Cooperative Interactions of Nucleotide Ligands Are Linked to Oligomerization and DNA Binding in Bacteriophage T7 Gene 4 Helicases†

Manju M. Hingorani and Smita S. Patel*

Department of Biochemistry, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210

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ABSTRACT: The equilibrium nucleotide binding and oligomerization of bacteriophage T7 gene 4 helicases have been investigated using thymidine 5′-triphosphate (dTTP), deoxythymidine 5′-(β,γ-methylenetriphosphate) (dTMP-PCP), thymidine 5′-diphosphate (dTDP), adenosine 5′-triphosphate (ATP), and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS). In the presence of nucleotide ligands, T7 helicases self-assemble into hexamers with six potential nucleotide binding sites that are nonequivalent both in the absence and in the presence of single-stranded DNA. All nucleotides tested bind with high affinity to three sites (K_d = 5 × 10^{-6} M, dTTP; 6 × 10^{-7} M, dTMP-PCP; 4 × 10^{-6} M, dTDP; 3 × 10^{-5} M, ATP; 2 × 10^{-6} M, ATPγS), while binding to the remaining sites is undetectable. Interestingly, nucleotide binding to the high-affinity sites exhibits positive cooperativity which is sensitive to protein concentration. This effect is a result of ligand binding-linked oligomerization wherein helicase oligomer equilibrium changes as a function of both nucleotide and protein concentration. A study of DNA binding shows that 1−2 NTPs bound per hexamer are sufficient for stoichiometric interaction between the helicase and DNA. Thus, the ring-shaped helicase hexamers assemble around DNA with one, two, or three NTPs bound to each hexamer. This study also examines the preferred use of dTTP for T7 helicase-catalyzed DNA unwinding by comparison with ATP, the more commonly used nucleotide ligand. ATP binds to the helicase with 6-fold weaker affinity than dTTP and promotes hexamerization as well as DNA binding. Nevertheless, DNA unwinding with ATP is at least 100-fold slower than with dTTP. Thus, the difference in ATP and dTTP utilization probably lies in a highly specific step in the coupling of NTP hydrolysis to DNA unwinding.

DNA helicases catalyze duplex DNA unwinding and provide transient single-stranded templates for various processes of DNA metabolism such as DNA replication, repair, recombination, and conjugation. Helicases bind and translocate on DNA using energy from nucleotide triphosphate (NTP)[1] binding and hydrolysis to effect processive DNA unwinding (Lohman, 1992, 1993). The molecular mechanism of helicase-catalyzed DNA unwinding is not clear; however, studies of different helicases have revealed several key features that appear to be important for helicase function. Most known helicases exist as oligomers, such as dimers (*Escherichia coli* Rep: Wong & Lohman, 1992; *E. coli* helicase II: Runyon *et al*., 1993) and hexamers (SV40 T antigen: Mastrangelo *et al*., 1989; phage T7 gene 4 helicases: Patel & Hingorani, 1993; *E. coli* Ruv B: Stasiak *et al*., 1994; *E. coli* Rho RNA/DNA helicase: Finger & Richardson, 1982; Gogol *et al*., 1991; phage T4 gene 41 helicase: Dong *et al*., 1995; *E. coli* DnaB: Reha-Krantz & Hurwitz, 1978a; Bujalowski *et al*., 1994). Oligomerization is considered an important property because it can provide helicases with multiple DNA binding sites that may be important for association with DNA during translocation and unwinding.

Another property that is essential for helicase-catalyzed unwinding is nucleotide binding and hydrolysis. The mechanism by which NTP binding and hydrolysis are coupled to DNA unwinding is not understood at the present time. It has been shown that nucleotide binding modulates interactions of helicases with DNA (*E. coli* Rep: Arai *et al*., 1981; Wong & Lohman, 1992; Wong *et al*., 1992; phage T7 gene 4 helicases: Hingorani & Patel, 1993; *E. coli* DnaB: Arai & Kornberg, 1981a,b; Nakayama *et al*., 1984). Equilibrium nucleotide binding studies have been performed with hexameric helicases such as *E. coli* Rho (Stitt, 1988; Geiselmann & von Hippel, 1992), *E. coli* DnaB (Arai & Kornberg, 1981b; Biswas *et al*., 1986; Bujalowski & Klonowska, 1993), and T7 4A′ helicase (Patel & Hingorani, 1995). The hexameric helicases share a striking feature that the six potential nucleotide binding sites are nonequivalent and can be clearly distinguished as three high-affinity and three low-affinity nucleotide binding sites. Although there are significant differences between the hexameric helicases and the ramifications of negative cooperativity are not clearly understood, it is likely that this cooperativity plays an important role in translocation and DNA unwinding.

In this paper, we investigate equilibrium nucleotide binding to phage T7 helicases and its effects on helicase oligomerization and DNA binding. Bacteriophage T7 gene 4 helicases provide duplex DNA unwinding activity during phage

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* To whom correspondence should be addressed.

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[1] Abbreviations: dTMP-PCP, deoxythymidine 5′-(β,γ-methylenetriphosphate); dAMP-PCP, 2′-deoxyadenosine 5′-(β,γ-methylenetriphosphate); AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); NTP, nucleotide triphosphate; NDP, nucleotide diphosphate; P-C-P, methylenediphosphonic acid; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); TEAB, triethanolamine bicarbonate; IPTG, isopropyl β-D-thiogalactoside; ssDNA, single-stranded DNA; PAGE, polyacrylamide gel electrophoresis; PEI, poly(ethylenimine).

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DNA replication. Gene 4 encodes two proteins, 4A and 4B (Bernstein & Richardson, 1988, 1989), that are synthesized in the same reading frame (Dunn & Studier, 1983). The full-length product is the 63 kDa 4A protein that has both DNA helicase and primase activities. The 56 kDa protein, 4B, is synthesized from an internal initiation site and has helicase activity alone. All the studies in this report have been performed with pure recombinant 4A′ and 4B proteins. 4A′ is an M64L mutant of 4A that has helicase and primase activities comparable to the wild-type protein (Rosenberg *et al*., 1992; Patel *et al*., 1992).

4A′ and 4B proteins assemble into stable hexamers in the presence of MgdTMP-PCP[1] and ssDNA. The hexamers are ring-shaped and bind around ssDNA in the presence of MgdTMP-PCP (Hingorani & Patel, 1993; Egelman *et al*., 1995). DNA binding is modulated by nucleotide ligands such that in the presence of MgdTTP and MgdTMP-PCP, 4A′ binds ssDNA with a higher affinity than in the presence of MgdTDP. No interaction between 4A′ and ssDNA has been detected in the presence of Mg^{2+} alone. (Hingorani & Patel, 1993).

An initial report has shown that only three dTTPs bind tightly to each 4A′ hexamer, which is sufficient for stoichiometric binding of ssDNA to 4A′ (Patel & Hingorani, 1995). The results described in this paper show that this extreme negative cooperativity in nucleotide binding persists over a wide range of protein concentrations and is observed with different nucleotides such as ATP, ATPγS, and dTDP. A closer study of DNA binding shows that one to two NTPs bound per hexamer are sufficient for tight interaction with ssDNA. Interestingly, the three high-affinity nucleotide binding sites exhibit significant positive cooperativity. We demonstrate here that positive cooperativity in nucleotide binding is sensitive to protein concentration and protein oligomerization is sensitive to nucleotide concentration.

Bacteriophage T7 helicases bind and hydrolyze a variety of NTPs such as dTTP, ATP, dATP, and dGTP. Both 4A′ and 4B proteins have the lowest K_m for dTTP, which appears to be the preferred nucleotide for helicase and primase activity (Matson *et al*., 1983; Matson & Richardson, 1983, 1985; Patel *et al*., 1992). This is very curious since most known helicases utilize ATP to power unwinding. In this study, we demonstrate that 4A′ helicase binds ATP and forms hexamers that bind ssDNA in the presence of ATP. However, the rate of helicase-catalyzed DNA unwinding remains significantly lower with ATP when compared to unwinding with dTTP.

EXPERIMENTAL PROCEDURES

Nucleotides and Other Reagents. dTMP-PCP and dAMP-PCP were purchased from Amersham Life Science Inc. [α-^{32}P]dTTP (3000 Ci/mM), [α-^{32}P]ATP (3000 Ci/mM), and [γ-^{35}S]ATP (1000 Ci/mM) were purchased from ICN Biochemicals Inc. Methylenediphosphonic acid, trisodium salt (P-C-P), dTTP, dTDP, ATP, AMP-PCP, and ATPγS were purchased from Sigma Chemicals Co. Nitrocellulose (BA-83; 0.2 μm) and DEAE membranes (NA 45) were obtained from Schleicher & Schuell Inc. DEAE-Sephadex A-25 was purchased from Pharmacia LKB Biotechnology. Biogel P-30 resin was purchased from Bio-Rad Laboratories Inc., and PEI−cellulose TLC plates were purchased from EM Separations Technology.

DNA. Oligodeoxynucleotides used in the synthesis of radiolabeled dTMP-PCP and binding assays were synthesized at the Biochemical Instrument Center at Ohio State University. Their sequences are as follows: 25-mer, 5′-GCCTC GCAGC CGTCC AACCA ACTCA; 30-mer, 5′-AGCTT GCATC ATAGT GTCAC CTGTT ACGTT; 36-mer, 5′-CGGAG CGTCG GCAGG TTGGT TGAGT AGGTC TTGTT T. The DNAs were purified by denaturing polyacrylamide gel electrophoresis (18% polyacrylamide/7 M urea), electroeluted from the gel (Elutrap, Schleicher & Schuell Inc.), ethanol-precipitated with sodium acetate, and reconstituted in distilled water. DNA concentrations were determined from absorbance measurements at 260 nm in Tris-HCl/7 M urea using the molar extinction coefficients of 25-mer (249 040 M^{-1} cm^{-1}), 30-mer (305 010 M^{-1} cm^{-1}), and 36-mer (377 000 M^{-1} cm^{-1}).

Buffers. The binding buffer consisted of 50 mM Tris−acetate, pH 7.5, 40 mM sodium acetate, 10 mM magnesium acetate, and 10% glycerol. Membrane wash buffer was 50 mM Tris−acetate, pH 7.5, 40 mM sodium acetate, and 10 mM magnesium acetate. Nucleotide synthesis buffer was 50 mM Tris−acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, and 0.1 mg/mL BSA.

Enzymes. *(A) A Modified Protocol for Fast Purification of Bacteriophage T7 Gene 4 Proteins*. Bacteriophage T7 gene 4 proteins 4A′ and 4B were overexpressed in *E. coli* and purified using a modified procedure from the one described earlier (Patel *et al*., 1992). The cell lines BL21-(DE3)/pAR5018 (4A′) and BL21(DE3)/pAR5019 (4B) (Rosenberg *et al*., 1992) were grown at 37 °C, induced with IPTG, harvested, and lysed by freeze−thaw cycles as described (Patel *et al*., 1992). In the modified procedure, the poly(ethylenimine) and ammonium sulfate precipitation steps following cell lysis have been eliminated. Instead, lysed cells were centrifuged at a relative centrifugal field of 257000*g* for 1 h (4 °C) to pellet the cell debris. The supernatant was collected and diluted with buffer B (20 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol) to bring the conductance of the crude extract to that of buffer B + 50 mM NaCl. The solution was loaded directly on a phosphocellulose column previously equilibrated with buffer B + 50 mM NaCl. Phosphocellulose column chromatography and the remaining purification steps were performed as described (Patel *et al*., 1992). The new procedure reduces the time required for purification and increases the yield by 10−20%. Protein purity remains greater than 95% as determined from Coomassie-stained SDS−PAGE. Protein concentrations were determined both by the Bradford method (Bradford, 1976) and from absorbance measurements at 280 nm in 100 mM Tris−HCl (pH 7.5)/8 M urea buffer, using the extinction coefficients 76 100 M^{-1} cm^{-1} for 4A′ and 67 850 M^{-1} cm^{-1} for 4B proteins.

(B) HIV-1 Reverse Transcriptase. Purified HIV-1 reverse transcriptase enzyme used for the synthesis of radiolabeled dTMP-PCP was a gift from Dr. Karen Anderson (Yale University).

Preparation of Radiolabeled Nucleotides. *(A) Synthesis of [α-^{32}P]dTMP-PCP*. [α-^{32}P]dTMP-PCP was prepared by HIV-1 reverse transcriptase-catalyzed pyrophosphorolysis of 3′-[α-^{32}P]dTMP containing partially duplex DNA as described earlier (Patel & Hingorani, 1995). The procedure involves transfer of [α-^{32}P]dTMP from DNA to P-C-P, resulting in formation of dTMP-PCP. Reverse transcriptase

was chosen as the catalyst for [α-^{32}P]dTMP-PCP synthesis because it can use P-C-P as a substrate for the pyrophosphorolysis reaction more efficiently than other DNA polymerases tested. The enzyme-catalyzed pyrophosphorolysis reaction transfers about 40−50% of [α-^{32}P]dTMP from the primer/template DNA to P-C-P. Purification of [α-^{32}P]-dTMP-PCP by anion-exchange chromatography in a disposable syringe column is convenient as it confines radioactivity and minimizes loss of synthesized nucleotide. Purified dTMP-PCP has a specific activity of about 20 mCi/mM and can be used directly in membrane binding assays to measure dTMP-PCP binding to 4A′ and 4B proteins.

(B) Synthesis of [α-^{32}P]dTDP. [α-^{32}P]dTDP was prepared by 4A′-catalyzed hydrolysis of [α-^{32}P]dTTP. The reaction (20 μL) contained [α-^{32}P]dTTP (30 μCi), 0.05 μM single-stranded M13mp18 DNA, and 0.2 μM 4A′ in nucleotide synthesis buffer. The solution was incubated at 37 °C for 2 h and subjected to anion-exchange chromatography to separate [α-^{32}P]dTDP from [α-^{32}P]dTTP and ssM13 DNA. The sample was loaded on a 1 mL DEAE-Sephadex column previously equilibrated with 0.05 M TEAB, pH 8.0, and the nucleotides were eluted with a linear gradient (8 mL) of 0.05−1 M TEAB, pH 8.0. Fractions (∼60 μL) of the eluate were collected, and 1 μL aliquots were spotted on a PEI−cellulose TLC plate and developed in 0.3 M potassium phosphate buffer, pH 3.4, to identify the nucleotide in each fraction. The initial fractions containing pure [α-^{32}P]dTDP were combined and extracted with phenol/chloroform to remove any contaminating 4A′ protein. The solution was dried under vacuum and washed several times with methanol followed by drying. [α-^{32}P]dTDP was reconstituted in water and used directly in equilibrium binding assays.

Equilibrium Binding of Nucleotide Ligands to Gene 4 Proteins: Measurement of dTTP Binding. Equilibrium nucleotide binding assays were performed at constant protein and increasing dTTP concentrations using a nitrocellulose membrane binding assay. dTTP binding to 4A′ or 4B was measured at 22 and 4 °C as described earlier (Patel & Hingorani, 1995). The assays were performed at 5 and 20 μM protein concentrations and 0−150 μM dTTP, both in the absence and in the presence of 30-mer DNA (5 μM). The molar fraction of dTTP bound to 4A′ protein was determined and plotted *versus* dTTP concentration.

Equilibrium Binding of dTMP-PCP to 4A′ Protein. dTMP-PCP binding to gene 4 proteins was measured at constant protein concentration (4A′: 0.1, 0.5, 5, 10, and 20 μM; 4B: 20 μM) and increasing concentrations of [α-^{32}P]dTMP-PCP (0−50 μM), using the membrane binding assay described above. dTMP-PCP binding to 4A′ (17 μM) and 4B (20 μM) was measured also in the presence of 30-mer ssDNA (4 μM).

Equilibrium Binding of dTMP-PCP Measured by Protein Fluorescence Change. dTMP-PCP binding to 4A′ was measured also by fluorescence titrations that were performed at 22 °C using an SLM SPF-500C spectrofluorometer (SLM Instruments Inc.). dTMP-PCP (0−50 μM) was added incrementally to 4A′ protein (10 μM) in buffer (50 mM Tris−acetate, pH 7.5, 50 mM sodium acetate, 3 mM magnesium acetate, and 10% glycerol). Nucleotide binding was measured by monitoring the quenching of protein tryptophan fluorescence (λ_{ex} = 300 nm; λ_{em} = 335 nm). The fluorescence intensity at each dTMP-PCP concentration was corrected for the inner filter effect as described (Lohman & Mascotti, 1992); photobleaching and dilution effects were found to be negligible. The ratio of fluorescence quenching to the maximum quenching detected was plotted *versus* total dTMP-PCP concentration.

Competitive Binding of dTMP-PCP and dTDP to 4A′ Protein. Equilibrium binding of [α-^{32}P]dTDP to 4A′ was measured first in the absence of dTMP-PCP. A constant amount of 4A′ (20 μM) was titrated with dTDP + [α-^{32}P]-dTDP (0−200 μM) in binding buffer, and the assay was performed at room temperature as described for dTTP. Competitive binding of dTDP and dTMP-PCP to 4A′ protein was measured by adding a constant amount of 4A′ (20 μM) to a preincubated mixture of dTMP-PCP (20 μM) and dTDP + [α-^{32}P]dTDP (0−200 μM). The same experiment was also performed with [α-^{32}P]dTMP-PCP and unlabeled dTDP. All titrations were quantitated by measuring the radioactivity on the membranes, and the molar amount of nucleotide bound to 4A′ protein was calculated and plotted *versus* total dTDP concentration.

Equilibrium Binding of ATP and ATPγS to 4A′ Protein. ATP binding to 4A′ was measured at 4 °C at constant protein and increasing ATP concentrations. 4A′ protein (5 μM) and ATP + [α-^{32}P]ATP (0−200 μM) were mixed in binding buffer and filtered through nitrocellulose membranes within 30−40 s as described above for dTTP. ATPγS binding was measured similarly by titrating 4A′ (0.2 μM) with ATPγS + [γ-^{35}S]ATP (0−25 μM). The molar amount of nucleotide bound to 4A′ was determined and plotted *versus* total nucleotide concentration.

Equilibrium Binding of ssDNA to 4A′ and 4B Proteins at Increasing Nucleotide Concentrations. DNA binding to gene 4 proteins was measured at increasing concentrations of nucleotides such as dTMP-PCP, ATPγS, AMP-PCP, and dAMP-PCP. Samples (15 μL) were prepared in binding buffer and contained 4A′ (17 μM with dTMP-PCP, 12 μM with ATPγS, AMP-PCP, and dAMP-PCP), 5′-^{32}P-radiolabeled 30-mer DNA (4 μM), and either dTMP-PCP (0−20 μM), ATPγS (0−400 μM), AMP-PCP (0−80 μM), or dAMP-PCP (0−80 μM). The above mixture was incubated at 22 °C for 20 min, and DNA binding was measured using the nitrocellulose−DEAE membrane assembly as described previously (Hingorani & Patel, 1993). Equilibrium binding of 5′-^{32}P-radiolabeled 30-mer (4 μM) to 4B protein (20 μM) was measured similarly at increasing dTMP-PCP concentrations (0−20 μM). Quantitation of radioactivity on the nitrocellulose membrane gave a measure of protein-bound DNA, and quantitation of the DEAE membrane provided free DNA concentration. The fraction of DNA bound per 4A′ hexamer was determined and plotted *versus* the nucleotide concentration.

High-Pressure Gel Filtration Chromatography of 4A′ Protein. Oligomerization of 4A′ was examined in the presence of different nucleotides such as dTTP, dTDP, and ATP at various protein and nucleotide concentrations using small-zone gel filtration chromatography. Chromatography was performed using a Bio-Sil SEC 400 gel filtration column as described earlier (Patel & Hingorani, 1993). The experiments were performed with dTTP (10, 30, 100 μM) and dTDP (30 and 200 μM) at 4A′ concentrations of 1 and 20 μM, and with ATP (0.2, 0.5, and 1 mM) at 5 μM 4A′. The eluted protein species were assigned molecular masses based on the elution profiles of standard marker proteins as described (Patel & Hingorani, 1993).

Curve Fitting. The equilibrium nucleotide binding isotherms were fit to a hyperbola or to the quadratic solution for 1:1 binding of ligand to a macromolecule (eq 1)

$$[\mathrm{ML}] = (K_\mathrm{d} + L_\mathrm{T} + M_\mathrm{T})/2 - \{(K_\mathrm{d} + L_\mathrm{T} + M_\mathrm{T})^2/4 - (M_\mathrm{T}L_\mathrm{T})\}^{1/2} \quad (1)$$

where K_d is the dissociation constant, L_T is the total ligand concentration (dTTP, dTMP-PCP, dTDP, ATP, and ATPγS), M_T is the total protein concentration (monomer), and ML is the amount of ligand bound to protein.

The equilibrium binding isotherms were fit to the Hill equation (eq 2) to estimate the cooperativity in ligand binding

$$Y_\mathrm{L} = [\mathrm{L}]^n/K + [\mathrm{L}]^n \quad (2)$$

where Y_L is the fraction of detectable ligand binding sites on the protein (fractional saturation), [L] is the free ligand concentration, K is the dissociation constant, and n is the Hill coefficient. All curves were fit by a nonlinear least-squares fitting program using KaleidaGraph (Synergy Software, Reading, PA).

RESULTS

Equilibrium Binding of MgdTTP. Nucleotide triphosphate binding and hydrolysis activities are essential for helicase-catalyzed unwinding of duplex DNA. The T7 gene 4 helicases 4A and 4B hydrolyze nucleotide substrates such as dTTP, dATP, and ATP in the presence of single-stranded DNA with varying specificity (Patel *et al*., 1992). dTTP is believed to be the nucleotide of choice primarily because of its lower K_m for hydrolysis. We study here the interactions of both 4A′ and 4B proteins with various nucleotide cofactors such as dTTP, dTDP, dTMPPCP, ATP, and ATPγS to understand the similarities and differences in their binding to the helicases. The equilibrium binding of radiolabeled nucleotides was measured using nitrocellulose membrane binding assays. The use of radiolabeled ligands provided directly the molar amounts of protein-bound ligand that were used to determine both the stoichiometry and the equilibrium dissociation constants of nucleotides bound to the hexameric helicase.

The equilibrium ligand binding experiments in this study have been carried out at constant protein and increasing ligand concentrations. Figure 1A shows the titration of a constant amount of 4A′ (5 μM monomer concentration) with increasing MgdTTP. The assay was performed at 4 °C to minimize dTTP hydrolysis during the course of the experiment (k_cat of dTTP hydrolysis is 0.03 s^{-1} at 22 °C, and hydrolysis is undetectable at 4 °C up to 2 h). The MgdTTP binding data in Figure 1A fit well to a quadratic equation (eq 1) with an equilibrium dissociation constant K_d of (4.9 ± 0.5) × 10^{-6} M. Interestingly, the binding reaches a plateau with dTTP bound to only half the molar amount of 4A′ monomers present in the reaction.

Equilibrium binding of MgdTTP was measured at a higher protein concentration to determine directly the stoichiometry of dTTP binding and to investigate if the three remaining nucleotide binding sites on the hexamer can be occupied at higher protein concentrations. Figure 1B shows the titration of 20 μM 4A′ with increasing MgdTTP. As above, binding saturates with three dTTP molecules bound per 4A′ hexamer.

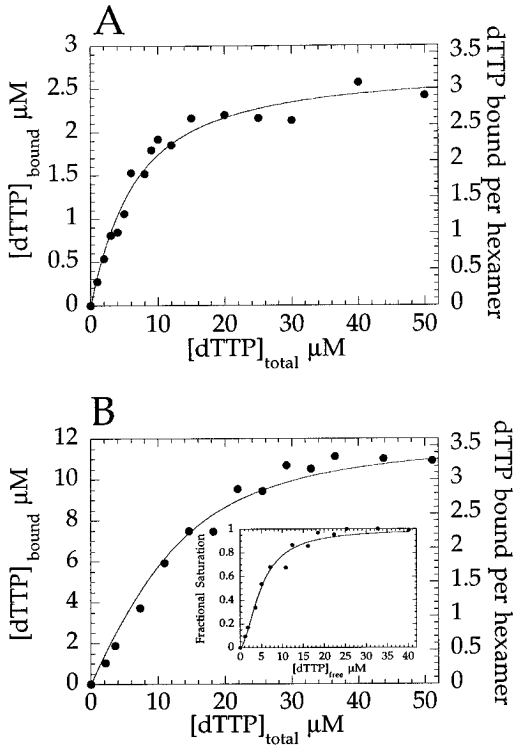


FIGURE 1: Equilibrium binding of MgdTTP to 4A′. dTTP binding to 4A′ protein was measured at 4 °C using nitrocellulose membrane binding assays as described under Experimental Procedures. Panel A shows binding of [α-^{32}P]dTTP to 5 μM 4A′ at increasing dTTP concentrations (0−50 μM). dTTP binding fits to a quadratic equation (eq 1) with a K_d of 4.9 × 10^{-6} M. Panel B shows the curve for a similar titration performed at 20 μM 4A′ with increasing dTTP (0−40 μM) fit to a quadratic equation. The second y-axis shows the number of dTTP bound per 4A′ hexamer. At both 4A′ concentrations, binding saturates at three molecules of dTTP bound per hexamer. The inset panel B shows the isotherm at 20 μM 4A′ fit to the Hill equation with a coefficient of 1.73 ± 0.15.

The half-site binding of MgdTTP was observed also in an experiment performed at 22 °C (data not shown). The absence of nucleotide binding to the remaining three sites on the hexamer even at very high concentrations of dTTP (200 μM) and protein (20 μM) suggests that those sites either do not bind dTTP or bind with a weaker affinity that may be unstable to detection by the nitrocellulose binding assay.

The protein concentration, 20 μM, used in the above dTTP binding experiment is well above the K_d of dTTP equal to 5 μM. Under these conditions, binding is expected to be stoichiometric, and the inflection points in the binding isotherms should provide the stoichiometry of dTTP binding. The inflection point in the dTTP binding isotherm at 20 μM 4A′ (Figure 1B) does not correspond to a stoichiometry of 3 dTTP per 4A′ hexamer. Thus, the isotherm does not fit well to a quadratic equation, but fits to the Hill equation with a Hill coefficient of 1.7 (see inset in Figure 1B). The measured Hill coefficient indicates that dTTP binds to 4A′ at higher concentrations with a positive cooperativity (Hill coefficient at 5 μM 4A′ = 1.1). The same results were obtained with the 4B helicase protein (data not shown). The above results indicate that the hexameric gene 4 proteins show both positive and negative cooperativity in dTTP binding. The three tight binding sites show positive cooperativity at high protein concentrations, and the lack of nucleotide binding to the remaining three sites suggests extreme negative cooperativity in nucleotide binding.

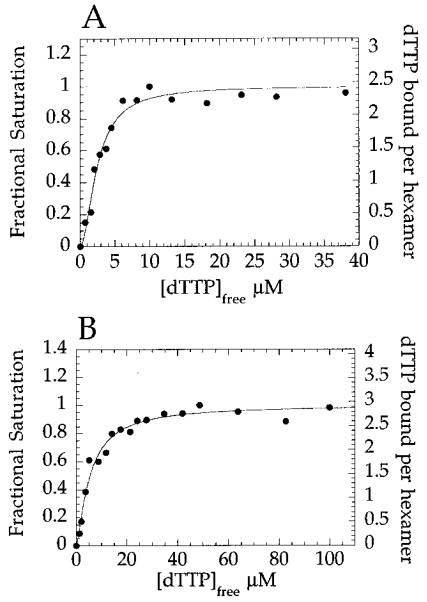


FIGURE 2: Equilibrium binding of MgdTTP to 4A′ in the presence of ssDNA. Binding of dTTP to a constant amount of 4A′ was measured at 4 °C in the presence of 30-mer ssDNA (5 μM) using the nitrocellulose binding assays. Panel A shows binding of [α-^{32}P]-dTTP to 5 μM 4A′ in the presence of 30-mer DNA, and panel B shows a similar titration at 20 μM 4A′ concentration. The binding isotherms fit to the Hill equation with coefficients of 1.8 ± 0.2 and 1.3 ± 0.1 for 5 μM and 20 μM 4A′, respectively. Binding saturates with three dTTP bound per 4A′ hexamer as shown by the second *y*-axis.

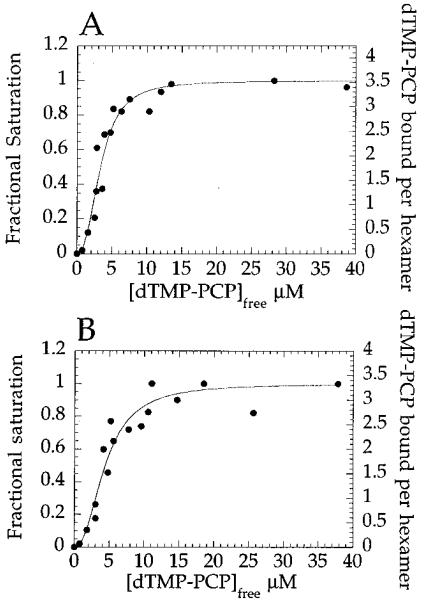


FIGURE 3: Equilibrium binding of MgdTMP-PCP to gene 4 proteins at constant protein concentration. [α-^{32}P]dTMP-PCP binding to 4A′ and 4B proteins was measured at 22 °C using the nitrocellulose binding assays. Panel A shows binding of [α-^{32}P]dTMP-PCP to 4A′ (20 μM) at increasing dTMP-PCP concentrations (0−40 μM). Panel B shows a similar titration performed under the same conditions with the 4B protein. The binding isotherms fit to the Hill equation with coefficients of 2.47 ± 0.4 and 2.26 ± 0.4 for 4A′ and 4B, respectively. Binding saturates with a maximum of 3−4 dTMP-PCP molecules bound per hexamer.

Equilibrium Binding of MgdTTP in the Presence of ssDNA. We have shown previously that 4A′ binds ssDNA only in the presence of nucleotide triphosphate (Hingorani & Patel, 1993; Egelman *et al*., 1995). The binding of dTTP to 4A′ was measured in the presence of 30-mer ssDNA to investigate the effect of DNA on the K_d and the cooperativity in dTTP binding. The concentration of 30-mer was chosen to be high enough to assure one DNA bound per protein hexamer. Figure 2A and Figure 2B show MgdTTP binding to 5 μM and 20 μM 4A′ protein, respectively, in the presence of 30-mer ssDNA. The experiment was performed at 4 °C to minimize dTTP hydrolysis (k_{cat} of dTTP hydrolysis in the presence of 30-mer is 0.0017 s^{-1} at 4 °C). The presence of DNA does not seem to significantly affect the binding of dTTP to 4A′. In the presence of the 30-mer, a maximum of 3 dTTP ligands bind to 4A′ at both protein concentrations. The isotherm fit to the Hill equation with Hill coefficients of 1.8 and 1.3 (at 5 and 20 μM 4A′, respectively), indicating positive cooperativity in dTTP binding in the presence of ssDNA.

Equilibrium Binding of MgdTMP-PCP. The radiolabeled analog [α-^{32}P]dTMP-PCP prepared via a novel enzyme-catalyzed reaction (see Experimental Procedures) was used to study the equilibrium interactions of gene 4 proteins with dTMP-PCP. The use of a nonhydrolyzable analog of dTTP facilitated nucleotide binding measurements at room temperature (22 °C) and avoided complications from nucleotide hydrolysis. Equilibrium binding of a constant amount of dTMP-PCP to increasing 4A′ protein has been described previously (Patel & Hingorani, 1995). Binding occurs with a ratio of two 4A′ monomers to one dTMP-PCP molecule or three dTMP-PCP per hexamer, both in the absence and in the presence of DNA. This is consistent with the results shown in Figures 1 and 2 where three dTTP molecules bind per 4A′ hexamer with high affinity. To avoid complications due to changing oligomeric states of 4A′ as its concentration is increased, dTMP-PCP binding was measured at constant protein and increasing ligand concentrations both in presence and in the absence of DNA. Figure 3A and Figure 3B show, respectively, binding of MgdTMP-PCP to 20 μM 4A′ and 20 μM 4B protein in the absence of ssDNA. A maximum of 3−4 dTMP-PCP molecules bind 4A′ and 4B hexamers at saturation. No additional dTMP-PCP molecules were found to bind even at nucleotide concentrations as high as 200 μM (data not shown). The dTMP-PCP binding isotherms do not fit to a hyperbola but fit to the Hill equation with Hill coefficients of 2.5 and 2.3 for 4A′ and 4B proteins, respectively. The 3−4 dTMP-PCP molecules therefore interact with both 4A′ and 4B proteins with a high positive cooperativity. dTMP-PCP binding isotherms remain unchanged in the presence of 30-mer ssDNA (see Figure 6A,B). No more than 4 dTMP-PCP bind per hexamer in the presence of ssDNA with Hill coefficients of 2.5 and 3.1 for 4A′ and 4B, respectively.

It is known that oligomerization of gene 4 proteins is dependent both on protein concentration and on the presence of nucleotide ligands (Patel & Hingorani, 1993). A series of dTMP-PCP binding experiments were performed at protein concentrations ranging from 0.1 to 20 μM 4A′ to investigate the dependence of positive cooperativity on protein concentration. Table 1 summarizes the results of dTMP-PCP binding at increasing 4A′ protein concentrations. At 0.1 μM 4A′, dTMP-PCP binding fit to a hyperbola with a K_d of (2.2 ± 0.3) × 10^{-6} M and a maximum binding of 3 dTMP-PCP per hexamer. The Hill coefficient for dTMP-PCP binding to 0.1 μM 4A′ is 1.4. At 0.5 μM 4A′, the K_d tightens to (7 ± 0.14) × 10^{-7} M with a maximum binding of 2.5 ligands per hexamer and a Hill coefficient of 1.2. At

Table 1: Stoichiometry, K_d, and Hill Coefficient Values for dTMP-PCP Binding to 4A′ Protein

4A′ (μM)	dTMP-PCP per hexamer	K_d ($\times 10^{-6}$ M)	Hill coefficient
0.1	2.9	2.23 ± 0.31	1.36 ± 0.09
0.5	2.4	0.69 ± 0.14	1.2 ± 0.19
5	2.9	0.3 ± 0.09	1.87 ± 0.19
10	3.35	0.6 ± 0.16	1.95 ± 0.26
20	4.2	4.18 ± 0.9	2.47 ± 0.38

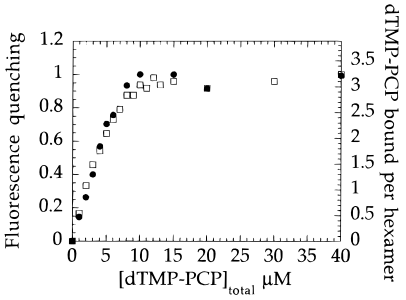


FIGURE 4: dTMP-PCP binding measured by protein fluorescence change. 4A′ protein (10 μM) was titrated with dTMP-PCP (0−40 μM), and the resulting quenching of protein fluorescence was measured as described under Experimental Procedures. Fluorescence quenching was normalized to 1 and plotted against total dTMP-PCP concentration (□). For comparison, binding of [α-^{32}P]-dTMP-PCP to 4A′ was measured under exactly the same conditions by the membrane binding assay and plotted *versus* total dTMP-PCP concentration (●).

5 μM 4A′, binding of dTMP-PCP shows increased sigmoidicity and saturates at 3 molecules of dTMP-PCP bound per hexamer with a Hill coefficient of 1.9, whereas at 10 μM 4A′ maximum binding is 3.4 and the Hill coefficient is 2.0. At 20 μM 4A′, the maximum binding of dTMP-PCP increases to 4 molecules per hexamer and the Hill coefficient is 2.5. These results clearly show that positive cooperativitiy increases with increasing protein concentrations. This behavior is consistent with nucleotide binding linked to oligomerization of gene 4 proteins. In this system, different oligomeric species of 4A′ must bind nucleotides with different affinities. Therefore, as the oligomer equilibrium shifts with protein and nucleotide concentrations, the affinity of 4A′ for the nucleotide changes, resulting in the observed cooperativity. Accordingly, a simple isotherm with one K_d value cannot be used to describe the binding isotherms especially at higher protein concentrations (Table 1).

The observed half-site binding of dTTP and dTMP-PCP nucleotides at all protein concentrations suggests either that the remaining three sites do not bind nucleotides or that the ligands are bound with a weaker affinity such that they dissociate during filtration through the nitrocellulose membranes. We have used a separate fluorescence-based assay to measure the equilibrium binding of dTMP-PCP. dTMP-PCP binding to 4A′ results in about 10% decrease in the intrinsic fluorescence of 4A′. This signal was used to measure the binding of dTMP-PCP at constant protein and increasing nucleotide concentrations as shown in Figure 4. It is difficult to obtain the molar amounts of nucleotide bound to 4A′ hexamer from the fluorescence change-based isotherm, because the effects of ligand binding linked to oligomerization result in a shifted curve (Lohman & Mascotti, 1992). Therefore, a binding isotherm measured under the same conditions using radiolabeled dTMP-PCP is plotted to show that the two isotherms overlap quite well at all concentrations of dTMP-PCP. These results indicate that the observed half-site binding is not an artifact of nonequilibrium conditions during the membrane binding assay. Another reason for half-site binding may be that the remaining three sites are occupied by tightly-bound nucleotides copurified during protein preparation. In order to test this possibility, 4A′ was treated with a high concentration of EDTA (5 mM) for 1−2 h to promote dissociation of any tightly-bound nucleotides (nucleotide binding is weaker in the absence of Mg^{2+}; unpublished results). 4A′ thus treated still bound only three dTMP-PCP nucleotides per hexamer with a tight affinity (data not shown). Furthermore, half-site binding does not result from slow binding as no additional dTMP-PCPs bind 4A′ hexamer even after incubation for 3 h.

Gel Filtration of 4A′ in the Presence of Various Nucleotide Ligands. We have demonstrated earlier using gel filtration chromatography that 4A′ forms stable hexamers in the presence of MgdTMP-PCP at protein concentrations as low as 0.2 μM (Patel & Hingorani, 1993). We investigate here the effects of varying concentrations of 4A′ and nucleotides on protein oligomerization to better understand the ligand binding linked oligomerization process. The gel filtration results shown here indicate that hexamers are formed in the presence of dTTP, dTDP, and ATP similar to those in the presence of dTMP-PCP. However, the protein and ligand concentrations required to form hexamers stable to gel filtration vary depending on the type of ligand. As shown in Figure 5A, at lower MgdTTP concentrations (10 and 30 μM), 4A′ at 1 and 20 μM elutes as a broad peak between monomer/dimer and hexamer positions. At a higher MgdTTP concentration (100 μM), 4A′ at both 1 and 20 μM elutes as a sharp peak at the hexamer position. A similar effect on 4A′ oligomerization is seen also in the presence of MgdTDP as shown in Figure 5B. At lower dTDP concentration (30 μM), 4A′ at 1 and 20 μM elutes as a mixture of dimer and hexamer, but at 200 μM MgdTDP, 4A′ elutes as a stable hexameric species. The only difference in 4A′ oligomerization in the presence of dTDP versus dTTP appears to be in the kinetics of interconversion of dimer and higher oligomers. 4A′ hexamers appear to be in rapid equilibrium with lower oligomers in the presence of dTTP. However, in the presence of dTDP, the hexamer does not rapidly equilibrate with the lower oligomers, and hence the protein migrates as distinct monomer/dimer or hexamer species. 4A′ behaves in the same manner in the presence of MgATP as shown in Figure 5C. At lower ATP concentrations (200 μM), 4A′ elutes as a mixture of dimers and hexamers, but at higher ATP concentrations (1 mM), 4A′ does form stable hexamers. High ATP concentrations are required in these experiments because ATP binding to 4A′ is weaker than dTTP or dTDP binding (see below).

Hexamers with 1−2 dTMP-PCP Bound Are Capable of Binding ssDNA. Gene 4 proteins bind ssDNA only in the presence of NTP, and stable DNA binding has been observed only in the presence of MgdTMP-PCP (Hingorani & Patel, 1993; Egelman et al., 1995). The question asked here is how many NTPs bound per hexamer are sufficient to promote stable ssDNA binding. To determine the number of dTMP-PCP molecules per hexamer required for ssDNA binding, we have measured the equilibrium binding of radiolabeled 30-mer DNA to 4A′ as a function of MgdTMP-PCP. DNA binding was quantitated using the double-membrane binding assay, and Figure 6A and Figure 6B show

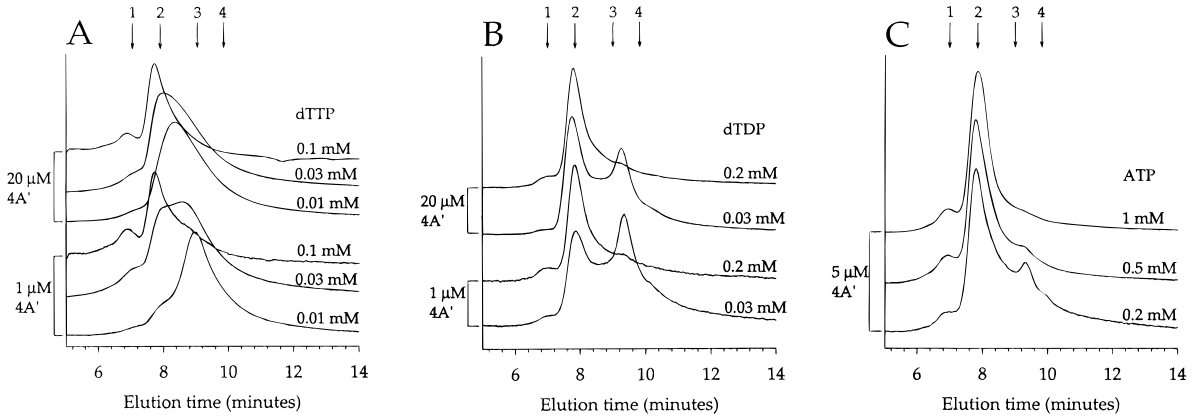


FIGURE 5: HPLC gel filtration of 4A′ at various nucleotide and protein concentrations. 4A′ protein oligomers were resolved by high-pressure gel filtration chromatography performed at 22 °C at a flow rate of 1 mL/min. Various concentrations of 4A′ (shown on the profile) were preincubated with nucleotides for 1−2 min before injection, and the eluted protein species were detected by protein fluorescence. Gel filtration experiments were performed with dTTP (profile A), dTDP (profile B), and ATP (profile C), that were present in the elution buffer at the concentrations indicated in the figure. The two clearly separated peaks are 4A′ dimer species eluting at 9−9.3 min and the hexamer species eluting at 7.8 min. Elution times of the standard gel filtration markers (BioRad) are indicated as follows: 1, thyroglobulin dimer (670 000 Da); 2, thyroglobulin monomer (335 000 Da); 3, IgG (150 000 Da); 4, ovalbumin (43 000 Da).

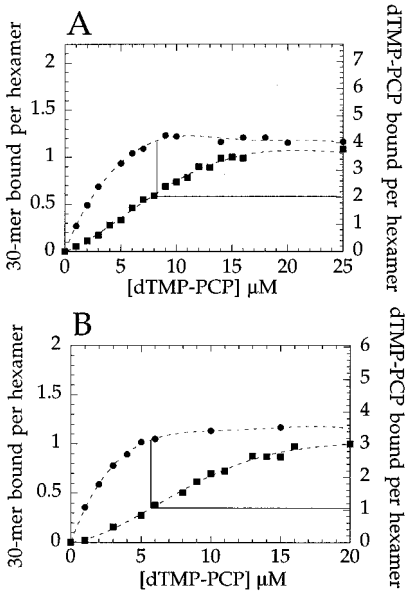


FIGURE 6: Measurement of DNA binding to 4A′ and 4B hexamers as a function of dTMP-PCP concentration. Equilibrium binding of DNA to 4A′ and 4B proteins was measured at 22 °C using radiolabeled DNA and the nitrocellulose−DEAE membrane binding assays. The corresponding binding of [α-^{32}P]dTMP-PCP under the same conditions was measured using the nicrocellulose binding assays. Panel A shows binding of radiolabeled 30-mer DNA (4 μM) to 4A′ (17 μM) at increasing dTMP-PCP concentrations (●), as well as the binding of [α-^{32}P]dTMP-PCP to 4A′ under the same conditions in the presence of unlabeled 30-mer (■). The isotherms show complete 30-mer DNA binding (one 30-mer per hexamer) when only two dTMP-PCP molecules are bound per 4A′ hexaimer. Panel B shows the results of identical assays of DNA binding (●) and dTMP-PCP binding (■) with the 4B protein (20 μM). DNA binding to 4B saturates with one dTMP-PCP molecule bound per hexamer.

results of such an experiment with 17 μM 4A′ and 20 μM 4B protein, respectively. The inflection points of the DNA binding isotherms provide the minimum concentration of dTMP-PCP necessary for DNA binding. For comparison, we also show the number of dTMP-PCP molecules bound to 4A′ under the exact conditions of the DNA binding assay. Interestingly, complete DNA binding to 4A′ is observed (one 30-mer bound per hexamer) with an average of two dTMP-PCP molecules bound per hexamer (Figure 6A). DNA binding to 4B is complete with an average of one dTMP-PCP bound per hexamer (Figure 6B). These experiments are highly informative because the results suggest that, first, 4A′ and 4B hexamers can be assembled with only one to two dTMP-PCP molecules bound per hexamer and, second, these partially-filled hexamers are capable of binding DNA. These results are consistent with an earlier finding that mixed hexamers of 4A′ and an inactive mutant 4A′/K318A (defective in DNA binding in the presence of dTMP-PCP) can bind ssDNA with the same stoichiometry as hexamers of pure 4A′ (Patel *et al*., 1994).

Interaction of MgdTDP with 4A′ Protein. During hydrolysis of dTTP by the gene 4 protein hexamers, there may be intermediate states where the hexamers have both dTTP and dTDP bound to various subunits simultaneously. To investigate if the half-site binding that we observe is simply an artifact of only the triphosphate nucleotide present in our assay, we have examined the interaction of 4A′ with dTDP in the absence and presence of dTMP-PCP. Equilibrium binding of MgdTDP alone to 4A′ was measured directly using [α-^{32}P]dTDP which was prepared by 4A′-catalyzed hydrolysis of [α-^{32}P]dTTP (see Experimental Procedures). Figure 7A shows binding of [α-^{32}P]dTDP to a constant amount of 4A′. dTDP binding fits to a quadratic equation with a K_d of $(4.17 \pm 0.97) \times 10^{-6}$ M and shows a maximum binding of three dTDP molecules per hexamer. MgdTDP binds 4A′ with an affinity very similar to that of MgdTTP, suggesting that the removal of a phosphate group does not significantly affect recognition and binding of the nucleotide cofactor to 4A′ protein. We did find, however, that the interaction of dTDP to 4A′ is sensitive to Mg^{2+} concentration in the reaction because at low Mg^{2+} (3 mM) dTDP binding is 6-fold weaker than at 10 mM Mg^{2+} (data not shown). This effect is not observed with dTTP and is specific to dTDP perhaps due to the lower stability of the MgdTDP complex relative to MgdTTP.

To determine if more than three nucleotide binding sites on 4A′ are occupied in the presence of both dTDP and dTMP-PCP, binding experiments were performed at constant protein + dTMP-PCP and increasing MgdTDP concentrations. The binding of both dTDP and dTMP-PCP was measured in separate experiments using [α-^{32}P]dTDP and

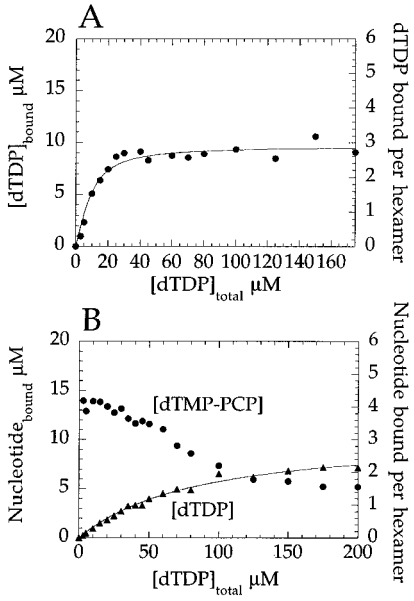


FIGURE 7: Equilibrium binding of MgdTDP to 4A′ protein in the absence and presence of dTMP-PCP. dTDP binding to 4A′ was measured at 22 °C using [α-^{32}P]dTDP and nitrocellulose membrane binding assays. Panel A shows [α-^{32}P]dTDP binding to 4A′ (20 μM) at increasing dTDP concentrations (0−200 μM). Three dTDP's bind per 4A′ hexamer, and the isotherm fits to a quadratic equation with a K_d of (4.17 ± 0.97) × 10^{-6} M. Panel B shows binding of [α-^{32}P]dTDP and [α-^{32}P]dTMP-PCP to 4A′ (20 μM) at increasing dTDP concentrations (0−200 μM). (▲) shows [α-^{32}P]-dTDP binding to 4A′ in the presence of 20 μM unlabeled dTMP-PCP, and (●) shows [α-^{32}P]dTMP-PCP (20 μM) binding to 4A′ at increasing concentrations of unlabeled dTDP. In the presence of dTMP-PCP, dTDP binds 4A′ with an apparent K_d of (76.6 ± 6.8) × 10^{-6} M.

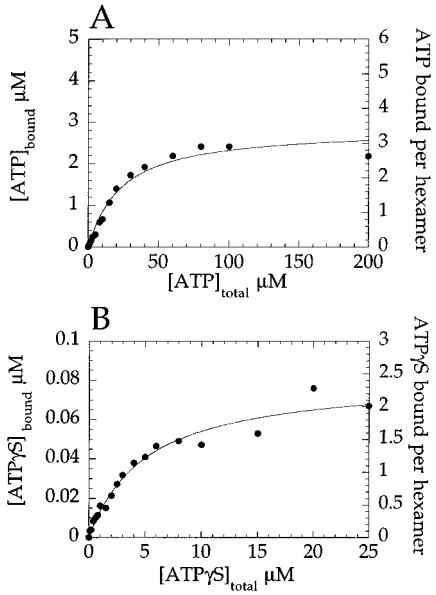


FIGURE 8: Equilibrium binding of ATP and ATPγS to 4A′ protein. Binding of ATP and ATPγS to 4A′ was measured at 4 °C using nitrocellulose membrane binding assays. Panel A shows binding of [α-^{32}P]ATP to 5 μM 4A′ protein at increasing ATP concentrations (0−200 μM). The binding isotherm fits to a quadratic equation with a K_d of 2.9 × 10^{-5} M, and binding saturates with a maximum of three ATP bound per hexamer. Panel B shows the binding of [γ-^{35}S]ATP to 4A′ (0.2 μM) at increasing ATPγS concentrations (0−25 μM). The binding isotherms fits to a quadratic equation with a K_d of 5 × 10^{-6} M and saturates at three ATPγS bound per 4A′ hexamer.

[α-^{32}P]dTMP-PCP, respectively. As shown in Figure 7B, dTDP binds to 4A′ in the presence of dTMP-PCP with an apparent K_d of 76 ± 6.8 μM, which is 18-fold weaker than the K_d in the absence of dTMP-PCP. Under the same conditions, the number of dTMP-PCP bound per hexamer decreases as the dTDP concentration is increased. At all concentrations of dTDP, no more than four nucleotides bind 4A′. Thus, dTDP appears to compete for the same tight binding sites on 4A′ hexamer as dTMP-PCP.

Interaction of 4A′ with ATP and ATPγS. Studies in the literature indicate that bacteriophage T7 gene 4 helicases preferentially use dTTP as the nucleotide cofactor during duplex DNA unwnding. Gene 4 proteins hydrolyze dTTP with the lowest K_m value (Patel *et al*., 1992; Matson & Richardson, 1983), and dTTP has been noted to promote optimal DNA binding and unwinding by the gene 4 proteins (Matson *et al*., 1983; Matson & Richardson, 1985). Most well-studied helicases such as *E. coli* helicases [DnaB (Reha-Krantz & Hurwitz, 1978b; Arai & Kornberg, 1981a−c; Lebowitz & McMacken, 1986), Rep (Yarranton & Gefter, 1979; Wong & Lohman, 1992), and transcription terminator Rho (Lowery & Richardson, 1977a,b)], use ATP hydrolysis to power their unwinding activity. Therefore, it is curious that gene 4 proteins prefer to use dTTP as their substrate. To confirm that the use of ATP for DNA unwinding is less favorable compared to dTTP, we have measured DNA unwinding more carefully with a short-forked DNA substrate in the presence of saturating concentrations of dTTP (0.25, 5 mM) and ATP (0.4, 1, 8 mM). DNA unwinding in the presence of MgATP was found to be at least 100-fold slower than in the presence of MgdTTP (unpublished data). It is curious that ATP is hydrolyzed 5-fold faster by 4A′ than dTTP, yet it does not promote optimal DNA unwinding (Patel *et al*., 1992).

We study here the interactions of ATP and ATPγS (the nonhydrolyzable analog of ATP) with 4A′ to understand why gene 4 proteins discriminate against ATP. Equilibrium binding of ATP and ATPγS to 4A′ was measured using the nitrocellulose membrane assays. As shown in Figure 8A, ATP binds to 4A′ with a K_d of 2.9 × 10^{-5} M, which is 6-fold weaker than the K_d of 4A′·MgdTTP. Figure 8B shows binding of ATPγS to 4A′ with an apparent K_d of 1.7 × 10^{-6} M, which is 2−3-fold weaker than the K_d of 4A′·MgdTMP-PCP. As observed with dTTP, binding of both ATP and ATPγS saturates with a maximum of three NTPs bound per hexamer. At high protein concentrations (20 μM 4A′), four ATPγS bind per hexamer, similar to dTMP-PCP binding to 4A′ (data not shown). It must be pointed out that 4A′ protein also contains a primase active site that catalyzes RNA primer synthesis initiating specifically with ATP. It is therefore possible that ATP and ATPγS bind to the primase sites rather than the helicase NTP binding sites. A competition binding experiment was performed with ATPγS and dTMP-PCP to investigate the above possibility. The results showed that ATPγS and dTMP-PCP both compete for the same sites on 4A′ hexamer (data not shown). Thus, ATP is unable to serve as an optimal cofactor for DNA unwinding even though it binds to the same NTP binding sites on the helicase as dTMP-PCP and dTTP.

Effect of ATP Analogs on ssDNA Binding to 4A′. DNA binding to 4A′ was studied in the presence of the nonhydrolyzable analogs of ATP to continue our investigation of the preferential use of dTTP by T7 gene 4 helicases. Interestingly, DNA binding is not detectable in the presence

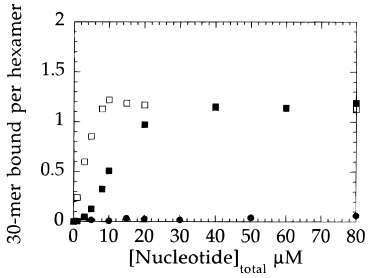


FIGURE 9: DNA binding to 4A′ in the presence of various ATP analogs. DNA binding to 4A′ was measured at 22 °C using the nitrocellulose−DEAE membrane binding assay. 4A′ (12 μM) was incubated with 5′-^{32}P-radiolabeled 30-mer (4 μM), and the mixture was titrated with ATPγS (●), AMP-PCP (■), or dAMP-PCP (□). DNA binds to completion only in the presence of AMP-PCP and dAMP-PCP, and no DNA binding is detectable in the presence of ATPγS.

of ATPγS (Figure 9), even at an ATPγS concentration as high as 1 mM (data not shown). When the same experiment was performed with the nonhydrolyzable analogs, AMP-PCP and dAMP-PCP, complete DNA binding was observed. DNA binding in the presence of AMP-PCP is sigmoidal perhaps due to the weaker affinity of AMP-PCP for binding 4A′. DNA binding in the presence of dAMP-PCP is similar to that in the presence of dTMP-PCP.

DISCUSSION

Negative Cooperativity in Nucleotide Binding. DNA helicases require binding and hydrolysis of nucleotide cofactors in order to catalyze duplex DNA unwinding. We have examined the interactions of various nucleotides with T7 4A′ and 4B hexameric DNA helicases to quantitate the equilibrium nucleotide binding parameters and to better understand the effect of nucleotide binding on protein oligomerization. Nucleotide binding was measured both by a direct assay using radiolabeled ligands and by measuring change in protein fluorescence upon ligand binding. The results indicated that both 4A′ and 4B proteins bind nucleotides with extreme negative cooperativity. Accordingly, at equilibrium, only three nucleotides bind per hexamer. Because gene 4 helicases are hexamers of identical subunits and have six potential nucleotide binding sites, half-site binding of nucleotides may mean either that the remaining three sites are unoccupied or that nucleotide binding is much weaker at those sites. Fluorescence titration experiments that measure binding at equilibrium provide the same results as the nitrocellulose binding experiments. Therefore, it is clear that if any interaction occurs with the remaining three sites, it must be at least 50-fold weaker (for dTTP) compared to binding at the high-affinity sites.

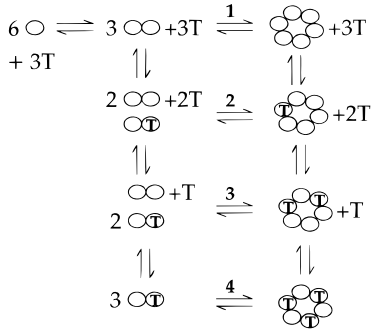
The extreme negative cooperativity in nucleotide binding was found to be a property of all the nucleotides tested, including dTTP, ATP, dTDP, and the nonhydrolyzable analogs of dTTP and ATP. We have investigated the possibility that the observed half-site binding may be simply an effect due to the presence of either the triphosphate or the diphosphate alone in our binding assays. Equilibrium binding assays in the presence of both dTMPPCP and dTDP showed that the hexamer does not bind more than four nucleotides at any given concentration of nucleotide. The three weak binding sites do not show any preference for binding dTDP, but it appears that both dTMPPCP and dTDP compete for the same tight binding sites on the hexamer. Also, ssDNA has little effect on the observed extreme negative cooperativity in nucleotide binding. It is important to note that the same half-site binding of nucleotides is observed at low protein concentrations, conditions under which protein is predominantly in the monomeric/dimeric state. These results suggest that negative cooperativity in nucleotide binding is a property of the dimer and is retained even at the hexamer level.

Negative cooperativity in nucleotide binding has been observed with other hexameric helicases as well, although it is not as striking as in the T7 helicases. The *E. coli* DnaB helicase shows negative cooperativity in ATP binding with three high-affinity and three low-affinity binding sites. It has been reported that negative cooperativity in DnaB helicase is sensitive to temperature. At low temperatures (0 °C), all six ATP bind to the DnaB hexamer with similar affinity such that negative cooperativity among binding sites is almost undetectable (Arai & Kornberg, 1981b; Bujalowski & Klonowska, 1993). This is not the case with the gene 4 proteins because binding isotherms at 4 and at 22 °C are identical and show only three dTTP molecules bound per 4A′ or 4B hexamer. ATP binding studies with *E. coli* Rho, which is a transcription terminator protein with RNA/DNA helicase activity, showed that Rho has three tight ATP binding sites (Stitt, 1988). A later report by Geiselmann and von Hippel (1992) showed that at high concentrations of Rho and ATP, additional ATPs bind with a 20−30-fold lower affinity relative to the tight binding sites.

The mechanistic significance of negative cooperativity in hexameric helicases remains unclear at the present time. It is possible that the extreme negative cooperativity of the helicase hexamer for binding three additional nucleotides is indicative of "half-site reactivity". Many enzymes that exhibit negative cooperativity also exhibit half-site reactivity (Seydoux *et al*., 1974). In such a case, while a hexamer of identical subunits is a favored structure for helicase activity, NTP binding and hydrolysis by only three subunits may be necessary for efficient translocation and DNA unwinding, while the other three subunits may be catalytically silent. Alternatively, interaction between the other three subunits and nucleotide may serve a different purpose, perhaps structural or regulatory function. An analogous situation has been found for F_1-ATPase hexamer where extensive biochemical study and high-resolution structure determination have shown that even though six ATP binding sites exist, only three are catalytically active (Abrahams *et al*., 1994). Once the molecular mechanism of the coupling between nucleotide binding/hydrolysis and DNA unwinding in helicases is better understood, the reason for the observed negative cooperativity should become clear.

Ligand-Linked Oligomerization. Gene 4 proteins have been shown previously to form stable oligomers only in the presence of MgdTMP-PCP (Patel & Hingorani, 1993). Here we show the same effect with dTTP, dTDP, and ATP, all of which promote hexamer formation. From high pressure gel filtration experiments, it is clear that 4A′ oligomerization is sensitive to the concentration of the nucleotide ligand. Reciprocally, from nucleotide binding isotherms, it is evident that nucleotide binding is sensitive to protein concentration. The three nucleotides bind both 4A′ and 4B proteins with a positive cooperativity that is most pronounced with the nonhydrolyzable analog dTMP-PCP. The positive cooper-

Scheme 1



ativity in dTMP-PCP binding increases with increasing protein concentrations. This suggests that cooperativity may result from ligand-linked oligomerization of the gene 4 proteins (shown in Scheme 1) if hexamers bind nucleotides tighter than monomers or dimers. A diagnostic test for cooperativity arising from ligand-induced oligomerization is the dependence of the magnitude of cooperativity on protein concentration. At low and high protein concentrations, where the predominant species would be either monomer/dimer or hexamer, respectively, there should be no cooperativity in ligand binding (Wong & Lohman, 1995). Our results show that at low concentrations of 4A′, where the predominant species are monomers or dimers, the binding isotherms are hyperbolic. At higher protein concentrations, the isotherms begin to show increasing cooperativity and do not fit to a hyperbola with the same K_d value. Because stable hexamer formation in the absence of nucleotides requires close to 100 μM 4A′ protein (Patel & Hingorani, 1993), we are unable to verify if the cooperativity disappears at high protein concentration. Therefore, it is possible that 4A′ hexamers bind nucleotides with an intrinsic positive cooperativity.

Scheme 1 shows qualitatively the ligand-linked oligomerization process in gene 4 proteins. At low protein concentrations, monomers and dimers are in equilibrium, and in the presence of nucleotide ligands, dimers with one nucleotide bound are formed. For simplicity, we have shown that three dimers associate to form hexamers via pathways 1−4. It is equally likely that dimers associate with monomers and form hexamers from intermediate species such as trimers, tetramers, and pentamers. To observe positive cooperativity in nucleotide binding due to ligand-linked oligomerization, hexameric species with less than three dTMPPCP molecules bound must be formed. Therefore, under conditions where hexamers are formed via pathway 1 or 4 (high and low protein concentrations, respectively), no cooperativity in nucleotide binding should be detected. At the protein concentrations employed in our experiments (20 μM), hexamers are most likely not formed via pathway 1. In this proposed oligomerization process, it is the tighter binding of dTMP-PCP to empty sites on the partially-filled hexamers that is responsible for the observed positive cooperativity in nucleotide binding. Evidence for the presence of these partially-filled hexameric species was provided by an independent experiment performed to measure DNA binding as a function of dTMPPCP concentration. It has been shown previously that DNA binds stably only to the hexameric species (Hingorani & Patel, 1993). Therefore, measurement of DNA binding at increasing dTMPPCP concentrations provided the amount of hexamers present at each dTMPPCP concentration. Comparison of dTMPCP binding and DNA binding under identical conditions confirmed the presence of partially-filled hexamers that were formed with an average of one to two nucleotides bound per hexamer.

Thus, in addition to modulating ssDNA binding to the helicase, nucleotide cofactors modulate the formation of active hexamers via ligand-linked oligomerization. NTP binding very likely causes changes in the regions of contact between subunits, leading to changes in the equilibrium between oligomeric species. Native PAGE studies show that T7 gene 4 helicases form a ladder of oligomers in the absence of ligands (unpublished data). The fact that no oligomers are missing or overly stable in the ladder indicates a propensity for "head-to-tail" interaction between the subunits. Nucleotide binding changes this oligomer equilibrium to favor stable hexamer species. A simple way for nucleotide binding to change intersubunit contacts would be to bind at the interface of the subunits as found in F_1-ATPase (Abrahams *et al*., 1994) and many other allosteric proteins that are oligomeric (Traut, 1994; Neet, 1995). The binding of nucleotides at the interface can also explain the observed negative cooperativity or half-site binding to the dimer species, and the fact that hexamer formation appears necessary for nucleotide binding to more than three sites (a fourth dTMP-PCP or ATPγS molecule binds to the hexamer only at high 4A′ concentrations).

The negative cooperativity in nucleotide binding could also be induced due to conformational changes caused by nucleotide binding itself. The effect of nucleotide binding to one unit may be communicated to the adjacent unit, resulting in its lower nucleotide binding affinity. Nucleotide-induced conformational changes and communication through subunits are the cornerstones of all proposed mechanisms of helicase activity. For example, a model for translocation and helicase activities of the *E. coli* transcription termination protein Rho proposes a conformational "switch" of the subunits linked to ATP hydrolysis (Geiselmann *et al*., 1993). This switching causes the subunits to bind RNA with alternating high and low affinity, resulting in translocation. In general, dynamic changes in nucleotide binding affinity through all subunits of the hexamer coupled with cycles of NTP binding, hydrolysis, and product dissociation would result in changing affinity of each subunit for DNA. Accordingly, the helicase can cycle through conformational states that effect translocation and processive DNA unwinding. Various forms of this alternating "DNA bind−release" mechanism have been envisioned for other helicases (Lohman, 1992, 1993). Our results from the titration of 4A′• ssDNA complexes with dTMP-PCP demonstrate that the helicase binds stably to ssDNA with only one to two NTPs bound per hexamer. In addition, previous studies using mixed hexamers of active and inactive subunits have shown that the hexamer can bind ssDNA by interaction with one to two active subunits (Patel *et al*., 1994). Therefore, if one or a few of the six subunits hydrolyze dTTP at any given time resulting in low affinity for DNA, interaction with the other dTTP-bound subunits allows helicase to remain associated with DNA, and this cycle of dTTP hydrolysis can continue in a sequential manner resulting in processive translocation.

ATP as a Cofactor for T7 Gene 4 Proteins. Most known helicases use ATP preferentially as the nucleotide cofactor to catalyze DNA unwinding. Bacteriophage T7 gene 4 proteins are unusual in their requirement for dTTP as the

nucleotide cofactor of choice. Our measurements indicate that 4A′ unwinds duplex DNA in the presence of ATP with at least a 100-fold slower rate than dTTP. In an effort to understand why ATP is unsuitable as a cofactor for the helicase activity, we investigated oligomerization of 4A′ in the presence of ATP and the binding of ATP and ATPγS to 4A′. Stable hexamer formation requires higher concentrations of ATP compared to dTTP. The interaction of ATPγS with 4A′ is comparable to that of dTMP-PCP, and ATP binds to 4A′ only about 6-fold weaker than dTTP. A study of DNA binding to 4A′ revealed that although no DNA binding was detectable in the presence of ATPγS, another ATP analog, AMP-PCP, supports stable DNA binding. Furthermore, ATP hydrolysis is stimulated by ssDNA as reported previously (Patel *et al*., 1992); therefore, we assume that 4A′ does bind DNA in the presence of ATP. The only clear difference between dTTP and ATP appears to be in their affinities for binding 4A′ and their differences in promoting oligomerization, both of which can be overcome simply by increasing the concentration of ATP (comparable to *in vivo* conditions). However, high ATP concentrations do not seem to correspondingly increase the ability of ATP to promote DNA unwinding at an optimal rate. These observations lead us to believe that there is an intrinsic difference between ATP and dTTP perhaps related to specific steps involved in the coupling of NTP hydrolysis to DNA unwinding.

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