicate half-site binding or negative cooperativity in NTP binding by the hexamer. Interestingly, binding of three NTP molecules to the hexamer was sufficient for stoichiometric binding of a single-stranded oligodeoxynucleotide. Similar negative cooperativity in NTP binding has also been observed for other helicases, suggesting that it may be a general feature of hexameric helicases. The significance of half-site binding, however, is not understood at the present time.

INTRODUCTION

DNA helicases are ubiquitous proteins that catalyze unwinding of duplex DNA into single-stranded DNAs using energy from nucleoside triphosphate (NTP) hydrolysis (Kornberg and Baker, 1992). Helicases are therefore analogous to many motor proteins such as kinesin and dynein that utilize ATP hydrolysis to move unidirectionally on microtubule polymer. Interestingly, most helicases that have been studied to date appear to form oligomers, self-assembling either as dimers or hexamers (Lohman, 1992, 1993) also similar to motor proteins that are oligomeric. The DNA helicase and helicase-primase proteins of bacteriophage T7, the subject of our study, are required for phage DNA replication (Dunn and Studier, 1983) and have been shown to form stable hexamers in the presence of thymidine 5′-(β,γ-methylenetriphosphate) (dTMP-PCP), the nonhydrolyzable analog of deoxythymidine triphosphate (dTTP) (Patel and Hingorani, 1993).

The hexameric structure of the gene 4 proteins was established using protein-protein cross-linking, gel filtration, and electron microscopy studies (Patel and Hingorani, 1993). In addition, analyses of 4A′ protein by native PAGE have shown that hexamer formation is necessary for DNA binding (Hingorani and Patel, 1993). The 4A′ protein has a high affinity for single-stranded DNA, and each hexamer protects about 25 bases of single-stranded DNA. Moreover, the single-stranded DNA appears to interact with at least five subunits of the hexamer (Patel et al., 1994).

Like many motor proteins, helicases use NTP hydrolysis to translocate on single-stranded DNA and to unwind DNA. NTP hydrolysis somehow modulates protein-DNA interaction that allows the helicase to move on the DNA. We are interested in understanding how the hexamer uses NTP hydrolysis to translocate on single-stranded DNA. To this end, we have quantitated equilibrium binding of dTTP and its nonhydrolyzable analog dTMP-PCP to 4A′. We report here that NTP binds to the hexamer with a negative cooperativity such that each hexamer appears to bind only three NTP molecules.

MATERIALS AND METHODS

Materials

Bacteriophage T7 4A′ protein was overexpressed in *E. coli* from pAR5018 and purified as described previously (Rosenberg et al., 1992, Patel et al., 1992). dTMP-PCP was purchased from U.S. Biochemical Corp. (Cleveland, OH), [α-^{32}P] dTTP (3000 Ci/mM) and [γ-^{32}P] ATP (>4000 Ci/mM) were purchased from ICN Biochemicals. Methylene diphosphonic acid trisodium salt (P-C-P) was purchased from Sigma Chemical Co. (St. Louis, MO). The single-stranded DNAs: 25-mer, 5′-GCCTC GCAGC CGTCC AACCA ACTCA; 30-mer, 5′-AGCTT GCATC ATAGT GTCAC CTGTT ACGTT; and 36-mer, 5′-CGGAG CGTCG GCAGG TTGGT TGAGT AGGTC TTGTT T were synthesized at the Biochemical Instrument Center at Ohio State University, and purified as described (Hingorani and Patel, 1993). Nitrocellulose (BA 83) and DEAE (NA45) membranes were purchased from Schleicher and Schuell (Keene, NH). DEAE-Sephadex A-25 was purchased from Pharmacia (Uppsala, Sweden), and Biogel P-30 resin was purchased from Bio-Rad Laboratories (Richmond, CA). Reverse transcriptase was a gift from Dr. Karen Anderson (Yale University).

Methods

Synthesis of [α-^{32}P] dTMP-PCP

The complementary DNAs 25-mer (2 μM) and 36-mer (2 μM) were mixed in buffer containing 50 mM Tris-acetate, pH 7.5, 50 mM sodium acetate, 1 mM DTT, 0.1 mg/ml BSA, and 10 mM magnesium acetate, to form the partially duplex 25/36-mer. The solution consisting of the 25/36-mer primer template, dTTP (20 μM), [α-^{32}P] dTTP (40 μCi), and 0.3 μM reverse transcriptase was mixed in a-100 μl total volume and incubated at 37°C for 15 min to incorporate [α-^{32}P] dTMP at the 3′ end of the 25-base primer. The solution was spun through a 0.5-ml Biogel P-30 column to remove unreacted [α-^{32}P] dTTP. The filtrate with radiolabeled primer-template was mixed

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with 8 mM P-C-P in the above buffer containing 20 mM magnesium acetate. Reverse transcriptase (1 μM) was added, and the pyrophosphorolysis reaction was continued at 37°C for 2 h to form [α-^{32}P] dTMP-PCP. The reaction solution was loaded on a 1-ml DEAE-Sephadex column, the column was washed with 1 ml water, and [α-^{32}P] dTMP-PCP was eluted with 1 M TEAB, pH 8.0. Three- to four-drop fractions were collected and an aliquot of each fraction was spotted on a polyethylene imine thin-layer chromatography plate and developed in 0.3 M potassium phosphate buffer, pH 3.4. Fractions containing high cpm of radiolabeled dTMP-PCP uncontaminated by DNA were pooled, dried under vacuum, and washed repeatedly with methanol. [α-^{32}P] dTMP-PCP was reconstituted in water and used directly in equilibrium binding assays.

Equilibrium binding of dTMP-PCP to 4A′ protein

Equilibrium binding assays were performed at constant dTMP-PCP and increasing protein concentrations and vice versa using nitrocellulose membranes and a glass microanalysis filter assembly (Fisher Scientific, Pittsburgh, PA). Nitrocellulose membrane circles (25 mm) were washed with 0.5 N sodium hydroxide, rinsed with water, and equilibrated in membrane wash buffer (50 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate and 5 mM sodium acetate) before use. Titration of dTMP-PCP with increasing 4A′ protein was carried out both in the absence and presence of 30-mer DNA (3.3 μM). The reactions contained 10 μM radiolabeled dTMP-PCP, 4A′ (0–35 μM) in binding buffer (50 mM Tris-acetate, pH 7.5, 3 mM magnesium acetate, 50 mM sodium acetate and 10% glycerol) in a total volume of 15 μl. The reactions were incubated for 10 min at 23°C and 10 μl volumes of each sample were filtered through the nitrocellulose membrane assembly. The membranes were washed before and after filtration with 0.5 ml of membrane wash buffer. One-microliter aliquots of each sample were spotted on a separate nitrocellulose membrane to quantitate total dTMP-PCP. The radioactivity on the membranes was quantitated on a Betascope 603 blot analyzer (Betagen, Waltham, MA). The molar amount of dTMP-PCP bound to 4A′ protein was determined and plotted versus 4A′ concentration.

Equilibrium binding assays at constant protein and increasing dTMP-PCP concentrations were performed in the absence and presence of 30-mer DNA (3.3 μM). The reactions contained 20 μM 4A′ protein and 0–50 μM radiolabeled dTMP-PCP in binding buffer. The assays were performed as described above, and the number of dTMP-PCP molecules bound per 4A′ hexamer was determined and plotted versus dTMP-PCP concentration. Nonspecific dTMP-PCP binding to nitrocellulose was measured by replacing 4A′ protein with buffer in the above experiments and was corrected in all titrations reported.

Equilibrium binding of dTTP to 4A′ protein

Equilibrium binding assays of 4A′ protein and dTTP were performed at constant protein and increasing dTTP concentrations using the filter assembly described above. The reactions contained 0–150 μM radiolabeled dTTP incubated on ice in binding buffer (50 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, 40 mM sodium acetate, and 10% glycerol) in the absence or presence of 5 μM 30-mer. 4A′ protein was added to a final concentration of 20 μM, and the samples were filtered through the assembly in 35 s. The membranes were washed with 0.5 ml ice-cold wash buffer before and after filtration. The reactions were quantitated as described above for the dTMP-PCP assays, and the number of dTTP molecules bound per 4A′ hexamer was determined and plotted versus dTTP concentration.

Equilibrium titration of 4A′: DNA interaction with dTMP-PCP

DNA binding to 4A′ protein was monitored in the presence of increasing concentrations of dTMP-PCP. The reactions (15 μl) contained 4A′ (10 μM), 5′-^{32}P radiolabeled 30-mer DNA (4 μM) and dTMP-PCP (0–15 μM) in binding buffer (50 mM Tris-acetate, pH 7.5, 3 mM magnesium acetate, 50 mM sodium acetate and 10% glycerol). The samples were incubated at 23°C for 5 min and assayed using a nitrocellulose-DEAE membrane assembly as described (Hingorani and Patel, 1993). Single-stranded DNA bound per 4A′ hexamer was quantitated and plotted versus dTMP-PCP concentration.

RESULTS

We have used quantitative nucleotide binding studies to investigate how many NTPs bind to each hexamer. It has been shown that the T7 DNA helicase protein hydrolyzes dTTP with the lowest K_m (Patel et al., 1992). Therefore, NTP binding studies have been carried out with dTMP-PCP, the nonhydrolyzable analog of dTTP. Fig. 1 *A* shows titration of radiolabeled dTMP-PCP with increasing concentrations of 4A′ protein. The binding isotherm appears stoichiometric, suggesting that dTMP-PCP binds 4A′ with a high affinity. However, 8 μM dTMP-PCP requires ~16 μM 4A′ monomers for complete binding. The same result was obtained also in the presence of a 30-mer single-stranded DNA (Fig. 1 *B*). These results indicate that each 4A′ hexamer binds only three dTMP-PCP molecules. To determine whether the hexamer can bind additional dTMP-PCP molecules, we titrated a constant amount of 4A′ with increasing concentrations of dTMP-PCP. As shown in Fig. 2, *A* and *B*, each hexamer still appears to bind only three nucleotides both in the absence and in the presence of DNA.

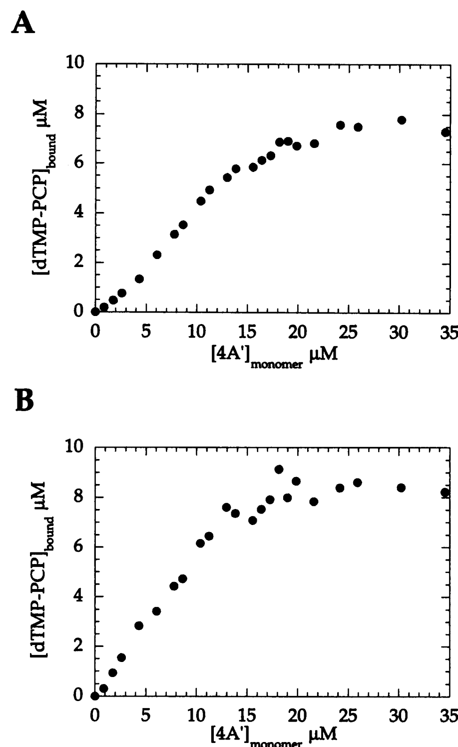

FIGURE 1 Titration of dTMP-PCP with 4A′ protein. Equilibrium dTMP-PCP binding was measured using a nitrocellulose membrane assay as described in Materials and Methods. (*A*) Titration of constant dTMP-PCP (10 μM) with increasing 4A′ protein concentrations (0–35 μM) in the absence of DNA. (*B*) Titration of dTMP-PCP (10 μM) with increasing 4A′ protein concentrations in the presence of 3.3 μM 30-mer DNA. Molar amount of dTMP-PCP bound to 4A′ protein was quantitated and plotted versus 4A′ concentration. Saturation of dTMP-PCP binding at 8–9 μM requires 4A′ protein concentration of 15–16 μM.

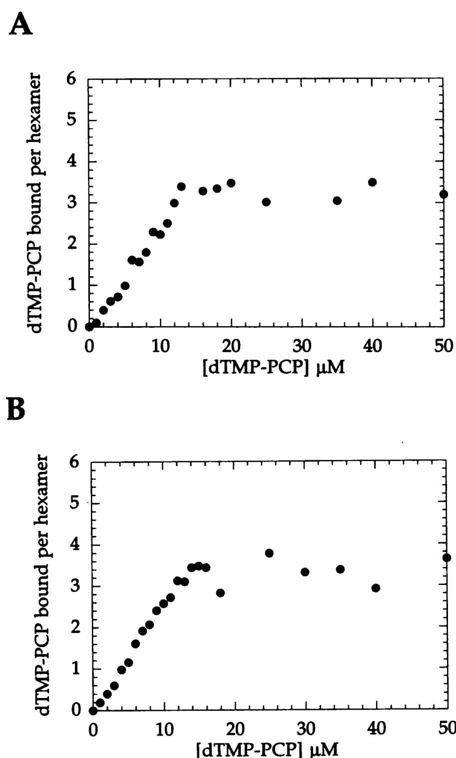

FIGURE 2 Titration of 4A′ protein with increasing concentrations of dTMP-PCP. 4A′ protein (20 μM) was titrated with radiolabeled dTMP-PCP (0–50 μM) as described in Materials and Methods. (*A*) dTMP-PCP bound per 4A′ hexamer in the absence of 30-mer DNA plotted versus dTMP-PCP concentrations. (*B*) Plot of the same titration performed in the presence of 3.3 μM 30-mer DNA. Both in the absence or presence of single-stranded DNA, three dTMP-PCP molecules appear to bind tightly to each 4A′ hexamer.

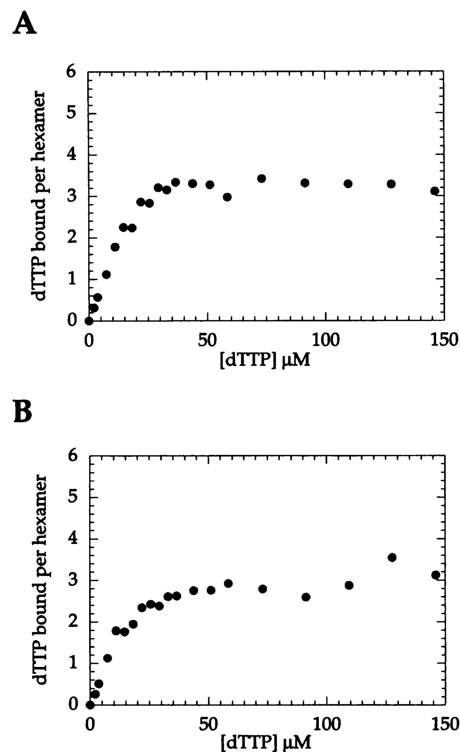

FIGURE 3 Titration of 4A′ protein with increasing concentrations of dTTP. A constant amount of 4A′ protein was titrated with increasing amounts of radiolabeled dTTP in the absence or presence of 30-mer DNA as described in Materials and Methods. (*A*) Plot of dTTP bound per 4A′ hexamer versus dTTP concentration shows that three dTTP molecules are bound per protein hexamer in the absence of 30-mer DNA. (*B*) Similar result for the titration in the presence of DNA.

In the above studies, we have used the nonhydrolyzable analog of dTTP to avoid complications due to substrate hydrolysis. To determine whether the half-site binding was characteristic of only the analog, we have repeated the equilibrium binding titrations using dTTP. As dTTP is hydrolyzed by 4A′ with a rate constant close to 0.03 s^{-1} in the absence of DNA at 22°C, we performed titrations at 4°C where hydrolysis does not occur in the time scale of the binding experiment. Fig. 3, *A* and *B* shows titration of a constant amount of 4A′ with increasing dTTP in the absence and presence of single-stranded 30-mer DNA, respectively. The results clearly show that each hexamer binds only three dTTP molecules, analogous to dTMP-PCP binding described above.

As 4A′ hexamers bind DNA only in the presence of MgdTMP-PCP (or dTTP), the question is, are three NTP binding sufficient for DNA binding? To investigate this we measured radiolabeled 30-mer DNA binding to 4A′ hexamer in the presence of increasing concentration of MgdTMP-PCP. DNA binding was quantitated by nitrocellulose-DEAE membrane assays. The results (Fig. 4) show that three dTMP-PCP binding to each hexamer are sufficient for stoichiometric binding of 30-mer DNA. These results are consistent with the above nucleotide binding studies and indicate that each hexamer binds three dTMP-PCP molecules tightly, and this half-site binding is sufficient for DNA binding to the helicase.

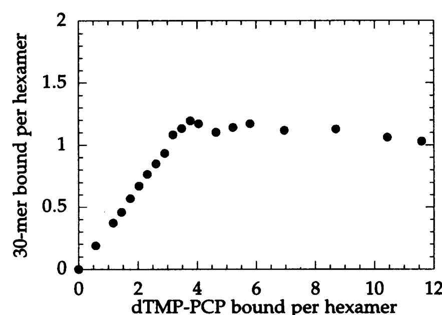

FIGURE 4 DNA binding in the presence of increasing concentration of dTMP-PCP. Equilibrium binding of 4A′ protein to radiolabeled 30-mer DNA was measured at increasing concentrations of dTMP-PCP as described in Materials and Methods. The plot of 30-mer DNA bound per 4A′ hexamer shows that binding of 3 dTMP-PCP per 4A′ hexamer is sufficient for tight binding interaction between the hexamer and 30-mer DNA.

DISCUSSION

The half-site binding of dTTP and dTMP-PCP to the helicase hexamer is interesting, and it may be a general feature of hexameric helicases. Another hexameric helicase, *E. coli*

DnaB, has been shown also to bind three ATP molecules tightly and three more with a weaker affinity (Bujalowski and Klonowska, 1993). Although we have observed only three dTTP binding, we cannot rule out additional weak NTP binding sites on 4A′ hexamer as its K_d may be >50–100 μM, and therefore beyond our experimental detection limit. The significance of the observed half-site binding or the negative cooperativity in NTP binding remains unclear at the present time. Recent electron microscopy studies have shown that the hexameric subunits of the helicase are arranged in a ring-like structure. The three strong NTP binding sites, therefore, may be arranged either in an alternating or consecutive manner.

Like most motor proteins, helicases have to translocate on the DNA unidirectionally in order to unwind DNA. NTP hydrolysis must therefore be used to modulate protein-DNA interactions that can promote movement. Previous quantitative DNA binding studies have shown how NTP hydrolysis may modulate DNA binding (Hingorani and Patel, 1993). We have found that 4A′ hexamers bind single-stranded DNA with a high affinity in the presence of MgdTMP-PCP, the nonhydrolyzable analog of dTTP, but the affinity of the helicase for the DNA is much weaker in the presence of MgdTDP. These results suggest that NTP binding to the hexamer promotes DNA binding and NTP hydrolysis leads to dissociation of the helicase from the DNA.

Given that the gene 4 proteins can translocate on single-stranded DNA in a processive manner (Tabor and Richardson, 1981), the question is, how does the helicase stay on the DNA through cycles of NTP hydrolysis if it dissociates from the DNA after each NTP hydrolysis? We have found that the ring-like hexamer binds single-stranded DNA through its hole (Egelman et al., 1995). This provides a mechanism for a highly processive helicase and translocase activities of the protein. Yet, it does not explain how the helicase may translocate on the DNA. To explain movement, we postulate that only a few subunits of the hexamer may hydrolyze NTP at any given time. This would allow the helicase to remain bound to the DNA at all times via the subunits not hydrolyzing NTP. The hexameric ring may use NTP hydrolysis to cause conformational changes in the protein that can allow the ring to move on the DNA in a rotating manner. The ability of the DNA to bind the helicase by interacting with only a few subunits is supported by the finding that mixed hexamers of 4A′ and an inactive mutant of 4A′ (4A′/K318A, which is defective in DNA binding in the presence of MgdTMP-PCP) can bind DNA with the same stoichiometry as hexamers of 4A′ (Patel et al., 1994). Detailed single-turnover dTTPase kinetic studies will need to be carried in order to determine whether only a few subunits hydrolyze NTP at any given time.

Hexameric helicases are emerging as a class of DNA helicases that may unwind DNA by similar mechanisms involving translocation on single-stranded DNA. We have shown that the T7 gene 4 helicase hexamer has three strong NTP binding sites whose exact function is unknown. NTP binding and hydrolysis is, however, used to bind and dissociate from the DNA. We postulate that the helicase contacts DNA via a few of its subunits at any given time, and thus DNA binding to consecutive subunits provides a mechanism for processive translocation.

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DISCUSSION

Session Chairperson: Steven Block
Scribe: Kevin Hacker

STEVEN M. BLOCK: Why doesn't the helicase spin instead of DNA? What keeps that from turning?

SMITA PATEL: You can either spin the DNA or the helicase. If you keep the DNA tied down it is probably the helicase that is spinning. If you keep the protein tied down the DNA is probably spinning. I don't think we can distinguish the two. The DNA may be consecutively binding to each of the subunits or the protein may be rotating. Do I understand your question?

BLOCK: Well, if the enzyme just keeps spinning that won't lead to any unwinding.