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ROLE OF HEXAMERIC STRUCTURE AND dTTPase ACTIVITY IN
BACTERIOPHAGE T7 HELICASE-CATALYZED UNWINDING OF DOUBLE-
STRANDED DNA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the
Degree Doctor of Philosophy in the Graduate School of
The Ohio State University

By
Manju M. Hingorani, B.Pharm.

*****
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To my family
ACKNOWLEDGEMENTS

Thank you Smita Patel for your guidance and encouragement. And of course many thanks to you my colleagues and friends, Todd Washington, Kristen Moore, Yiping Jia, Peter Ahnert, Amar Kumar, and Anish Konkar, for the marvelous discussions and cat-fights; I will miss them.
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

PART A: INTRODUCTION TO DNA HELICASES

DNA helicases are enzymes that catalyze unwinding of double-stranded DNA to form single-stranded DNA products. In vivo DNA exists stably as a double helix and it has to be unwound, transiently, to provide ssDNA templates required for various processes of DNA metabolism. All DNA helicases studied to date bind and hydrolyze nucleotide triphosphates, and they catalyze duplex DNA unwinding by an NTPase-dependent mechanism (reviews: Matson & Kaiser-Rogers, 1990; Matson, 1991; Lohman, 1992; 1993). DNA helicases apparently play an important role in the functions of life, because they have been found in a variety of organisms ranging from bacteriophages and viruses (e.g., Bacteriophage T4: Nossal, 1979; Morris et al., 1979; Herpes simplex virus: Crute et al., 1989) to bacteria (e.g, E. coli: Matson & Kaiser-Rogers, 1990, review), and eukaryotes (e.g., S. cerevisiae: Li et al., 1992; mouse: Seki et al., 1986; 1988; frog: Poll & Benbow, 1988). Since they were first discovered in 1976 (Abdel-Monem et al., 1976), the number of known helicases has risen dramatically, and it has been speculated that
helicase genes comprise as much as 1% of the total number of genes in both prokaryotic and eukaryotic genomes (Gorbalenya & Koonin, 1993).

Many researchers are now studying DNA helicases to determine their function and importance in DNA metabolism, and also to solve their mechanism of action. A brief discussion of DNA helicases, classified according to their associated function, is included below.

DNA Replication.

A number of DNA helicases are known to unwind duplex DNA with high processivity and have been found essential for DNA replication by in vivo and in vitro studies. In bacteriophage T4, gene 41 protein (gp41) functions as a helicase (as part of a primosome complex) to facilitate leading strand and lagging strand DNA synthesis (reviews: Nossal, 1994; Cha & Alberts, 1988). In E. coli, DnaB helicase (an essential replication protein; LeBowitz & McMacken, 1986), and Helicase II (Taucher-Scholz, 1983) are but two of the helicases thought to be involved in DNA replication, and Rep helicase (which may also play a role in E. coli replication) is required for replication of f1 and φX174 ssDNA phages (Lane & Denhardt, 1975). An important and well studied eukaryotic helicase is the SV40 large T antigen, which is essential for replication of the SV40 chromosome as well as for regulation of viral and cellular
genes (Stahl et al., 1986; Stillman, 1989; DeCaprio et al., 1988; Levine et al., 1991). Mutation or deletion of replicative helicases results in inhibition of DNA synthesis; although, it appears that many higher organisms have more than one helicase, and these enzymes may compensate for one another.

DNA Repair.

Various processes of DNA repair need DNA unwinding activity which is provided by DNA helicases. For example, E. coli UvrA and UvrB proteins that are involved in nucleotide excision repair (for uv- and chemical-damaged DNA) have helicase activity (Oh & Grossman, 1987; 1989). Helicase II (UvrD) is required for methyl-directed mismatch repair (Lahue et al., 1989; Modrich, 1989) as well as for excision repair (Kuemmerle & Masker, 1983; Selby & Sancar, 1988). The E. coli Rep protein has also been implicated in DNA repair (Bridges & von Wright, 1981). An example of a eukaryotic DNA repair helicase is the RAD3 protein of S. cerevisiae, which is also essential for cell viability (Sung et al., 1987a, b; Higgins et al., 1983; Naumovski & Friedberg, 1983).

DNA Recombination.

The E. coli RecBCD enzyme complex has helicase activity, which is responsible for generating ssDNA strands used in RecA-mediated homologous recombination (Taylor,
1988; Smith, 1990). This complex functions in genetic recombination as well as in DNA recombination repair; therefore, mutations of RecA and the RecBCD complex can make cells sensitive to uv-induced DNA damage.

Conjugation.

*E. coli* Helicase I, the first helicase identified (Abdel-Monem et al., 1976a, b), has extremely processive unwinding activity (Lahue & Matson, 1988), as well as nicking activity (Traxler & Minkley, 1987; 1988). These activities, coupled with the fact that Helicase I is encoded by the *traI* gene on the F plasmid and is not essential for *E. coli* DNA replication, indicate that Helicase I-catalyzed DNA unwinding is involved in transfer of the F plasmid from donor to recipient cell in bacterial conjugation (Review: Willets & Shurray, 1987).

Common Features of DNA Helicases.

Polarity of DNA Unwinding.

Unwinding activity requires that helicases translocate on DNA and destabilize the base-pairing within duplex DNA regions. Most helicases preferentially unwind DNA in one direction, 5' to 3' (e.g., *E. coli* DnaB) or 3' to 5' (e.g., SV40 large T antigen). It is not known yet what precisely determines directionality in helicase-catalyzed unwinding, but it has been speculated that interaction with the duplex
region at the DNA fork may result in biased movement of the helicase along DNA (E. coli Rep: Wong & Lohman, 1992). It is also possible, however, that polarity in helicase structure coupled with the inherent polarity of ssDNA confers the bias. A number of studies have been performed to determine whether helicases translocate unidirectionally on ssDNA or whether they can translocate in both directions but unwind DNA in only one direction (Phage T4 gene 41 helicase: Young et al., 1994; Phage T4 Dda helicase: Raney & Benkovic, 1995); however, there is no conclusive evidence, so far, of unidirectional translocation of helicases on ssDNA.

DNA Binding Properties of Helicases.

Helicases may interact with ss or dsDNA, or both, at the fork-junction during DNA unwinding. For a number of DNA helicases, interaction with DNA is independent of the DNA sequence (E. coli Rep: Wong et al., 1992; T7 gene 4 helicases: Matson & Richardson, 1985); in fact, the SV40 large T antigen appears to interact with mainly the sugar phosphate backbone of ssDNA (SenGupta & Borowiec, 1992). This property may be important for processivity because it allows the helicase to maintain equivalent contacts with DNA of varying sequences during translocation and unwinding.

In order to move on DNA it appears that helicases must possess at least two DNA binding sites, so that they can bind and release DNA, simultaneously, to move forward and
stay associated with DNA during unwinding. Most helicases studied to date form oligomers (dimers or hexamers), which is a simple means to obtain multiple DNA binding sites. SV40 large T antigen (Mastrangelo et al., 1989) and E. coli DnaB (Reha-Krantz & Hurwitz, 1978a, b), among other helicases, form hexamers, and E. coli Rep (Wong & Lohman, 1992) and Helicase II (Runyon et al., 1993) form dimers. Helicases assemble into stable oligomers under conditions where they unwind duplex DNA; therefore, it is likely that these oligomers are the active forms of the helicases (Review: Lohman & Bjornson, 1996).

Nucleotide Binding and Hydrolysis.

Helicase-catalyzed DNA unwinding is an NTPase-dependent activity, and helicases utilize energy from NTP binding and hydrolysis to unwind duplex DNA. It has been proposed that helicases cycle through a series of conformational states, driven by NTP binding, hydrolysis, and product release, to translocate on DNA and catalyze DNA unwinding (Yarranton & Gefter, 1979; Hill & Tsuchiya, 1981). There is substantial preliminary evidence that favors the above model: NTP binding and hydrolysis modulate the interactions of a number of helicases with DNA (e.g., DnaB: Nakayama et al., 1984; Rep: Wong & Lohman, 1992), which may be linked to movement of the helicase on DNA. Also, there are nucleotide-dependent changes in helicase structure (e.g., DnaB: Nakayama et al.,
1984; Rep & Helicase II: Chao & Lohman, 1990) that may be the basis for the changing affinities of helicases for DNA, or may reflect different conformations of the helicase during unwinding. The coupling between NTPase and unwinding activity, however, has not been explicitly solved for any helicase, and it remains to be seen whether conformational changes caused by NTP binding and hydrolysis are sufficient for helicase activity, or whether NTPase activity serves other functions in DNA unwinding.

Sequence-Based Classification of Helicases.

A recent sequence-comparison study of helicases shows that these enzymes can be broadly classified into large families (Gorbalenya & Koonin, 1993). All helicases have the nucleotide binding motif (Walker A sequence), but there appear to be no other motifs that are absolutely conserved among all the helicases. Within each family, however, there are regions of homology in helicases that may form domains with similar functions. The authors suggest that in all helicases there are motifs, organized in a core, that are responsible for unwinding function, and there are other sequences and domains that provide accessory functions resulting in the diversity of helicases; for example, the primase-helicase proteins of bacteriophage T7 and P4 have N-terminal domains with primase activity, while their C-terminal domains are DnaB-like helicases (Gorbalenya &
Koonin, 1993). These are interesting observations, because even though there is a wide variety of helicases with multiple functions, there are several common features among helicases that appear essential for unwinding activity, as described previously. These common features may reflect sequence homology among helicases within a family, or may be due to convergent evolution among helicases from different families that do not share primary sequence homology but have similar protein structure and function.

Why Study Helicases?

DNA helicases are involved crucial processes of cellular metabolism, including DNA replication and repair. A careful study of all components of the DNA replication machinery, including DNA helicases, is in progress to understand the mechanism of replication of several organisms, from viruses to humans. The potential benefits of understanding this basic process of life are many, ranging from efficient targeting of viral diseases to more effective therapy of cancer which is a direct consequence of defective DNA replication.

Mutations are the primary cause of cancer and heritable disease; therefore, cells have complex and efficient systems that repair DNA to maintain the integrity of their genome. There are a number of diseases that are associated with defective DNA repair, including xeroderma pigmentosum (XP),
Cockayne's syndrome (CS), and trichothiodystrophy (TDD). It is now known that a deficiency in DNA helicases associated with the nucleotide excision repair system can produce symptoms of CS and TDD (Reviews: Sancar, 1994; Hanawalt, 1994; Lehmann, 1995). Clearly, DNA helicases play an important role in the processes that maintain and propagate life and are worthy targets for detailed biochemical examination.

PART B: BACTERIOPHAGE T7 GENE 4 HELICASES

The research in this dissertation was aimed at investigating the mechanism of bacteriophage T7 gene 4 helicases, which are involved in T7 DNA replication (Studier, 1969). In vitro studies have shown that efficient leading strand and lagging strand synthesis, catalyzed by T7 DNA polymerase, requires T7 gene 4 proteins (along with gene 2.5 ssDNA binding protein, gene 6 exonuclease, and DNA ligase; Lechner & Richardson, 1983; Engler & Richardson, 1983; Richardson, 1983). T7 gene 4 proteins effect a marked stimulation of T7 polymerase-catalyzed DNA synthesis when added to reactions in vitro (Richardson et al., 1979), indicating their importance in phage DNA replication.

Gene 4 specifies two proteins (made in approximately equal amounts), 4A and 4B (Studier, 1972; Dunn & Studier, 1981). 4A is the full-length protein product (63 kDa) and 4B
(56 kDa) is the smaller protein, synthesized from an internal initiation site on the same mRNA (Dunn & Studier, 1983). 4A has both DNA helicase and primase activities (Kolodner & Richardson, 1977; Scherzinger et al., 1977; Romano & Richardson, 1979, Matson et al., 1983), and 4B protein, which is missing 63 amino acids from the N-terminus and a putative "zinc finger", has only helicase activity (Bernstein & Richardson, 1988a, b). The helicase activity catalyzes unwinding of duplex DNA ahead of the T7 polymerase during replication, and the primase synthesizes small RNA primers which are elongated to form Okazaki fragments during lagging strand synthesis (Bernstein & Richardson, 1989).

T7 gene 4 proteins were first purified in 1975 (Hinkle & Richardson, 1975), however, it was not possible to separate 4A and 4B proteins by chromatography because of their similar properties. Partial separation of the two proteins was achieved, some years later, by hydroxylapatite affinity chromatography (Bernstein & Richardson, 1989). Overexpression and complete separation of 4A and 4B was reported only recently (Rosenberg et al., 1992; Patel et al., 1992); the authors cloned gene 4 minus the 4A initiation codon to obtain pure 4B, and in another clone, changed methionine-64 of 4A (the initiation codon of 4B) to leucine to obtain pure 4A. The proteins were overexpressed using a pET vector system (transcription controlled by a T7 promoter; Novagen). Both proteins were purified and
characterized, and the primase and helicase activities of the M64L mutant, 4A', were shown to be unchanged from the wild-type gene 4 proteins.

As shown for other helicases, gene 4 proteins bind to ssDNA in the presence of NTPs and processively unwind duplex DNA in the 5' to 3' direction (Matson et al., 1983). T7 helicase-catalyzed DNA unwinding is dependent on NTPase activity, and dTTP is the preferred substrate of the gene 4 proteins (Matson & Richardson, 1983; Patel et al., 1992). Since 4A protein has both primase and helicase activities, it is not known why two forms of the protein are made by the T7 bacteriophage. Richardson and his colleagues (Nakai & Richardson, 1988) suggested a model for gene 4 protein activity, in which, 4A and 4B form a heterodimer which translocates on DNA till it encounters a primase recognition site. At this point 4A dissociates from the complex to synthesize the primer, and 4B continues to processively unwind duplex DNA ahead of the polymerase. This model is a qualitative description based on minimal knowledge of the DNA unwinding and primase mechanisms in bacteriophage T7. The true oligomeric state of the T7 helicases was not determined, nor was it known whether the primase activity translocated with the helicase or whether it was a completely separate process. Furthermore, it is more important, at the initial level, to dissect the helicase and primase activities, understand the molecular mechanism of
each activity, and then develop a comprehensive mechanism for the functions of the T7 gene 4 proteins.

PART C: RATIONALE AND AIM OF Ph.D. DISSERTATION RESEARCH

Bacteriophage T7 gene 4 helicases are essential for replication of phage DNA. The biochemical and structural properties of 4A' and 4B proteins were investigated in this research project as part of a broader objective of understanding the enzymatic mechanism of DNA helicases. T7 helicases bind DNA and hydrolyze dTTP to catalyze dTTPase-dependent unwinding of duplex DNA. In order to understand the parameters that control the mechanism of T7 helicase-catalyzed DNA unwinding, in vitro experiments were specifically designed to examine:

1. The oligomeric structure of 4A' and 4B proteins, to determine the active form of the helicases.

2. The preferred DNA substrates of 4A' and 4B and their equilibrium binding constants, to define the interactions of the helicases at the DNA fork-junction during the unwinding process.

3. The equilibrium nucleotide binding parameters, to determine the effect of nucleotide binding on interactions between helicase and DNA, as well as its effect on helicase oligomer formation.
4. The role of the Walker motif A, the conserved nucleotide binding sequence, to investigate its importance in nucleotide binding and hydrolysis during 4A' and 4B-catalyzed DNA unwinding.

5. The steady-state and pre-steady-state kinetic parameters of dTTP binding and hydrolysis, in the absence of DNA, as a first step to understanding how dTTPase activity is coupled to helicase-catalyzed DNA unwinding.
OLIGOMERIC STRUCTURE OF BACTERIOPHAGE T7 HELICASES

ABSTRACT

The oligomeric structure of bacteriophage T7 gene 4 proteins was studied in vitro using non-denaturing gel electrophoresis, high pressure gel-filtration chromatography and electron microscopy. On a non-denaturing gel, 4A′ protein formed a ladder of oligomeric species, up to hexamer and higher, in the absence of ligands. HPLC gel-filtration studies showed that in the absence of ligands the oligomeric equilibrium of 4A′ shifted from dimers to higher oligomers with increasing protein concentrations, and hexamers were the predominant species at about 100 μM protein. In the presence of nucleotide ligands (MgdTMP-PCP, MgdTTP, MgdTDP, and MgATP), much lower concentrations of protein were required to form stable hexamers. For example, in the presence of MgdTMP-PCP, 4A′ (and 4B) protein assembled into hexamers at concentrations of about 4 to 6 μM. Hexamer formation was dependent also on nucleotide concentration. 4A′ formed mainly dimers at low dTTP concentrations (10 μM),
and only hexamers were detectable at a 10-fold higher dTTP concentration. Addition of ssDNA further stabilized the hexamer; 4A' and 4B were hexameric at concentrations as low as 0.2 μM in the presence of ssDNA. Electron microscopy of negatively-stained 4A and 4B proteins was used to investigate structural features of the hexamer. Both 4A' and 4B form ring-shaped hexamers (130 Å diameter) with a hole in the center that is 25-30 Å in diameter. The stability and organized structure of helicase hexamers strongly suggests an important role for hexamerization in helicase-catalyzed duplex DNA unwinding.

INTRODUCTION

In vivo, the normally double-stranded DNA is transiently unwound to form single-stranded intermediates that are essential for various processes of DNA metabolism. The unwinding reaction is catalyzed by DNA helicases using energy from NTP binding and hydrolysis. A large number of helicases have been identified to date, and interestingly, many of these helicases form oligomeric structures. Helicases such as E. coli DnaB (Reha-Krantz & Hurwitz, 1978a, b; Arai et al., 1981; Bujalowski et al., 1994), Phage T4 gene 41 helicase (Dong et al., 1995), SV40 T antigen (Mastrangelo et al., 1989), E. coli Ruv B (Stasiak et al., 1994), and E.coli Rho transcription terminator, which has
RNA/DNA helicase activity (Finger & Richardson, 1982; Gogol et al., 1991), form hexamers. Other helicases including *E. coli* Rep (Wong & Lohman, 1992) and *E. coli* Helicase II (Runyon et al., 1993) form stable dimers. Oligomerization appears to be an important property shared among helicases. Current theories to explain the importance of oligomerization are based on the realization that in order to effect translocation and unwinding, a helicase must cycle through conformational changes powered by NTP binding and hydrolysis, which modulate its interaction with DNA (Hill & Tsuchiya, 1981; Lohman, 1992; 1993; Lohman and Bjornson, 1996). Thus, to move on DNA and maintain processivity, it is necessary for the helicase to bind DNA via at least two sites during translocation. Oligomerization is a simple means of providing helicases with multiple sites for DNA binding.

This study investigates the oligomeric structure of the T7 bacteriophage gene 4 helicases, which are essential for phage DNA replication. Earlier studies have suggested that gene 4 proteins form oligomers (Nakai & Richardson, 1988; Bernstein & Richardson, 1988); however, it has only recently been demonstrated that 4A' and 4B form dimers and higher order oligomers (Patel et al., 1992). The current study aims to establish the native/active structure of gene 4 proteins, and investigate any connection between the structure of the
helicase and its interactions with nucleotides and DNA that may be important for unwinding activity.

Experiments were performed in vitro with pure 4A' and 4B proteins. Oligomerization was studied as a function of protein concentration, both in the presence and in the absence of nucleotide ligands and ssDNA, and also as a function of nucleotide concentration. Electron microscopy was used to examine the structure of the helicase. The results of this study show that 4A' and 4B form ring-shaped hexamers in the presence of nucleotide ligands and ssDNA.

MATERIALS AND METHODS

Nucleotides and Other Reagents.

dTTP, dTDP, and ATP were purchased from Sigma Chemicals Co. and dTMP-PCP was purchased from Amersham Life Science Inc. 60-mer ssDNA was synthesized at the Biochemical Instrument Center at The Ohio State University (Appendix C). The 60-mer was purified on a 12% polyacrylamide /7 M urea gel prepared in TBE buffer (45 mM Tris-borate, pH 8.0, and 1 mM EDTA). DNA was recovered from the gel by electroelution (Elutrap, Schleicher & Schuell), ethanol precipitated and redissolved in water. DNA concentration was determined by measuring absorbance at 260 nm in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA)/7 M urea using the calculated extinction coefficient, 616,890 M⁻¹cm⁻¹. Uranyl acetate and carbon-coated
grids were obtained from Electron Microscopy Sciences. IPTG was purchased from Biosynth AG (Switzerland).

Enzymes.

Bacteriophage T7 gene 4 proteins 4A' and 4B were overexpressed in *E. coli* and purified to homogeneity as described below. BL21(DE3) cells were transformed with the clones pAR5018 (4A') or pAR5019 (4B), grown at 37 °C, induced with IPTG, harvested and lysed by freeze-thaw cycles as described (Patel et al., 1992). The earlier purification procedure was modified so as to eliminate poly(ethylenimine) and ammonium sulfate precipitation steps following cell lysis. Instead, the lysed cells were centrifuged (4 °C) at a relative centrifugal field of 257,000 (x g) for an hour, to pellet the cell debris. The supernatant was diluted with buffer B (20 mM potassium phosphate, pH 7.2, 1 mM DTT, 0.1 mM EDTA, and 10 % glycerol) to bring the conductance of the crude extract equal that of buffer B + 50 mM NaCl. The extract was loaded onto a phosphocellulose column, which was equilibrated with buffer B + 50 mM NaCl. The remaining purification procedure, including phosphocellulose and DEAE anion exchange chromatography followed by concentration of pure protein, was performed as described earlier (Patel et al., 1992). SDS-PAGE analysis (Laemmli, 1970) was used to check the yield and purity of the protein at each step of the process. Protein concentrations were determined using
the Bradford assay (Bradford, 1976), and from absorbance measurements at 280 nm in 100 mM Tris-Cl (pH 7.5)/8 M urea buffer, using the extinction coefficient 76,100 M\(^{-1}\)cm\(^{-1}\) for 4A' and 67,850 M\(^{-1}\)cm\(^{-1}\) for 4B protein.

Nondenaturing Polyacrylamide Gel Electrophoresis.

Native polyacrylamide gel electrophoresis (PAGE) was performed to examine the oligomeric properties of 4A' protein. A 6 % polyacrylamide gel was prepared in buffer containing 375 mM Tris-HCl, pH 8.8, and a 3 % stacking gel was prepared in buffer containing 125 mM Tris-HCl, pH 6.8. Samples (10 μL), containing 5 and 10 μM 4A' protein in binding buffer A (50 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, 5 mM sodium acetate, and 1 mM DTT) were mixed with native gel loading dye (0.25 % bromophenol blue/15 % Ficoll) and electrophoresed in Tris-glycine buffer (25 mM Tris and 200 mM glycine; pH 8.3) for 3 h, at a constant current of 18 mA. A similar experiment was carried out with 0.1, 0.2, and 0.4 μM 4A', except 100 μM dTMP-PCP and 10 mM magnesium acetate were added to the samples as well as the gel. 4A', at low concentration, was visualized by silver staining (Merril, 1990) and 4A', at 5 and 10 μM concentration, was detected by Coomassie Blue staining.
High Pressure Gel-Filtration Chromatography.

Oligomerization of 4A' was examined, in the absence of ligands, in the presence of nucleotides, and in the presence of nucleotide plus ssDNA, by small-zone gel-filtration chromatography. In the absence of ligands, increasing concentrations of 4A' (3.6 - 216 µM) were equilibrated for 2 min in standard elution buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, and 0.1 mM EDTA) prior to injection. Similar experiments were performed in the presence of nucleotide ligands as follows: with MgdTTP, 4A' (1 and 20 µM) was preincubated with elution buffer containing 10 mM MgCl₂ and dTTP (0.01, 0.03 and 0.1 mM); with MgdtMP-PCP, 4A' (0.2 - 50 µM) and 4B (0.5, 5 and 50 µM) were preincubated with elution buffer containing 10 mM MgCl₂ and 100 µM dTMP-PCP; with MgdTDP, 4A' (1 and 20 µM) was preincubated with elution buffer containing 10 mM MgCl₂ and dTDP (0.03 and 0.2 mM); with ATP, 4A' (5 µM) was preincubated with elution buffer containing ATP (0.2, 0.5 and 1 mM) prior to injection into the column. To determine the effect of ssDNA on oligomerization, 4A' (0.2 - 50 µM) and 4B (0.5, 5 and 50 µM) were preincubated in the standard elution buffer with 40 µM 60-mer in the presence of 10 mM MgCl₂, and 100 µM MgdtMP-PCP, and gel-filtration was carried out using elution buffer containing 10 mM MgCl₂, and 100 µM dTMP-PCP. Chromatography
was performed at 22 °C using a 30 cm HPLC gel-filtration column (Bio-Sil SEC 400, Bio-Rad) that was equilibrated in the appropriate elution buffer. Flow rates of 0.5 ml/min or 1 ml/min were used (indicated on the elution profiles) and 20 to 50 μl sample volumes were injected into the column from a 50-μl injection loop. A Waters model 625 LC system was used for chromatography with a Waters 470 scanning fluorescence detector (Millipore), and proteins were detected by monitoring fluorescence emission of tryptophan and tyrosine residues (340 nm) following excitation at 280 nm.

The following proteins were used as molecular mass standards (Sigma Chemicals Co.): α-crystallin (810 kDa), thyroglobulin dimer (670 kDa), β-galactosidase (520 kDa), urease (480 kDa), thyroglobulin monomer (335 kDa), β-amylase (201 kDa), alcohol dehydrogenase (150 kDa) and ovalbumin (43 kDa). A standard curve was prepared by plotting elution times versus log of molecular masses of the size standards. The apparent molecular masses of 4A' and 4B oligomers were determined from the linear range of the standard plot. Elution times of selected markers are shown on the profiles.

Negative Staining Electron Microscopy.

Oligomeric structures of 4A' and 4B were examined by negative staining electron microscopy using standard
procedures (Haschemeyer & Myers, 1972; Horne, 1965). 4A' or 4B protein (0.05 - 0.1 mg/ml) were each incubated for 10 min in binding buffer A containing 500 μM MgDTMP-PCP. A drop of the sample was placed onto a carbon-coated grid, rinsed with water after 3-5 min, and stained with a drop of 1 % uranyl acetate (pH 7.5). The grids were blotted dry and examined in a Zeiss high resolution electron microscope 10C/CR, at 60 kV. The images were photographed at 60,000 - 80,000 x magnification.

RESULTS

Purification of T7 gene 4 Proteins.

Pure 4A protein was prepared from a clone in which gene 4 was altered by replacing the 4B initiation codon with the codon for leucine (Rosenberg et al., 1992), a mutation that abolished synthesis of 4B protein. The mutant protein, 4A', was purified and tested for nucleotidase, helicase, and primase activities, and found to be comparable to the wild-type proteins (Patel et al., 1992). The smaller 4B protein was prepared from a clone that was missing the initiation codon for 4A protein. The modified procedure for purifying 4A' and 4B, described here, reduces the time required for purification by several hours. Protein yield is about 2-3 mg per liter of E.coli cell cultures and protein purity remains greater than 95 % as determined by SDS-PAGE. The Coomassie-
stained gel (Figure 1) shows 4A' protein at various steps of the purification process. Lane 2 shows 4A' prepared by the previous procedure, and lane 5, 4A' purified by the modified procedure. Preparation of 4B by the modified procedure also yields milligram quantities of pure protein.

Nondenaturing PAGE

Previous reports had indicated that gene 4 proteins formed dimers and possibly higher oligomers; however, oligomerization was not examined in detail (Patel et al., 1992). In the current study, oligomeric structure of 4A' has been tested with nondenaturing PAGE using a low percent gel to resolve large oligomeric species. Figure 2A shows a 6% Coomassie-stained gel with 5 μM (lane 1) and 10 μM (lane 2) 4A' protein. In the absence of ligands, 4A' forms ladder of oligomers in which species up to 7-mer and higher can be detected. Interestingly, the ladder appears to have no missing or highly concentrated species, indicating that all oligomers have similar stability. A second experiment, performed with 4A', in the presence of the nucleotide MgdTMP-PCP, shows that large oligomers are stable even at 0.1 μM or 0.2 μM protein concentration (Figure 2B; lanes 1 and 2). In the presence of MgdTMP-PCP, one oligomer (fifth band from the bottom) in the ladder appears more prominent than the remaining oligomers (compare with ladder in Figure 2A), as if stabilized by MgdTMP-PCP. The effect of MgdTMP-
Figure 1. Purity of 4A' protein analyzed on SDS-PAGE. 4A' protein was analyzed on a 10% SDS-polyacrylamide gel at various stages of the modified purification procedure described in Materials and Methods. Lane 1 shows the molecular size markers, lane 2, 4A' purified by the earlier procedure, lane 3, crude extract containing 4A' protein, lane 4, 4A' peak from the phosphocellulose column, lane 5, 4A' from the final purification step of DEAE column chromatography.
Figure 2. Nondenaturing polyacrylamide gel electrophoresis of 4A'. Panel A shows a Coomassie-stained native polyacrylamide gel with 5 μM and 10 μM 4A' in lanes 1 and 2, respectively. The 6% native gel was prepared without SDS as described in Materials and Methods. Both lanes show a large number of 4A' oligomeric species extending to hexamers and beyond. Panel B, silver-stained native gel with 0.1, 0.2 and 0.4 μM 4A' in lanes 1, 2, and 3, respectively. The 6% native gel was prepared in buffer containing 10 mM magnesium acetate and 100 μM dTMP-PCP. 4A' forms a mixture of oligomers and the fifth band from the bottom of the gel appears slightly predominant compared to the other species.
PCP and other nucleotide ligands on gene 4 protein oligomerization has been studied in greater detail by HPLC gel-filtration.

High Pressure Gel-Filtration of Gene 4 Proteins.

Following detection of large 4A' oligomers by native PAGE, the oligomeric structure of both 4A' and 4B proteins was investigated in greater detail by high pressure small-zone gel-filtration chromatography. Experiments were carried out at varying concentrations of protein, both in the absence and in the presence of nucleotide ligands and DNA. Initially, a standard curve of size markers was determined by plotting the elution times of various protein markers versus log of their molecular masses (Figure 3). The linear range of the standard curve was used to determine the masses of gene 4 protein oligomers, with the caveat that 4A' and 4B proteins are similar in shape to the size markers.

A. 4A' Oligomerization in the Absence of Ligands.

Oligomerization of increasing concentrations of 4A' in the absence of nucleotides was examined by gel-filtration chromatography (Figure 4). Two peaks were detected at the lowest 4A' concentration tested (3.6 μM); the mass of the fast moving peak (elution time = 15.5-16 min) was 480 ± 8 kDa, which corresponds to a 4A' heptamer or octamer (63 kDa monomer), and the mass of the larger, slow moving peak
Figure 3. Standard plot of molecular size markers for HPLC gel-filtration. Globular proteins of various molecular masses were analyzed on a gel-filtration column in standard elution buffer as described in Materials and Methods. Elution times of the proteins were measured and plotted versus log of their molecular masses to prepare a standard curve. Sizes of 4A' oligomers were determined from interpolation of the linear range of this curve.
Figure 4. Gel-filtration chromatography of 4A’ in the absence of nucleotide ligands. High pressure gel-filtration chromatography was performed using the standard elution buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA). 4A’ (3.6 to 216 μM) was incubated in the elution buffer and 15 μl volumes were injected into the column. Protein species were detected by monitoring intrinsic fluorescence of tryptophan residues (flow rate = 0.5 ml/min). Elution profiles of 4A’ protein show two major peaks: a slow-moving peak of mass 98 kDa and a fast-moving peak of mass 480 kDa.
Figure 4
(elution time = 18.5 min) was 98 ± 16 kDa, which corresponds to a 4A' monomer or dimer. The 98 kDa peak broadens and migrates toward the higher oligomer peak with increasing 4A' concentration. At 100 μM 4A' the two merge to form a broad peak centered around the heptamer/octamer position.

B. Oligomerization of Increasing Concentrations of 4A' and 4B in the Presence of Nucleotide Ligands.

Gel-filtration experiments with gene 4 proteins were performed also in the presence of nucleotide ligands. Previous reports had suggested that dTTP is the preferred nucleotide ligand for T7 gene 4 helicases (Matson et al., 1983; Matson & Richardson, 1985; Patel et al., 1992). Chromatography was initially performed with dTMP-PCP, the nonhydrolyzable analog of dTTP, to avoid possible artifacts due to helicase-catalyzed dTTP hydrolysis. Magnesium chloride was included in the elution buffer to enable nucleotide binding to the protein. Elution profiles of 4A' at varying concentrations (0.2-50 μM) are shown in Figure 5A. At the lowest concentration, 0.2 μM, 4A' elutes in two peaks of masses 395 ± 17 kDa and 93 ± 6 kDa, which correspond to 6.2 monomers (hexamer) and 1.5 monomers (monomer or dimer), respectively. At 6-8 μM 4A', the monomer/dimer species is completely converted to hexamers. A small amount of dodecamer is also visible at 1 μM and higher
Figure 5. High pressure gel-filtration chromatography of 4A' and 4B proteins in the presence of nucleotide ligands. The effects of various nucleotides on the oligomeric state of 4A' and 4B were examined by gel-filtration chromatography. The proteins were incubated with each nucleotide and chromatography was performed with elution buffer containing the nucleotide and 10 mM MgCl₂. A, shows elution profiles of 4A' at concentrations ranging from 0.2 to 50 μM and B, shows similar elution profiles of 4B protein at concentrations of 0.5, 5, and 50 μM (flow rate = 0.5 ml/min). In both cases, the protein elutes predominantly as a hexamer-sized peak (± monomer) of mass 395 kDa for 4A' and 293 kDa for 4B protein. C, shows the elution profiles of 4A' (1 and 20 μM) in the presence of dTTP (10, 30, and 100 μM); D, shows elution profiles of 4A' (1 and 20 μM) in the presence of dTDP (30 and 200 μM); E, shows 4A' (5 μM) in the presence of ATP (0.2, 0.5, and 1 mM), with chromatography performed at a rate of 1 ml/min in all three cases. With all three nucleotides, 4A' elutes as a mixture of oligomers, and as protein or nucleotide concentration is increased, the hexamer species is stabilized. The arrows on the profiles indicate elution times of selected gel-filtration markers: 1, thyroglobulin dimer (670,000 Da); 2, thyroglobulin monomer (335,000 Da); 3, alcohol dehydrogenase (150,000 Da); 4, ovalbumin (43,000 Da).
protein concentrations (752 ± 14 kDa); however, the hexamer peak is the predominant form at all high protein concentrations tested. Similar results were obtained with the 4B protein, as shown in Figure 5B. At 5 μM and higher concentrations, 4B elutes as a species of mass 293 ± 4 kDa, equivalent to a pentamer/hexamer of 4B (56 kDa monomer).

The experiment was carried out next in the presence of dTTP. Figure 5C shows the elution profiles of 4A', at 1 and 20 μM concentrations, in the presence of MgdtTP (10, 30 and 100 μM). In the presence of 100 μM MgdtTP, 4A' elutes as a sharp peak of 435 ± 5 kDa (hexamer or heptamer) at both protein concentrations tested. Both MgdtMP-PCP and MgdtTP induce stable hexamer formation, indicating that dTTP and its nonhydrolyzable analog have similar effects on the oligomeric equilibrium of gene 4 proteins. 4A' oligomerization was tested also in the presence of dTDP, the product of helicase-catalyzed dTTP hydrolysis. Figure 5D shows that at both 1 and 20 μM 4A', stable hexamers are formed in the presence of 200 μM MgdtDP. Finally, Figure 5E shows oligomerization of 4A' in the presence of MgATP, the ligand used most commonly as an energy source by helicases and other enzymes. 4A' (5 μM) elutes as a sharp hexamer peak in the presence of 500 μM ATP.
C. 4A' Oligomerization in the Presence of Increasing Concentrations of Nucleotides.

The effect of nucleotide concentration on the oligomer equilibrium was tested by observing helicase hexamer formation in the presence of increasing amounts of nucleotide ligands. At low Mg\textsubscript{d}dTTP concentrations (10 μM dTTP; Figure 5C), 4A' (1 and 20 μM) elutes as a broad peak of monomers or dimers. At the same protein concentration, when Mg\textsubscript{d}dTTP concentration is increased to 30 μM, the protein peak broadens and also elutes faster, indicating a mixture of higher oligomers. Finally, at 100 μM dTTP, the protein forms predominantly hexamers as mentioned above.

A similar effect is observed in the presence of Mg\textsubscript{d}TDP. At low dTDP concentration (30 μM), 4A' (1 and 20 μM) elutes as a mixture of dimers and hexamers, but at 200 μM dTDP, 4A' forms stable hexamers (Figure 5D). At low Mg\textsubscript{d}dTTP concentrations, 4A' oligomers (dimers to hexamers) appear to be in rapid equilibrium (eluting in broad peaks); whereas at low concentrations of Mg\textsubscript{d}TDP, 4A' migrates as clearly separated peaks of monomer/dimers or hexamers. Thus, there are some differences between the effects of Mg\textsubscript{d}TDP and Mg\textsubscript{d}dTTP on the oligomer equilibrium. The effect of nucleotide concentration on 4A' was also studied with ATP. Again, at low MgATP concentration (200 μM) 4A' (5 μM) elutes as a mixture of dimers and hexamers, while at higher MgATP
concentrations (500 μM and 1 mM) only hexamers are detectable (Figure 5E). Each nucleotide exhibits a different range of concentrations for optimal stabilization of hexamers, which may be due to differing affinities of the nucleotides for 4A' protein.

D. 4A' (and 4B) Oligomerization in the Presence of Mg’d TMP-PCP and ssDNA.

In order to study the effect of ssDNA on 4A' and 4B oligomerization, gel-filtration experiments were performed by preincubating the proteins with Mg’d TMP-PCP and 60-mer ssDNA, and eluting the protein with buffer containing MgCl₂ and dTMP-PCP. Figure 6A shows the gel-filtration profiles of increasing amounts of 4A' protein in the presence of Mg’d TMP-PCP and the 60-mer. Under these conditions, the lowest concentration of 4A' tested (0.2 μM) exists as a stable hexamer species. This is very different from the result with Mg’d TMP-PCP alone, where at 0.2 μM 4A' both dimer and hexamer species are detectable in approximately equal amounts. In the presence of ssDNA, hexamers are the predominant species at all protein concentrations; although, small amounts of dodecamers form at all 4A' concentrations higher than 1 μM. Also, the protein peaks appear sharp and symmetrical, in contrast to the broad peaks seen in the absence of ligands. This feature indicates that the protein peak is composed of
Figure 6. High pressure gel-filtration chromatography of 4A' and 4B in the presence of MgdTMP-PCP and single-stranded DNA. The effect of ssDNA on oligomerization of 4A' and 4B was studied by mixing the proteins with dTMP-PCP (100 μM) and 60-mer ssDNA (40 μM) and performing gel-filtration chromatography in elution buffer containing 10 mM MgCl₂ and 100 μM dTMP-PCP (flow rate = 0.5 ml/min). A, shows elution profiles of 4A' at 0.2 to 50 μM concentration; B, shows elution profiles of 4B at 0.5, 5, and 50 μM concentration. The fast moving peaks of 4A' and 4B have molecular masses of 395 kDa and 293 kDa, respectively, and the slow moving peak (marked with an asterisk) is free 60-mer DNA.
homogeneous hexamers in the presence of DNA, while in the absence of any ligands, the hexamer peak has a high proportion of other oligomers.

4B protein behaves similarly, when examined in the presence of ssDNA and MgdTMP-PCP (Figure 6B), forming stable hexamers at the lowest concentration tested (0.5 μM). Clearly, the equilibrium strongly favors hexamerization of 4A' and 4B under conditions that allow ssDNA binding (DNA binding properties of both 4A' and 4B will be discussed in the next chapter).


Following the discovery that 4A' and 4B form stable hexamers with nucleotides and DNA, it was possible to examine the helicase structure by electron microscopy because of the large size of the hexamer (378 kDa). Negative staining electron microscopy was performed with the gene 4 proteins under various conditions. Figure 7A shows an electron micrograph of 4A' protein in the presence of MgdTMP-PCP. A large number of ring-shaped particles can be seen, scattered evenly over the field. Due to relatively low resolution and high background noise, it is difficult to determine if the ring-shaped particles have six subunits. To determine the approximate mass of the rings, the diameter of about 20 particles was measured and averaged at about 120-130 Å. This measurement was used to calculate the
circumference of the ring (370-408 Å). According to this value, if the ring is made up of six spherical subunits, the diameter of each subunit can be approximated as 62-68 Å.

A theoretical estimate of the subunit diameter can be calculated from the molecular mass of the 4A' monomer (63 kDa). The hydrated volume of each monomer can be calculated as follows,

\[ V_h = \frac{M (V_i + 8 V)}{N_o} \]  

where \( V_h \) is the hydrated volume, \( M \) is the molecular mass of 4A' monomer (Da), \( N_o \) is Avogadro's number \((6.023 \times 10^{23})\) molecules/mole), \( V_i \) is the specific volume of a molecule in solution (0.73 cc/gm for proteins), 8 is hydration of the molecule (0.4 gm/gm for proteins), and \( V \) is the specific volume of water (1 cc/gm). Using the above equation, the volume of 4A' monomer is found to be \(1.18 \times 10^5\) Å³. If the monomer is assumed spherical, the diameter of the monomer is calculated as 61 Å. This value agrees well with the diameter of one subunit of a hexameric ring, estimated from the electron micrograph (62-68 Å; see above). Therefore, it appears that the rings in the electron micrograph are top-views of 4A' hexameric rings.

In the absence of MgTMP-PCP, 4A' appears to form aggregates rather than well defined rings (Figure 7B). The
Figure 7. Negative-staining electron microscopy of T7 gene 4 proteins. Oligomeric structures of 4A' protein were examined by electron microscopy as described in Materials and Methods. A, an electron micrograph of 4A' assembled in the presence of MgdTMP-PCP. Half-rings showing three lobes are marked by arrows (bar = 20 nm). B, 4A' protein in the absence of any ligands. A few particles are visible; they do not have any defined structure, in contrast to the rings seen in panel A, and cannot be identified at this resolution. C, ring-shaped oligomers of 4B in the presence of MgdTMP-PCP. D, an electron micrograph of 4A' prepared in the presence of MgdTMP-PCP and 60-mer ssDNA.
Figure 7 (continued)

B 20 nm
Figure 7 (continued)

C 20 nm
Figure 7 (continued)

D 20 nm
image has a noisy background, which is very different from the image in Figure 7A. Rather than an even distribution of ring-shaped hexamers, there are a few particles of various sizes that have an unstructured appearance. If there are any rings present, they are difficult to identify in the background. A search of several fields on the grid yielded the same results; therefore, the unstructured appearance of 4A' is not a localized phenomena or an artifact. The electron microscopy results are consistent with the nondenaturing PAGE and gel-filtration results showing that 4A', in the absence of ligands, exists as a mixture of oligomers rather than stable hexamers. 4B protein was also examined by negative staining electron microscopy. In the presence of MgTMP-PCP, 4B forms ring-shaped hexamers that appear identical to those formed by 4A' (Figure 7C). At this level of resolution, it is not possible to detect any difference between 4A' and 4B protein rings. The structure of 4A' was also examined following assembly in the presence of MgTMP-PCP and 60-mer ssDNA. As shown in Figure 7D, 4A' forms ring-shaped hexamers that appear identical to those seen in Figure 7A.

Since the electron micrographs suffer from a low signal to noise ratio, detailed structural information can be obtained only by image averaging. These studies were carried out by Dr. Edward H. Egelman and Dr. Xiong Yu at University of Minnesota. 4A' and 4B hexamers were examined by negative
Figure 8. Image analysis of 4A' and 4B protein rings. Following negative-staining electron microscopy, images of individual protein rings were aligned and averaged using a reference-free algorithm. A, shows an average of 1000 4A' rings, and B, shows an average of 800 4B rings (bar in B = 50 Å). No symmetry has been imposed by the averaging process, but the image of 4A' appears to have a six-fold rotational power. C, and D, show averages of 4A' and 4B, respectively, with an exact six-fold symmetry imposed. The hexamer ring is 130 Å in diameter with a hole in the center that is about 25-30 Å in diameter.
staining and image analysis techniques to obtain reliable views with greater structural details. The methods used for image averaging and analysis have been described (Egelman et al., 1995). Figures 8A and 8B show averages of 1000 images of 4A' rings and 800 images of 4B rings, respectively. Both proteins have the same structure, with six subunits arranged in a ring with a prominent hole in the center. The diameter of the ring is ~ 130 Å, in close agreement with the earlier measurement, and the diameter of the hole is 25-30 Å. Figures 8C and 8D show the averaged images of 4A' and 4B, respectively, with a six-fold symmetry imposed on them. The image of 4A' shows a slight "handedness", indicating that the ring may have C₆ symmetry. This C₆ symmetry in the hexamer is consistent with the ladder of oligomers seen on the native gel, and it suggests that 4A' monomers most likely interact in a "head-to-tail" fashion.

DISCUSSION

Oligomeric Structure of 4A' and 4B Proteins.

The results of our study clearly establish that both 4A' and 4B helicases form stable ring-shaped hexamers in the presence of nucleotide ligands and ssDNA. Nondenaturing PAGE showed that 4A' forms a ladder of oligomers in the absence of nucleotide ligands. The ladder may form as a result of dissociation of a large oligomer during electrophoresis, or
it may indicate that 4A' is a mixture of oligomers in solution. There are no exceptionally prominent or missing oligomers in the ladder. This pattern suggests that the subunits assemble via "head-to-tail" interactions to form an oligomer chain. In the presence of MgdtMP-PCP, 4A' has a high propensity for oligomer formation. Interestingly, one species in the ladder appears stabilized to some extent by the nucleotide. In the presence of nucleotide ligands, 4A' may undergo conformational changes to stabilize a particular oligomer that is important for helicase activity.

A detailed study of protein assembly using HPLC gel-filtration also showed that gene 4 proteins exist as a mixture of oligomers in the absence of ligands. Larger oligomers of 4A' are formed as protein concentration is increased. The protein peaks appear broad over the entire range of protein concentrations tested, indicating that 4A' is a mixture of oligomers even at concentrations greater than 100 μM, where a hexamer/octamer species is predominant. Thus, without nucleotide ligands, gene 4 proteins form large but unstable oligomers that are in rapid equilibrium during gel-filtration.

**Hexamer Formation in the Presence of Nucleotide Ligands.**

4A' and 4B form hexamers at low protein concentrations (4-6 μM) in the presence of MgdtMP-PCP. The protein peaks eluting from the column are narrow and symmetrical,
indicating a composition of mainly hexamers. MgΔTMP-PCP apparently induces or stabilizes hexamer formation in 4A' and 4B proteins. A similar experiment performed with MgΔTTP also showed that 4A' forms stable hexamers at low protein concentrations. Both dTTP and its nonhydrolyzable analog have similar effects on 4A' protein; therefore, while NTP binding is important, NTP hydrolysis appears unnecessary for hexamer formation.

Hexamerization of T7 helicases is linked to the presence of nucleotide ligands. Binding of nucleotide triphosphate may induce conformational changes in the protein that promote stabilization of the hexamer species. In support of this theory, oligomerization appears to be sensitive to nucleotide concentration, and more hexamers are formed as NTP concentration is increased. Other helicases oligomerize in the presence of nucleotide ligands; Polyoma large T antigen (Wang & Prives, 1991), bacteriophage T4 gene 41 helicase (Dong et al., 1995), SV40 T antigen (Mastrangelo et al., 1989), E. coli RuvB protein (Stasiak et al., 1994), and E. coli rho protein (Finger & Richardson, 1982), form stable hexamers in the presence of MgATP. Recent studies of the E. coli DnaB helicase indicate that DnaB requires magnesium ions for hexamerization, and interestingly, the helicase exists as a stable hexamer over a wide range of concentrations (0.1 to 10 μM) in the presence of magnesium ions alone (Bujalowski et al., 1994). This is not the case
with T7 gene 4 helicases, because gel-filtration chromatography shows that 4A' at 5 μM concentration forms only monomer/dimer species (100 kDa), in the presence of magnesium alone. Moreover, oligomerization of T7 helicases is sensitive to nucleotide concentration indicating a tight dependence of hexamer formation on MgNTP binding.

Nucleotide triphosphate binding has been shown to induce conformational changes in helicases like *E. coli* Rep (Chao & Lohman, 1990), and *E. coli* DnaB (Nakayama et al., 1984). T7 gene 4 proteins also undergo significant conformational changes in the presence of magnesium ions, MgdTMP-PCP, and MgdTMP-PCP plus DNA, as determined by changes in their proteolysis pattern (experiments performed by Peter Ahnert in the laboratory). These conformational changes could be related to the stabilization of hexamers that occurs on nucleotide binding to the proteins.

Hexamer Formation in the Presence of Nucleotides and ssDNA.

Gel-filtration studies show that MgdTMP-PCP plus ssDNA induce 4A' hexamer formation at even lower protein concentrations than with MgdTMP-PCP alone (0.2 μM). These results indicate that ssDNA further affects the stability of helicase hexamers. Similar effects of DNA have been observed on hexamerization of SV40 large T antigen (Mastrangelo et al., 1989; 1994), and DNA binding increases the dimerization constant of *E. coli* Rep helicase by at least a factor of 10^5.
(Chao & Lohman, 1991; Wong et al., 1992). Nucleotides and DNA, which are substrates for helicase-catalyzed unwinding, have a striking influence on hexamer formation. This strongly suggests that the hexamer species is the active form of the T7 helicases.


Bacteriophage T7 helicase hexamers are ring-shaped structures as seen from electron micrographs of negatively-stained proteins. Top views of both 4A' and 4B show that the hexameric ring has a central hole about 25-30 Å in diameter. This ring structure is common to other helicases including SV40 T antigen (Mastrangelo et al., 1989), E. coli Rho (Gogol et al., 1991), E. coli DnaB (Yu et al., 1996), phage T4 gene 41 protein (Dong et al., 1995), and E. coli Ruv B (Stasiak et al., 1994). The ubiquitous presence of the ring-motif among well-studied hexameric helicases is quite significant, given the fact that these helicases do not share much primary structure homology.

Significance of the Hexameric Structure.

The hexamer provides multiple nucleotide and DNA binding sites thought to be important for unwinding activity, while the ring-shape may provide optimal interactions with DNA during conformational changes that effect duplex DNA unwinding. A few possible modes of DNA binding by a ring-shaped protein have been depicted in
Figure 9. Possible modes of interaction between the hexameric ring and ssDNA. Single-stranded DNA may bind outside the ring or go through the hole in the ring. In either case, it may interact with all six subunits or only a few subunits of the hexamer.
Figure 9. The DNA may wrap around the ring (on the inside or the outside of the ring), interacting with all of the subunits, or it may bind only a few subunits at a time (on the inside or the outside of the ring). If DNA is wrapped around the ring, through the hole or on the outside, the helicase can translocate on DNA by a "rolling" mechanism, in which subunits exhibit changing affinity for DNA. The subunits can consecutively release DNA and bind another length to produce forward motion. A mechanism based on this principle has been proposed for *E. coli* Rho transcription-termination helicase (Geiselmann *et al.*, 1993), which unwinds RNA/DNA duplexes. Even if DNA interacts with only a few subunits, the helicase may employ a similar mechanism (perhaps a "walking" mechanism), in which only a few subunits consecutively bind and release DNA, as opposed to all six. It is not clear yet how the T7 helicase hexamer binds to DNA, and because only single-stranded DNA has been used in the oligomerization study, it is not known whether the helicase interacts with double-stranded DNA. Detailed information about the interaction of helicase with DNA is essential for understanding the mechanism of helicase-catalyzed DNA unwinding.
CHAPTER III
DNA BINDING PROPERTIES OF BACTERIOPHAGE T7 HELICASES

ABSTRACT

DNA binding to bacteriophage T7 helicase proteins was examined with small synthetic oligodeoxynucleotides of varying lengths. 4A' and 4B bind DNA with high affinity only in the presence of MgTMP-PCP, the nonhydrolyzable analog of dTTP. Binding was undetectable in the absence of nucleotide, and only about 20% binding was observed in the presence of dTDP. The stoichiometry was one 10-mer or 30-mer DNA bound per 6 to 8 monomers or one hexamer of 4A' protein. Native polyacrylamide gel electrophoresis showed that DNA binds predominantly to the hexameric form of 4A'. A 60-mer ssDNA is long enough to accommodate two 4A' hexamers, suggesting that one hexamer interacts with only about 30 bases of DNA. This was corroborated by nuclease protection experiments in which the smallest length of DNA protected by 4A' and 4B was 30 bases. Single-stranded DNA and gene 4 proteins form a tight complex that has a $K_d$ of approximately $1 \times 10^{-7}$ M. At high concentrations of DNA a second DNA binding site is detectable that has a $K_d$ of $1 \times 10^{-5}$ M. The duplex DNA
interacts weakly with the helicase and is easily competed off by ssDNA.

4A' binding to M13 DNA was investigated using protein-protein cross-linking and electron microscopy techniques. In the presence of Mg\textsuperscript{2+}-ATP-PCP, 4A' and 4B form ring-shaped hexamers on M13 ssDNA with the DNA passing through the hole in the center of the ring. Side views of the rings show that the hexamer has a two-tiered structure with a small-ring domain and a large-ring domain. This hexameric ring binds DNA with defined polarity such that the small domain points toward the 5'-end of the DNA. Further studies indicate that DNA binds specifically to only one or two subunits of the hexamer and nonspecifically to additional subunits.

INTRODUCTION

Helicases are enzymes that catalyze unwinding of duplex DNA \textit{in vivo} to generate single-stranded DNA for the processes of DNA replication, repair, recombination and conjugation. Helicases bind DNA and use energy from NTP hydrolysis to promote translocation on DNA, and DNA unwinding. Helicase-DNA interactions play an important role during the unwinding process. It has been proposed that NTP binding to the helicase and helicase-catalyzed hydrolysis modulates interactions between the DNA and helicase to effect translocation and unwinding (Lohman, 1992; 1993). In
order to understand the mechanism of helicase-catalyzed unwinding, it is essential to characterize the interactions between DNA and the helicase.

Bacteriophage T7 gene 4 helicases have been shown to form stable ring-shaped hexamers in the presence of nucleotide ligands. These hexamers are stabilized even further by ssDNA which suggests that hexamers may be the active form of the helicase. It has been shown earlier that gene 4 proteins bind DNA in the presence of nucleotides such as dTMP-PCP and dTDP (Matson & Richardson, 1985); however, the parameters of the interaction were not defined (e.g. stoichiometry, K_s), nor was there any quantitative examination of duplex DNA binding to the helicases. In light of the new finding that the T7 helicases form hexamers, it is necessary to determine which species binds DNA and to investigate the mode of interaction between the hexameric ring and DNA.

This study was designed to investigate the DNA binding properties of T7 helicases with ssDNA and dsDNA using both small synthetic oligodeoxynucleotides and long M13 DNA. DNA binding to the helicase in the presence of various nucleotides was examined by nitrocellulose membrane binding assay and native gel electrophoresis. The binding of DNA to the hexamer was also examined by protein-protein cross-linking, protein-DNA cross-linking, and electron microscopy. The results indicate that DNA binds predominantly to the
hexamer species, and binding is stable only in the presence of nucleotide triphosphate. DNA is bound through the hole of the hexameric ring with a specific polarity, and it specifically interacts with one subunit of the hexamer.

MATERIALS AND METHODS

Enzymes.

4A’ and 4B proteins were purified from overexpressing clones as described earlier (Hingorani & Patel, 1996; Chapter II). T4 polynucleotide kinase (10 U/ml), nuclease S7 (micrococcal nuclease), terminal deoxynucleotidyltransferase and DNA markers V were purchased from Boehringer Mannheim. Marker proteins for native gel electrophoresis were purchased as part of a nondenaturing gel molecular weight determination kit from Sigma Chemicals Co.

Oligodeoxynucleotides.

Small DNAs used in binding assays, native gel electrophoresis, and uv cross-linking experiments were synthesized at the Biochemical Instrument Center at The Ohio State University and at Macromolecular Resources, Colorado State University. The 10-mer, fluorescein-labeled 17-mer (17merF), (dT)$_{20}$, 26-mer, 30-mer, 40-merA, 40-merB, 60-mer single-stranded DNAs, and 20 base pair hairpin DNA (hp) were purified on 12 % (60-mer), 16 % (30-mer, hp duplex, 40-merA
and 40-merB), and 18 % (10-mer, 17-merF, (dT)$_{20}$, and 26-mer) polyacrylamide (TBE)/7 M urea gels. The oligos were
electroeluted from the gels (Elutrap; Schleicher & Schuell),
ethanol precipitated and reconstituted with water. DNA
concentrations were determined from absorbance measurements
at 260 nm in TE buffer/7 M urea using the following molar
extinction coefficients: 10-mer, 108,920 M$^{-1}$cm$^{-1}$; 17-merF,
195,410 M$^{-1}$cm$^{-1}$; (dT)$_{20}$, 168,000 M$^{-1}$cm$^{-1}$; 26-mer, 305,850 M$^{-1}$cm$^{-1}$;
30-mer, 305,010 M$^{-1}$cm$^{-1}$; 40-merA, 451,120 M$^{-1}$cm$^{-1}$; 40-merB,
420,240 M$^{-1}$cm$^{-1}$; 60-mer, 616,890 M$^{-1}$cm$^{-1}$; and hp, 447,260 M$^{-1}$cm$^{-1}$.
Single-stranded M13mp8 DNA used in protein-DNA cross-linking
and electron microscopy experiments was purified as
described (Lechner & Richardson, 1983). M13 ssDNA
concentration was calculated from absorbance measurements at
260 nm and by assuming 1 absorbance unit represents 33 µg/ml
ssDNA.

Nucleotides and Other Reagents.

The nucleotides dTTP and AMP-PCP were purchased from
Sigma Chemicals Co., and dTMP-PCP was purchased from
Amersham Life Science Inc. Radiolabeled nucleotides [$\gamma$-
$^{32}$P]ATP (4000 Ci/mM) and [$\alpha$-$^{32}$P]dTTP (>3000 Ci/mM) were
obtained from ICN Radiochemicals. Dimethyl suberimidate
(DMS) and triethanolamine (TEA) were purchased from Pierce
and Sigma Chemicals Co., respectively. Nitrocellulose (BA-S)
and DEAE membranes were purchased from Schleicher and Schuell. Biogel P-30 resin was purchased from BioRad and PEI-cellulose TLC plates were purchased from EM Separations Technology.

5'-32P Labeling of Oligodeoxynucleotides.

Oligodeoxynucleotides were 5'-labeled with [32P]phosphate using T4 polynucleotide kinase and [γ-32P]ATP. The reactions (100 μl) contained 0.1 to 1 μM DNA and [γ-32P]ATP (20-30 μCi) in kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 100 mM KCl and 1 mM 2-mercaptoethanol). The reaction was initiated by addition of 1 μl (10 Units) of kinase and incubated at 37 °C for 1 h. Unincorporated [γ-32P]ATP was removed by centrifugation through a 0.5 ml Biogel P-30 gel-filtration column (prepared in a 1 ml tuberculin syringe barrel with Biogel P-30 resin, previously equilibrated in 50 mM Tris-Cl, pH 7.5, 50 mM NaCl, and 1 mM EDTA). Aliquots of the reaction were spotted onto PEI-cellulose TLC plates and Whatman DE-81 filters before and after gel-filtration. The TLC plates were developed in 0.3 M phosphate buffer, pH 3.4, and the radioactivity was measured on a PhosphorImager (Molecular Dynamics) to test for [γ-32P]ATP contamination in the solution following gel-filtration. The DE-81 filters were washed in 0.3 M ammonium formate, pH 7.8, and the radioactivity was measured by
scintillation counting. Total radioactivity in the reaction was measured from unwashed filters, and the concentration of radiolabeled DNA was determined from the final volume of the reaction and the calculated specific activity (counts/mole).


Uniformly labeled poly(dT) was prepared by elongating a 30-mer oligodeoxynucleotide (0.7 μM) in a reaction containing [α-32P]dTTP (30 μCi), dTTP (2 mM), CoCl2 (2 mM), 20 units of terminal deoxynucleotidyltransferase (TdT), and DNA-tailing reaction buffer from the kit provided by Boehringer Mannheim. The reaction (50 μl) was incubated at 37 °C for 1 h and quenched by addition of EDTA to a final concentration of 50 mM. Unincorporated nucleotide was removed by gel-filtration through Bio-gel P-30 resin, as described above. The purity and homogeneity of radiolabeled poly(dT) was checked before use on an 8 % polyacrylamide (TBE)/7 M urea sequencing gel. Synthesized [α-32P]poly(dT) was found to migrate consistently as a tight band >500 nucleotides in length.

Synthesis of [α-32P]dTMP-labeled ssDNA.

Radiolabeled linear DNA of random sequence was prepared by elongating a 30-mer oligodeoxynucleotide with terminal deoxynucleotidyltransferase. The reaction mixture was
prepared with 0.7 μM 30-mer, 0.5 mM of each dNTP, [α-32P]dTTP (20 μCi), 1 mM CoCl2, and 35 units of TdT in DNA-tailing reaction buffer. The mixture was incubated at 37 °C for 2 h, and unincorporated nucleotides were removed by gel-filtration. The purity of the DNA was checked on a sequencing gel as described above for [α-32P]poly(dT), and the length was estimated to be around 200 nucleotides.

Equilibrium Binding of Radiolabeled DNA to 4A' (or 4B) Proteins:

Preparation of the Membrane Assembly.

Nitrocellulose membrane binding assays were performed on a 96-well dot-blot apparatus (BRL) using a modified version of a previously described procedure (Wong et al., 1992). Each well corresponded to one point in the assay. The membrane assembly was prepared by laying a Whatman No.1 filter paper on the bottom plate of the dot-blot apparatus, followed by a DEAE membrane and a nitrocellulose membrane on the top, taking care to remove air bubbles in between the membranes. Prior to use, the nitrocellulose and DEAE membranes were soaked for 10 min in 0.5 M NaOH, washed extensively with double-distilled water and equilibrated in the membrane wash buffer A (50 mM Tris-acetate, pH 7.5, 5 mM sodium acetate, 10 mM magnesium acetate) for at least 1 h at room temperature (22 °C).
Measurement of DNA Binding.

Equilibrium binding assays at constant DNA and increasing 4A' or 4B concentrations were performed as follows: 1) in the absence of nucleotide ligands; 2) in the presence of 1 mM MgdtMP-PCP; 3) in the presence of 5 mM dtTDP; 4) in the presence of 1 mM AMP-PCP. The samples (10 µl or 20 µl) contained 1 µM radiolabeled DNA and 0-30 µM 4A' or 4B in binding buffer A (see Appendix B). Binding assays at constant protein and increasing DNA were performed using 2 µM 4A' or 4B protein and 0-40 µM radiolabeled ssDNA in binding buffer in a total volume of 20 µl. The reactions were initiated by addition of protein, and the mixtures were incubated at room temperature for 10-20 min before filtration through the membrane assembly (in case of samples containing dtTTP, the reactions were incubated for 30-40 s only because of helicase-catalyzed dtTTP hydrolysis). The membranes were washed before and after filtration with 100 µl membrane wash buffer. After the samples were blotted, radioactivity on both membranes was measured on a Betagen 603 blot analyzer or a PhosphorImager (Molecular Dynamics). Radioactivity on the nitrocellulose membrane gave a measure of the protein-DNA complex, and that on the DEAE membrane yielded free DNA concentration. The fraction of total DNA bound to protein was determined from these measurements.
Nonspecific DNA binding to the membrane was determined for each experiment by filtering samples in which protein was replaced by water. Non-specific binding was 1 % of the total DNA in titrations at increasing protein concentrations, and ranged from 0.01 to 10 % in titrations with increasing DNA. All titrations reported have been corrected for nonspecific DNA binding.

Fluorescence-based Assay for DNA Binding.

DNA binding to increasing concentrations of 4A' protein was also assayed by measuring fluorescence quenching of a fluorescein-labeled 17-mer DNA. The sample, prepared in a 1 ml quartz cuvette, contained 0.3 μM 17-merF DNA and 0.5 mM MgTmPCP (or 2 mM dTTP) in binding buffer A which was filtered through a 0.2 μm membrane. Aliquots of 4A' protein were added to the cuvette to vary 4A' concentration from 0 to 3 μM. After each addition, the sample was incubated for 2 min (30 s with dTTP) before fluorescence was measured by exciting the sample at 488 nm and measuring emission at 520 nm. Fluorescence measurements were carried out at room temperature (22 °C) on an SPF-500C spectrofluorometer (SLM Instruments). Changes in volume and inner-filter effect, on addition of 4A', were negligible, and were not taken into consideration.
Competitive Binding of ssDNA and dsDNA at Increasing Protein Concentration.

Titration of radiolabeled 60-mer (0.2 µM) with increasing 4A' protein (0-10 µM) was performed in the absence and in the presence of hp duplex DNA (0.2 µM) with 1 mM MgDTMP-PCP in the binding buffer. Similarly, titration of 0.2 µM radiolabeled hp was carried out in the absence and in the presence of nonradiolabeled 60-mer DNA (0.2 µM). In titrations where both 60-mer and hp were included, the two DNAs were mixed in the sample prior to addition of protein. DNA binding was assayed on the nitrocellulose-DEAE membrane assembly as described above.

Competitive Binding of ssDNA and dsDNA at Increasing 60-mer Concentrations.

Competitive binding of the 60-mer and hp DNA to 4A' was tested by titrating 4A' with increasing amounts of radiolabeled 60-mer (0-10 µM) in the presence of hp duplex DNA (0.5 µM). The DNAs were mixed prior to addition of 4A' protein (2 µM) in samples containing 1 mM MgDTMP-PCP in binding buffer A. The samples were assayed for DNA binding, and the fraction of 60-mer DNA bound to 4A' in the presence of hp DNA was quantitated as described above. The titration was also carried out in the absence of hp duplex DNA. A
Complementary assay was performed with radiolabeled hp duplex (0.5 μM or 8 μM) and unlabeled 60-mer (0-10 μM) to measure hp DNA binding to 4A' in the presence of increasing amounts of 60-mer DNA.

Competitive Binding of ssDNA and dsDNA at Increasing hp Duplex Concentrations.

4A' was titrated with increasing concentrations of radiolabeled hp duplex (0-10 μM) in the presence of unlabeled 60-mer DNA (0.5 μM). 4A' (2 μM) was added to a reaction containing the two DNAs and MgTMP-PCP (1 mM) in binding buffer A, and duplex DNA binding was measured as described above. The assay was also performed in the absence of 60-mer DNA. A complementary experiment was carried out with radiolabeled 60-mer (0.5 μM) and unlabeled hp (0-10 μM) to measure 60-mer binding to 4A' in the presence of increasing amounts of hp duplex DNA.

Data Analysis.

Radioactivity on the nitrocellulose and DEAE membranes was quantitated to determine the molar amounts of 4A'•DNA complex and free DNA, respectively, for each point in the assay. Equilibrium binding titrations at constant DNA and increasing protein concentrations were analyzed by plotting the fraction of total DNA bound versus protein monomer
concentration. For the fluorescence titration at constant DNA and increasing 4A' concentration, saturation of fluorescence quenching was assigned a value of 100%, and the percent fraction of fluorescence quenching was plotted versus 4A' concentration to obtain the binding isotherm.

Titrations at constant 4A' and increasing DNA concentrations were analyzed by plotting the fraction of DNA bound per 4A' hexamer versus free DNA concentration. Error bars represent standard errors of the mean determined from three to five separate binding experiments. The dissociation constants were estimated by using nonlinear regression analysis (KaleidaGraph software; Synergy Software, Reading, PA, USA) to fit the binding data with equations derived for binding of two DNAs per 4A' hexamer:

\[
\begin{align*}
  E + D & \rightleftharpoons K_1 ED \quad (2) \\
  ED + D & \rightleftharpoons K_2 ED_2 \quad (3)
\end{align*}
\]

where E and D are 4A' hexamer and ssDNA, respectively, and \( K_1 \) and \( K_2 \) are the equilibrium binding constants for the first and second DNA binding to the hexamer, respectively. The fraction of DNA bound per hexamer \( (D_b / E_t) \) is derived from equations 2 and 3 as follows:

\[
\begin{align*}
  K_1 &= ED / E_t D_t \\
  K_2 &= ED_2 / (ED) D_t \\
  E_t &= E_t + ED + ED_2
\end{align*}
\]
\[ E_t = E_i + K_1 E_i D_t + K_2 K_1 E_i D_t^2 \]
\[ D_b = ED + 2ED_i \]
\[ D_b = K_1 E_i D_t + 2K_2 K_1 E_i D_t^2 \]
\[ D_b = K_1 D_t + 2K_2 K_1 D_t^2 \]
\[ E_t = \frac{1 + K_1 D_t + K_2 K_1 D_t^2}{E_i} \]

where \( E_t \) and \( E_i \) refer to concentration of total and free hexamer species, respectively, \( D_b \) and \( D_i \) are bound and free DNA, respectively. The 60-mer titration data was plotted as fraction of DNA bound per dodecamer, and the data were fit with \( E_t \) and \( E_i \) refering to total and free 4A' dodecamer concentrations.

Nondenaturing Polyacrylamide Gel Electrophoresis.

Native polyacrylamide gel electrophoresis was performed to examine formation of 4A' oligomers in the presence of nucleotide ligands and nucleotide plus ssDNA, and to identify the oligomer species that binds DNA. The 6% polyacrylamide gel was prepared as described in Chapter II, with 10 mM magnesium acetate and 100 \( \mu \)M dTMP-PCP. 4A' (12 \( \mu \)M) was mixed with 1 mM dTMP-PCP and 0-2 \( \mu \)M 5'-\( ^{32} \)P radiolabeled oligodeoxynucleotides in binding buffer A. The samples (10 \( \mu \)l) were mixed with 2 \( \mu \)l native gel loading dye (0.25% bromophenol blue, 15% Ficoll) and loaded on the gel. Electrophoresis was performed in Tris-glycine buffer,
pH 8.3, for 3 h at constant current (18 mA). Proteins were visualized by Coomassie Blue staining, and the radiolabeled DNAs were visualized by autoradiography.

The stable 4A' oligomer that predominantly bound DNA was identified by a Ferguson analysis (Ferguson, 1964; Hedrick & Smith, 1968). A number of standard proteins such as lactalbumin (14,200 Da), bovine serum albumin (monomer:66,000 Da; dimer:132,000 Da), chicken egg albumin (45,000 Da), carbonic anhydrase (29,000 Da), and urease (trimer:272,000 Da; hexamer:545,000 Da) were analyzed by a series of native polyacrylamide gel experiments. 4A' protein plus 100 μM MgdTMP-PCP (in the absence and in the presence of 2 μM DNA) and the size markers were electrophoresed on 5.5 %, 6 %, 6.5 %, and 7 % gels, with 3 % stacking gels (all gels contained 100 μM MgdTMP-PCP). Electrophoresis was carried out in Tris-glycine buffer as described above. The relative mobilities (Rf) of marker proteins and 4A' species that bound DNA were measured and the corresponding transformed values, 100[Log(Rf x 100)], were plotted versus % gel concentration for each protein. A standard curve was generated by plotting the log of the negative slope of the line obtained for each marker (from the previous plot) versus log of its molecular mass. The molecular mass of the relevant 4A' species was obtained by interpolation of the standard curve.
Nuclease Protection Assay.

The minimum length of single-stranded DNA covered by 4A' and 4B proteins was investigated by nuclease protection assays. [α-32P]poly(dT), which was used as DNA substrate in the assay, was synthesized as described above. 4A' (10-40 μM) and 4B (5-30 μM) were incubated with 0.2 μM labeled poly(dT) in nuclease buffer (22 °C), in the presence of Mg-dTMP-PCP (1 mM). Reactions were initiated by adding nuclease S7 to a final concentration of 1.6 μg/ml, and stopped with SDS (1 % final concentration) after 30 s. The samples were mixed with equal volumes of sequencing gel loading buffer (98 % deionized formamide, 10 mM EDTA, pH 8.0, 0.025 % xylene cyanol FF, 0.025 % bromophenol blue), heated to 90 °C for 2 min, and electrophoresed on a 20 % polyacrylamide (TBE)/7 M urea gel. 5'-32P radiolabeled 10-mer, 30-mer, 40-mer, 60-mer ssDNA, as well as DNA V markers (Boehringer Mannheim) that were dephosphorylated with calf intestinal phosphatase and radiolabeled using T4 polynucleotide kinase and [γ-32P]ATP, were used as size standards. Following electrophoresis, DNA markers and nuclease-protected DNA products were visualized by autoradiography. The sizes of the protected bands were determined from interpolation of standard curves prepared by
plotting log of masses of DNA markers versus distance migrated on the gel.

Cross-linking of T7 Helicase Hexamers Bound to DNA.

Interaction of 4A' and 4B protein hexamers with circular M13 ssDNA was examined by protein-protein cross-linking with the bifunctional cross-linking agent dimethyl suberimidate (DMS). A fresh solution of DMS was prepared immediately before use by dissolving 7 mg of DMS in 25 μl ice-cold TEA-HCl (0.15 M triethanolamine; pH adjusted to 8.2 with 1 M HCl), and the pH of the solution was adjusted to 8.2 with 1 M NaOH. 4A'(or 4B) protein (5, 15, and 30 μM) was assembled on M13 ssDNA (0.07 μM) in the presence of 200 μM MgTmP-PCP in TEA buffer (50 mM TEA, 10 mM MgCl₂, 100 mM NaCl, and 1 mM DTT; pH adjusted to 8.2 with 1 M HCl), and incubated at 22 °C, or cross-linked by addition of DMS (10 mg/ml final concentration). Following incubation for 12 min, the samples were quenched with an equal volume (10 μl) of 1 M glycine. 7 μl aliquots of each sample were mixed with native gel loading dye (see Appendix B) and loaded on a non-denaturing 1% agarose gel (8 cm x 5 cm) prepared with TAE buffer (40 mM Tris-acetate, pH 8.0 and 1 mM EDTA) containing 10 mM magnesium acetate and 80 μM MgTmP-PCP. Electrophoresis was carried out at 50 V constant potential
using TAE buffer. The above samples (7 μl) were also heat
denatured at 90 °C (3 min) in the presence of 1 % SDS and
0.5 mM DTT, mixed with SDS gel loading dye (50 mM Tris-HCl,
pH 6.8, 100 mM DTT, 2 % SDS, 0.1 % bromophenol blue, and 10
% glycerol), and electrophoresed on a 1 % agarose gel
prepared in TAE buffer containing 0.1 % SDS.

Cross-linking was also performed with linear [α-
\textsuperscript{32}P]ssDNA (synthesis described earlier in the Methods). 4A'
protein (15 and 30 μM) was assembled on the DNA (0.07 μM),
and cross-linking and gel electrophoresis (denaturing and
native) were carried out as described above. The DNA was
visualized by autoradiography.

Protein-DNA Cross-linking.

Interaction of 4B hexamers with ssDNA was investigated
by protein-DNA cross-linking. 4B (5 μM) was incubated with
MgdTMP-PCP (0.5 mM) and [5'-\textsuperscript{32}P](dT)\textsubscript{20} (3 μM) in binding
buffer A. The reaction mixture was spotted in 10 μl drops on
a Parafilm sheet placed on ice. The samples were irradiated
at 254 nm using a UVGL-25 Mineralight lamp from a distance
of 2 cm at 4 °C. 8 μl aliquots were removed at varying
times, from 0 to 8 min, and quenched with 5 μl SDS gel
loading dye. The protein-DNA cross-linked species were
resolved on a 3-10 % SDS polyacrylamide gradient gel. Each
oligomeric species linked to radiolabeled DNA was visualized and quantitated on a PhosphorImager and plotted as percent of hexamer cross-linked versus cross-linking time. The HopKINSIM kinetic simulation program (described in Chapter V) was used to simulate curves for rate of DNA linking to one subunit, followed by linking to another subunit and so on. The rate constants cross-linking of DNA to monomer, dimer etc. were determined from visual comparison of the data and the simulated curves.

Electron Microscopy of 4A'•DNA Complexes.

Complexes formed between 4A' or 4B protein and ssDNA were examined in various experiments by negative-staining electron microscopy and image analysis, performed by Dr. Edward H. Egelman and Dr. Xiong Yu at University of Minnesota. Electron micrographs were obtained for 4A' and 4B proteins bound to M13mp8 circular ssDNA to examine side views of the protein rings, and to determine the number of protein rings bound on the circular DNA. In another experiment, the polarity of 4B protein binding to DNA was investigated using an exonuclease III digest and negative-staining electron microscopy. Also, interaction between subunits of the 4B hexamer and 30-mer ssDNA was visualized by electron microscopy and image averaging and analysis. The above mentioned experiments have been described in detail in
the literature (Egelman et al., 1995; Yu et al., 1996: manuscript in preparation).

RESULTS

Nitrocellulose-DEAE Membrane Binding Assays.

Equilibrium interactions between T7 gene 4 proteins and DNA were investigated by nitrocellulose-DEAE membrane binding assays. The nitrocellulose membrane has a high affinity and capacity for binding protein, while the DEAE membrane, an anion exchanger, binds DNA tightly through ionic interactions. In this assay, the two membranes are assembled such that the nitrocellulose membrane lies on top and binds the helicase-DNA complex, and the DEAE membrane, which is at the bottom, retains free DNA. A sample blot showing titration of a constant amount of radiolabeled ssDNA with increasing 4A' protein is shown in Figure 10. As protein concentration increases all DNA in the reaction is bound and radioactivity (free DNA) on the DEAE membrane reduces to background level. Quantitation of radioactivity bound to the membranes yields the amount of bound and free DNA which is used to calculate the fraction of total DNA bound to protein in each assay point. The greatest advantage of this assay is that bound and free DNA concentration can be measured directly for each point in the assay. This increases accuracy in data collection because errors due to
Figure 10. A sample dot-blot of the nitrocellulose-DEAE membrane binding assay. A constant amount of DNA was titrated with increasing concentrations of 4A', and DNA binding was measured by a nitrocellulose-DEAE membrane binding assay as described in Materials and Methods. The membranes were analyzed on a PhosphorImager (Molecular Dynamics). Radioactivity on the nitrocellulose membrane reflects the amount of protein-DNA complex, which saturates with increasing protein (top two rows), and radioactivity on the DEAE membrane is the amount of free DNA (bottom two rows), which is depleted as protein concentration increases.
separate measurements of total DNA in the reaction are eliminated. The nitrocellulose-DEAE membrane binding assay has been described in detail in previously published literature (Wong & Lohman, 1993) and essentially the same procedure has been applied here, except for the dot-blot apparatus used. The 96-well dot-blot apparatus purchased from Bio-Rad for this study has been "machined" precisely to provide a tight seal, which effectively prevents lateral diffusion of samples on the membranes compared to the Minifold I apparatus (Schleicher and Schuell), which requires rubber O-rings on each well. The BioRad dot-blot apparatus avoids the possibility of interactions between the rubber rings and the samples during filtration.

Effect of Nucleotides on Equilibrium Interactions of ssDNA and T7 Gene 4 Proteins.

DNA binding to 4A' and 4B proteins was examined in the absence and in the presence of nucleotide ligands using nitrocellulose membrane binding assays. Figure 11A shows titration of 30-mer ssDNA with increasing concentrations of 4A' protein. In the absence of nucleotide, no interaction between 4A' and DNA can be detected. On the other hand, in the presence of MgTMP-PCP, DNA binding reaches saturation with all 30-mer ssDNA in the reaction bound to 4A' protein. The curve appears stoichiometric, suggesting that DNA binds to protein with high affinity at the concentrations used in
Figure 11. Single-stranded DNA binding to increasing concentrations of T7 gene 4 proteins. DNA binding to increasing amounts of 4A' and 4B proteins was tested with various oligodeoxynucleotides, and in the presence of various nucleotide ligands as described in the Materials and Methods. Panel A shows equilibrium binding of 5'-32P ss30-mer (1 μM) to 4A' protein (0-20 μM) in the presence of 1 mM MgδTMP-PCP (●), 5 mM MgδTDP (♦) and in the presence of Mg2+ alone (▲). Panel B shows the same experiment performed with 4B protein (0-20 μM) in the presence of 1 mM MgδTMP-PCP. Panel C shows titration of 1 μM 5'-32P ss10-mer with 4A' protein (0-20 μM) in the presence of 1 mM MgδTMP-PCP, and panel D shows the same experiment performed with 5'-32P ss60-mer (1 μM) and 4A' (0-30 μM). As shown in panel E, binding was also measured with a 26-mer DNA (1 μM), without the primase recognition site, in the presence of 1 mM MgδTMP-PCP (●), 5 mM dTDP (□), and 5 mM dTTP (♦). Panel F shows the titration of 5'-32P ss30-mer DNA (1 μM) with 4A' protein (0-20 μM) in the presence of 1 mM AMP-PCP. All binding isotherms show fraction of total DNA bound plotted versus 4A' monomer concentration.
Figure 11
Figure 11 (continued)

C

\[
\frac{[10\text{mer}]_{\text{bound}}}{[10\text{mer}]_{\text{total}}}
\]

\[
\frac{[60\text{mer}]_{\text{bound}}}{[60\text{mer}]_{\text{total}}}
\]

D

\[
[4A']_{\text{monomer}} \mu M
\]

\[
[4A']_{\text{monomer}} \mu M
\]
Figure 11 (continued)

E

\[
\frac{[26\text{mer}]_{\text{bound}}}{[26\text{mer}]_{\text{total}}} \quad vs. \quad [4A']_{\text{monomer}} \ \mu M
\]

F

\[
\frac{[30\text{mer}]_{\text{bound}}}{[30\text{mer}]_{\text{total}}} \quad vs. \quad [4A']_{\text{monomer}} \ \mu M
\]
the assay. In the presence of dTDP, only about 20% of the 30-mer binds to 4A' protein indicating weak complex formation. These results show that Mg\(\text{dTMP-PCP}\) is required for complete binding of ssDNA to 4A' protein. A similar experiment with 4B protein yielded a stoichiometric DNA binding curve in the presence of Mg\(\text{dTMP-PCP}\) (Figure 11B). DNA binding was examined also in the presence of dTTP. DNA binding appears weak in the presence of dTTP compared with dTMP-PCP, but at high protein concentrations up to 40% 30-mer ssDNA is bound. The apparent weak binding may be explained by the fact that in the presence of 30-mer ssDNA 4A' hydrolyzes dTTP at a rate of about 0.3 s\(^{-1}\) at 22 °C, and during hydrolysis, interactions between 4A' and DNA may not be as stable as with the nonhydrolyzable analog.

Equilibrium binding experiments were carried out with oligodeoxynucleotides of various lengths to define the length of DNA required for binding to 4A'. Titrations of 10-mer, 30-mer and 60-mer ssDNAs were performed with increasing amounts of 4A' protein in the presence of Mg\(\text{dTMP-PCP}\). Figure 11C shows binding of 10-mer ssDNA to 4A'. The binding isotherm is stoichiometric, and 10-mer binding (1 \(\mu\)M) saturates with about 6 to 8 \(\mu\)M of 4A' protein (monomer) in the reaction. The same ratio is observed for 30-mer ssDNA as shown in Figure 11A. Titration of 60-mer ssDNA shows that 1 \(\mu\)M 60-mer requires 12 to 14 \(\mu\)M 4A' protein to saturate
binding (Figure 11D). Thus, with 10-mer and 30-mer ssDNAs, stoichiometry of binding appears to be one DNA per 6 to 8 monomers, while one 60-mer binds 12 to 14 protein monomers. Also, 30-mer binds 4B protein with a stoichiometry of one DNA per 6 to 8 4B monomers (Figure 11B).

Previously described experiments using gel-filtration and electron microscopy have demonstrated that 4A' and 4B proteins form stable hexamers in the presence of MgдTMP-PCP and ssDNA (Patel & Hingorani, 1993; Egelman et al., 1995; Chapter II). Those results, along with the 1:6 stoichiometry for DNA binding to 4A', determined here, show that hexamer is the active, DNA-binding form of the helicase. Each 4A' hexamer binds one DNA about 10 to 30 nucleotides in length, and the longer 60-mer DNA binds two hexamers or one 4A' dodecamer (HPLC gel-filtration showed stable 4A' and 4B dodecamer species in the presence of MgдTMP-PCP and ss-DNA).

The stoichiometry of the 4A' hexamer-DNA complex was confirmed by other assays. HPLC gel-filtration experiments were carried out with a constant amount of 30-mer DNA (2 μM) which was titrated with 4A' protein. The free DNA peak eluting from the gel-filtration column was monitored as 4A' concentration was increased. 30-mer binding was found to saturate (disappearance of free DNA peak) at a ratio of one DNA to one 4A' hexamer. Similar results were also obtained from a nondenaturing PAGE assay which will be described later.
In addition to helicase activity, 4A' also has primase activity that catalyzes synthesis of small RNA primers as substrates for the DNA polymerase. So far, all ssDNAs used to examine DNA binding to 4A' contain the primase-recognition sequence, 5'-GTC-3'. To determine whether this site plays any role in the stoichiometry of DNA binding, a titration of a 26-mer DNA without "GTC" was carried out with increasing concentrations of 4A' in the absence of ligands, in the presence of MgTTP, and in the presence of MgTTP-PyC. As shown in Figure 1E, 26-mer binding reaches saturation at a ratio of 1 DNA per 7 to 8 4A' monomers; thus, even without the primase-recognition site, DNA binds 4A' with high affinity in the presence of MgTTP-PyC. This result is corroborated by the fact that 4B protein, which lacks primase activity and the zinc finger required for primase recognition, also binds one 30-mer per helicase hexamer. 26-mer binding cannot be detected in the absence of nucleotide ligands, as in the case of 30-mer DNA. Interestingly, in the absence of the primase site, interaction between 4A' and DNA in the presence of dTTP is further weakened so that no binding can be detected by the membrane binding assay. The DNA-stimulated dTTPase rate of 4A' is the same with the 26-mer and the "CTG"-containing 30-mer DNA. The difference probably lies in the fact that the zinc finger in the primase domain of 4A' increases stability
of the complex between 4A' and "CTG"-containing DNA, when binding is measured in the presence of dTTP.

Equilibrium DNA binding assays were also performed in the presence of AMP-PCP, the nonhydrolyzable analog of ATP. As shown in Figure 11F, 30-mer DNA binding reaches saturation with a stoichiometry of 1 DNA per 4A' hexamer. Thus, T7 helicases can form hexamers and bind DNA in the presence of ATP and its analogs (Hingorani & Patel, 1996; Chapter II).

Equilibrium ssDNA Binding Measured by Change in Fluorescence.

DNA binding to 4A' was examined by fluorescence-based assays using a 17-mer ssDNA, prepared with a fluorescein tag at the 3'-end. Binding of 4A' protein to the 17-merF DNA resulted in 23 % quenching of fluorescence. A constant amount of the DNA was titrated with increasing 4A' protein as shown in Figure 12. As expected, in the presence of MgDTMP-PCP, fluorescence quenching reaches saturation at a ratio of one 17merF to one 4A' hexamer (0.3 μM 17-merF : 1.8-2 μM 4A' monomer). The assay was also performed in the presence of dTTP. Similar to the results obtained from the nitrocellulose binding assay, in the presence of dTTP, the 4A'•DNA complex is weaker, and a maximum of only 60 % DNA
Figure 12. Fluorescence-based assay for DNA binding to 4A' protein. Titration of fluorescein-labeled ssDNA with 4A' was performed by measuring fluorescence quenching that occurs on DNA binding to protein. 17-merF ssDNA (0.3 μM) was titrated with increasing concentrations of 4A' protein (0-3 μM) in the presence of 0.5 mM Mg2TMP-PCP (●), or 2 mM Mg2TTP (□). The maximum fluorescence quenching was assigned a value of 100 %, and percent fraction of fluorescence quenching during the titration was plotted versus protein concentration (monomer).
binding is detectable. In the absence of nucleotide, no significant fluorescence quenching can be detected.

Native Polyacrylamide Gel Electrophoresis of 4A'.

Gel-filtration experiments have shown that 4A' forms stable hexamers in the presence of MgdTMP-PCP and ssDNA. In this study, native polyacrylamide gel electrophoresis has been used to identify the oligomeric species that binds DNA. Native PAGE was performed in the absence and in the presence of 10-mer, 30-mer and 60-mer single-stranded DNAs, and the 20-base pair hairpin duplex DNA. Binding was assayed in the presence of MgdTMP-PCP, which was added to the gel to maintain the stability of the 4A'•DNA complex during electrophoresis. Figure 13A shows that in the absence of DNA a well-resolved ladder of 4A' species is formed (lane 2). When 10-mer ssDNA is added in substoichiometric amounts (<1 DNA per hexamer), one oligomer becomes more prominent (lanes 3 and 4). As ssDNA concentration is increased above saturation, all lower oligomers disappear and the concentration of the prominent oligomer increases further (lane 5). A similar effect is observed with the 30-mer and 60-mer ssDNA in lanes 6 and 7, respectively.

If the oligomers in the ladder are counted, assigning the bottom one as a monomer, the predominant species appears to be a pentamer. The molecular mass of the prominent species was determined by a Ferguson analysis (Ferguson,
Figure 13. Native polyacrylamide gel electrophoresis of 4A'. Panel A shows a Coomassie-stained 6 % native polyacrylamide gel of 4A' protein. The hexamer species, indicated on the gel, was identified from the Ferguson plot shown in panel B (Materials and Methods). Panel C is an autoradiogram of the gel in A, and it shows the labeled DNA bound to 4A' oligomers. The lanes in panels A and C represent the following samples: lane 1, 30-mer ssDNA (2 μM); lane 2, 4A' protein (12 μM); lane 3, 4A' + 10-mer (0.1 μM); lane 4, 4A' + 10-mer (1 μM); lane 5, 4A' + 10-mer (2 μM); lane 6, 4A' + 30-mer (2 μM); lane 7, 4A' + 60-mer (2 μM); lane 8, 4A' + hp duplex (2 μM). In lanes 3-8, 4A' concentration is 12 μM.
Figure 13
Figure 13 (continued)

B

\[ y = -86.109 + 20.3x \quad R = 0.99409 \]

- slope

Log (MW)

4A' hexamer
Figure 13 (continued)

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<th>5</th>
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<th>8</th>
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<td>C</td>
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Hexamer

Dodecamer
4A' was electrophoresed on a set of nondenaturing polyacrylamide gels (5.5 - 7 %) along with molecular mass markers. The relative mobilities of 4A' and the markers in each gel were determined and plotted versus % gel concentration for each protein (see Materials and Methods). The slopes of the lines were determined, and the log of the negative slope was plotted versus log of the molecular masses of the marker proteins (Figure 13B). From this standard curve, molecular mass of the predominant species on the native gel was found to be 389 ± 10 kDa, which is closest to the calculated mass of a 4A' hexamer (378 kDa). Thus, as DNA concentration is increased to saturating conditions, the smaller protein oligomers associate stably to form hexamers.

On the native gel, the presence of only four bands below the hexamer indicates that one species between monomer and pentamer is missing. If the monomer is missing, the fact that it cannot be detected even at concentrations as low as 0.1 μM 4A' (Chapter II; Figure 2B) implies that 4A' forms dimers or even higher oligomers with an association constant tighter than $1 \times 10^7 \text{ M}^{-1}$. It is also likely that the missing species is the pentamer. This may be possible by two means: a) if the hexamer ring (with dTMP-PCP and DNA) is more compact than the hexamer chain (six subunits in 'head-to-tail contact in the absence of ligands), it may migrate faster on the gel and merge with the pentamer-chain so that
the two species appear as one band; b) the pentamer may simply have a higher propensity for hexamer formation compared to the other oligomers, in the presence of nucleotides and DNA, and thus be undetectable as a stable species.

The DNAs used in the nondenaturing PAGE experiment were radiolabeled, and the autoradiogram of the native gel is shown in Figure 13C. All DNAs bind mainly to the hexameric form of 4A'. Smaller oligomers do not appear to bind DNA, but species larger than the hexamer exhibit some DNA binding. Most notable is a band that appears to be the dodecamer species, as seen from other gels on which bands higher than a hexamer can be clearly counted. Despite saturating conditions for DNA binding, a large fraction of the DNA migrates as free DNA at the dye front. The large fraction of free 10-mer in the gel may be due to relatively facile dissociation of the small 10-mer DNA from 4A' under gel-electrophoresis conditions as compared to the 30-mer ssDNA. There is also a large amount of free 60-mer DNA when compared to the 30-mer. This may be because the concentration of 4A' is not high enough to saturate 60-mer binding (60-mer requires twice as much 4A' protein for complete binding relative to the 30-mer; Figure 11D).

Lane 8 of the native gel shows 4A' hexamerization and DNA binding in the presence of the hairpin duplex DNA. In contrast to ssDNA, the hp duplex does not appear to induce
significant amount of 4A' hexamer formation (Figure 13A). Furthermore, the autoradiogram shows a very small amount of duplex DNA bound only to the hexamer among the various oligomer species (Figure 13B, lane 8). Thus, interaction of 4A' with double-stranded DNA is clearly different than with ssDNA. Interaction with duplex DNA has been investigated further by membrane and fluorescence binding assays, and is discussed later.

Titration of 4A' Protein with Increasing DNA Concentrations.

Titration of ssDNA with increasing amounts of 4A' protein has shown that one DNA binds stoichiometrically to a 4A' hexamer or a dodecamer as in the case of 60-mer DNA. Because of the apparent high affinity of the interaction, to measure the dissociation constant for DNA binding it is necessary to measure binding at lower DNA concentrations. To test if more than one DNA can bind to the hexamer, and to determine the $K_d$ of the interaction, titrations were performed at constant 4A' protein and increasing DNA concentrations. Figure 14A shows equilibrium binding of 4A' to 10-mer ssDNA. The curve is biphasic, and at high DNA concentrations, 2 10-mer strands are bound per hexamer. The first phase of the curve shows tight binding of 1 DNA to the 4A' hexamer (inset in Figure 14A), and the second phase shows weaker binding of a second DNA strand to the 4A' hexamer. Similar results are obtained with the 30-mer ssDNA:
Figure 14. Titration of 4A' and 4B with increasing concentrations of ssDNA. A constant amount of protein was titrated with increasing amounts of ssDNA of varying lengths. Titrations were carried out in the presence of MgTMP-PCP as described in Materials and Methods. Panel A shows titration of 4A' (2 μM) with 0-40 μM 10-mer ssDNA, panel B shows the same experiment performed with 0-40 μM 30-mer ssDNA, panel C shows titration of 4A' (2 μM) with 60-mer ssDNA, and panel D shows titration of 4B protein (2 μM) with 30-mer ssDNA. In each case the fraction of DNA bound per 4A' or 4B hexamer is plotted versus free DNA concentration. The insets in the panels show the initial tight binding phase. The error bars represent standard errors of the mean determined from 3 or more independent experiments. The solid lines are the best fits to the data, of equations defined for a model in which two 10-mer or 30-mer DNAs bind per hexamer, and two 60-mer DNAs bind per dodecamer (see Materials and Methods). For both 4A' and 4B proteins, the two binding phases for 10-mer or 30-mer DNAs fit to $K_d$ values of about $1 \times 10^{-7}$ M and $1 \times 10^{-5}$ M, and the initial phase of 60-mer binding fits to a $K_d$ of $1.4 \times 10^{-7}$ M.
Figure 14

Panel A: 

\[
\frac{[10\text{mer}]_{\text{bound}}}{[4A']_{\text{hexamer}}} \]

Panel B: 

\[
\frac{[30\text{mer}]_{\text{bound}}}{[4A']_{\text{hexamer}}} \]
Figure 14 (continued)

C

![Graph showing [60mer]_bound / [4A]_hexamer vs. [60mer]_free \(\mu M\).]

D

![Graph showing [30mer]_bound / [4B]_hexamer vs. [30mer]_free \(\mu M\).]
one 30-mer binds tightly per hexamer followed by weaker binding of a second DNA (Figure 14B). Figure 5C shows the titration of 4A' protein with 60-mer ssDNA. One 60-mer binds tightly to 2 hexamers (0.5 DNA / 1 hexamer, shown in inset to 14C), which is consistent with the results of the titration of 60-mer DNA with increasing 4A' concentrations (Figure 11D). The final stoichiometry of 60-mer binding is the same as that for 10-mer and 30-mer DNA binding, with two 60-mer DNAs bound per 4A' hexamer at high DNA concentrations. Figure 14D shows the titration of 4B protein with 30-mer ssDNA. As seen for 4A', two ssDNAs bind per 4B hexamer, one with higher affinity than the other.

The binding data for 10-mer and 30-mer DNAs were fit to equations derived for a model in which two DNAs bind per 4A' hexamer with differing affinities (Eqn. 2, 3; Materials and Methods). The fit to the 10-mer titration data provided dissociation constants of 1.3 x 10^{-7} M and 1.3 x 10^{-5} M, for the high-affinity and low-affinity DNA binding site, respectively (Table 1). Similar dissociation constants were obtained from curves fit to the 30-mer binding data (1 x 10^{-7} M and 1.4 x 10^{-5} M). These results indicate that one DNA binds 4A' hexamer with about 100-fold higher affinity than a second DNA strand. The 60-mer data were fit to a model assuming DNA binding to a dodecamer rather than a hexamer. The $K_a$ for one 60-mer bound to 4A' was estimated to be 1.4 x 10^{-7} M (the same as for the interaction of one 10-mer or 30-
Table 1: Equilibrium constants for ssDNA binding to 4A' protein

<table>
<thead>
<tr>
<th>DNA</th>
<th>$K_d$ (Site 1) (M)</th>
<th>$K_d$ (Site 2) (M)</th>
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<tbody>
<tr>
<td>10-mer</td>
<td>$1.3 \times 10^{-7}$</td>
<td>$1.3 \times 10^{-5}$</td>
</tr>
<tr>
<td>30-mer</td>
<td>$1.0 \times 10^{-7}$</td>
<td>$1.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>60-mer</td>
<td>$1.4 \times 10^{-7}$</td>
<td>N/A</td>
</tr>
</tbody>
</table>
mer DNA with 4A’). The subsequent Kₐ values were difficult to determine because of the lack of clear transition points between phases in the 60-mer binding isotherm.

Nuclease Protection of ssDNA.

Titrations performed with various lengths of single-stranded DNA indicate that the stoichiometry of DNA binding to 4A’ changes when the length is increased from 30 to 60 bases. The 30-mer binds one hexamer with high affinity, while the 60-mer binds two 4A’ hexamers or one dodecamer. Thus, it appears that the DNA binding site on the hexamer is centered around a length of 30 bases. The size of the DNA binding site was directly determined by S7 nuclease protection of a uniformly radiolabeled poly(dT) DNA bound to 4A’ or 4B protein. The protected DNA lengths were analyzed on a denaturing 20 % polyacrylamide gel shown in Figure 15. The smallest length of DNA protected by 4A’ protein (Figure 15A) is 30 ± 5 nucleotides (determined from a standard plot of the markers; Figures 15C and 15D for 4A’ and 4B, respectively), and the next band is 53 ± 5 bases in length. 4B protein also protects a minimal length of 30 ± 5 bases and the next band is 55 ± 5 bases in length (Figure 15B). Thus, both 4A’ and 4B proteins have a binding site about 25-35 bases in length, which likely reflects binding of one hexamer (30-mer DNA binds hexamer with a 1:1 ratio). The subsequent band of protected DNA reflects binding of two
Figure 15. Nuclease protection of poly(dT) by 4A' and 4B proteins. Nuclease protection of a uniformly radiolabeled poly(dT) DNA (0.2 μM) by 4A' and 4B proteins (in the presence of 1 mM MgdtMP-PCP) was assayed as described in Materials and Methods. Panel A shows DNA protected from S7 endonuclease by 4A' protein, analyzed on a 20 % polyacrylamide/7 M urea gel. The products were visualized by autoradiography, and the lanes correspond to the following: lane 1, DNA markers V (lengths indicated on left of gel); lane 2, DNA markers: 30-mer, 40-mer, and 60-mer; lane 3, full length poly(dT); lanes 4-7, nuclease-protected DNA products from reactions containing 10, 20, 30, and 40 μM 4A' protein. Panel B shows DNA protected by 4B protein. The lanes represent the following samples: lane 1, DNA markers V; lanes 2-4, DNA markers: 10-mer, 30-mer, and 60-mer; lanes 5-8, nuclease-protected DNAs from reactions containing 5, 10, 20, and 30 μM 4B protein. Panel C, and panel D show standard curves with relative mobilities of marker DNAs plotted versus log of the molecular sizes, for the gels in panels A, and B, respectively. The smallest length of DNA protected by both 4A' and 4B proteins is 30 ± 5 nucleotides. The subsequent DNA is 53 ± 5 bases and 55 ± 5 bases in length, for 4A' and 4B, respectively.
Figure 15 (continued)

C

\[ y = 69.721 + -33.294x \quad R = 0.98396 \]

D

\[ y = 50.723 + -25.274x \quad R = 0.99261 \]
hexamers next to each other on the DNA. The gels show that hexamers or dodecamers are capable of stacking on the long poly(dT) DNA resulting in formation of a ladder of protected DNA species.

Binding of Duplex DNA to 4A' Protein.

DNA helicases catalyze DNA unwinding at the fork junction where they may bind the single-stranded or double-stranded regions of the fork, or both. There is evidence that *E. coli* Rep and DnaB helicases function by interacting with both ss and ds DNA (review: Lohman, 1993). In order to test whether T7 helicases also interact with dsDNA, a hairpin duplex was titrated with increasing concentrations of 4A' protein, under various conditions, as shown in Figure 16A. In the absence of nucleotide ligands, duplex DNA does not bind 4A' to any measurable extent, which is analogous to the interaction between ssDNA and 4A'. In contrast to ssDNA binding, however, in the presence of Mg\textsubscript{2+}TMP-PCP a maximum of only 20% of hairpin DNA is bound at protein concentrations as high as 20 \muM. In the presence of Mg\textsubscript{2+}TDP, duplex DNA does not appear to bind to 4A' protein. It is not possible to determine the stoichiometry of duplex DNA binding to 4A' from this assay because of incomplete DNA binding.

The hairpin binding isotherm is puzzling because it saturates at 20% rather than increase gradually with increasing 4A' concentration, as expected for a weak binding
Figure 16. Duplex DNA binding to increasing concentrations of 4A' protein. Interaction of 4A' with double stranded DNA was investigated by equilibrium binding assays carried out with hairpin duplex DNA and double-stranded 40-mer DNA, in the presence and absence of nucleotides (Materials and Methods). Panel A shows 5'-32P hp duplex DNA (1 μM) titrated with increasing concentrations of 4A' protein (0-20 μM) in the presence of 1 mM MgTDP-PCP (●), 5 mM dTDP (◆), and in the presence of Mg2+ alone (O). Panel B shows titration of 0.2 μM 40-mer dsDNA with 4A' (0-12 μM) in the presence of 1 mM MgTDP-PCP. The binding isotherms show fraction of total DNA bound, plotted versus 4A' monomer concentration. DNA binding was also measured by fluorescence quenching of a fluorescein-tagged duplex DNA upon binding to 4A'. The assay was performed with 17-merF ssDNA (●), and 26-merF duplex DNA (◆), and fluorescence quenching was plotted as percent of maximum quenching versus protein concentration.
complex. It almost seems that only a fraction of 4A' or DNA is capable of interaction, while the remaining species cannot undergo binding. To test the possibility that there are various populations of 4A', due to differences in assembly of hexamers in the reaction, the assay was performed by mixing the components of the reaction in two different ways: 4A' was mixed with DNA first and then MgdTMP-PCP was added to initiate binding, or 4A' was added last to a mixture of DNA and MgdTMP-PCP in solution. Both experiments yielded identical binding isotherms with 20% maximum hp binding.

The hairpin duplex has a 3-nucleotide loop which may introduce an artifact in binding. The assays were performed with a duplex of 40-base-long complementary ssDNAs to eliminate the 3-base loop structure. Again, only about 20% binding was observed for the 40-mer duplex (Figure 16B), indicating that the loop does affect the interaction of 4A' with hairpin DNA. The hairpin duplex forms a 1:1 ratio of hairpins to intermolecular hybrids (shown in Figure 16A) in the reaction. To eliminate this difference in DNA populations, a homogenous hairpin stock solution was prepared by heating and slowly cooling hp DNA at a low concentration (0.1 μM). Native PAGE was used to confirm that the DNA was purely in hairpin form. Nitrocellulose membrane binding assays performed with the pure hairpin DNA showed the same extent of binding (15-20%) as seen previously,
indicating that 4A' does not prefer binding to one form of the duplex over the other. It is possible that the 4A'*duplex DNA complex dissociates during filtration through the nitrocellulose membrane or during the washing process, although elimination of washing did not alter the binding isotherm.

Duplex DNA binding to 4A' was also measured by fluorescence binding assays. A 26-mer DNA with a 3'-fluorescein tag was annealed to a complementary non-fluorescent 26-mer DNA to form fluorescently labeled duplex DNA. The DNA was titrated with 4A' protein using the same conditions as for the assay with fluorescent single-stranded DNA. A maximum of only 10 % of duplex DNA fluorescence was quenched on addition of 4A' protein, as compared to 23 % fluorescence quenching with ssDNA (Figure 16C). This may mean that duplex DNA binding saturates with only about 50 % of DNA bound by 4A'. It is also possible that the difference in the extent of quenching is due to differing interactions of 4A' with dsDNA and ssDNA. If so, 10 % quenching could reflect complete binding of duplex DNA to 4A' protein.

Although the interaction between 4A' and duplex DNA is not clearly understood from these experiments, it is obvious that 4A' binds duplex DNA either nonspecifically or with a lower affinity compared to ssDNA. Therefore, competitive binding of dsDNA and ssDNA to 4A' has been examined to
directly measure the relative affinities of 4A' protein for ssDNA and dsDNA.

Competitive Binding of ssDNA and dsDNA at Constant DNA and Increasing 4A' Concentrations.

Competitive binding of 60-mer ssDNA and hp duplex DNA to 4A' protein is shown in Figure 17. Radiolabeled 60-mer and radiolabeled hp DNA were used in separate experiments to monitor the 4A'•60-mer or 4A'•hairpin complex, respectively. Radiolabeled 60-mer was titrated with 4A' in the absence and in the presence of an equimolar amount of unlabeled hairpin DNA. The two 60-mer binding curves are indistinguishable, indicating that under these conditions, the hairpin does not compete with 60-mer DNA for binding to 4A' protein. Radiolabeled hairpin DNA was titrated similarly, in the absence and presence of an equimolar amount of 60-mer ssDNA (Figure 17). In the absence of 60-mer, 4A' binds 20-25 % of hairpin duplex as expected from the earlier results (Figure 16A). However, in the presence of 60-mer DNA, the hairpin binding isotherm is sigmoidal. Comparison of the curves reveals that 4A' binds duplex DNA only after 60-mer binding is saturated. Thus, under the conditions of this assay, hairpin duplex does not compete with 60-mer ssDNA for the same binding site. About 20 % of duplex DNA, however, does bind to 4A' simultaneously with ssDNA.
Figure 17. Competitive binding of ssDNA and dsDNA to increasing concentrations of 4A' protein. Binding assays were performed in the presence of 1 mM MgTMP-PCP, as described in Materials and Methods. The binding curves, (O) and (▲), show titrations of 5'-32P 60-mer ssDNA (0.2 µM) and 5'-32P hp duplex (0.2 µM) with increasing amounts of 4A' protein (0-10 µM), respectively. Competitive binding of 60-mer and hp DNA was measured by preincubating the two DNAs prior to addition of 4A' protein. (□), shows binding of 5'-32P 60-mer (0.2 µM) to 4A' protein, in the presence of nonradiolabeled hp duplex (0.2 µM). Similarly, (●), shows competitive binding of 5'-32P hp duplex (0.2 µM) to 4A' protein in the presence of unlabeled 60-mer ssDNA (0.2 µM).
Competitive Binding of ssDNA and dsDNA at Constant 4A' and Increasing DNA Concentrations.

4A' was titrated with increasing concentrations of hairpin in the presence of a constant amount of 60-mer DNA. The experiment was performed with radiolabeled hairpin to monitor the 4A'•hairpin complex and with radiolabeled 60-mer to monitor the 4A'•60-mer complex. Titration of 4A' with increasing hp DNA showed that up to four hairpin bind per hexamer (Figure 18A). When the same titration was carried out in the presence of a constant amount of unlabeled 60-mer, hairpin binding had a distinct sigmoidal character compared to binding in the absence of 60-mer. The complementary experiment performed with radiolabeled 60-mer showed that 4A' binds the 60-mer to completion (one 60-mer per dodecamer), even at 20-fold higher hp concentrations. Similar experiments carried out with 30-mer ssDNA yielded identical results, confirming that ssDNA is an effective competitor of duplex DNA.

Competitive DNA binding titrations at constant 4A' and increasing 60-mer concentrations, in the absence and in the presence of a constant amount of hairpin DNA, are shown in Figure 18B. 60-mer DNA binding is unaffected by the presence of hairpin DNA, while the hairpin is easily competed off by increasing concentrations of 60-mer.

The above results indicate that duplex DNA binds at the same site on 4A' as ssDNA and thus, is readily competed off by ssDNA, or they may indicate that ssDNA binding induces a
Figure 18. Competitive binding of ssDNA and dsDNA at constant 4A' concentrations. Competitive binding assays, at constant 4A' and increasing DNA concentrations, were conducted in the presence of 1 mM MgđTMP-PCP as described in Materials and Methods. Panel A shows titrations of 4A' (2 μM) with increasing concentrations of 5'-32P hp DNA (0-10 μM) in the absence of 60-mer (Ο) and in the presence of unlabeled 60-mer (□), and the complementary titration of 4A' with increasing amounts of unlabeled hp DNA in the presence of 0.5 μM 5'-32P 60-mer (●). Similarly, panel B shows titrations of 4A' (2 μM) with increasing concentrations of 5'-32P 60-mer in the absence of hp DNA (Ο), and in the presence of 0.5 μM unlabeled hp DNA (□), and the titration of 4A' (2 μM) with increasing concentrations of unlabeled 60-mer DNA in the presence of 0.5 μM 5'-32P hp DNA (●). Panel C shows titration of 2 μM 4A' protein with 0-10 μM unlabeled 60-mer DNA in the presence of 8 μM 5'-32P hp duplex DNA. In each case, the fraction of labeled DNA bound per hexamer is plotted versus total hp or total 60-mer DNA concentration.
Figure 18
conformational change in the protein that is unfavorable for stable interaction between duplex DNA and 4A'. The affinity of 4A' for duplex DNA is much lower than that for ssDNA, however, simultaneous binding of ssDNA and dsDNA suggests that duplex DNA may bind to other sites on 4A' in the presence of ssDNA (Figure 17 and Figure 18A). An experiment, similar to that shown in Figure 18B, was performed to investigate if the four hairpins per hexamer, observed in Figure 18A, were bound specifically at DNA binding sites on the hexamer. All four hairpin DNA bound to 4A' (hp = 8 μM, the concentration at which four strands bind per hexamer; Figure 18A) were competed off with increasing amounts of 60-mer DNA (Figure 18C). Binding of three hairpin DNAs was blocked by 0.5 μM 60-mer DNA, and the fourth hairpin DNA was competed off with about 2 μM 60-mer. At these concentrations only one ssDNA strand binds per hexamer (Figure 14D).

It appears that single-stranded DNA binding is strongly favored at the high affinity site (all hp are competed off by one strand of 60-mer DNA), and duplex DNA binding to 4A' may be a nonspecific interaction. It is also possible that duplex DNA binds to the second weak binding site detected in the titration of 4A' with increasing amounts of ssDNA (Figure 14). Four or more duplex DNA bind to the hexamer, indicating possible weak interaction with six sites on the hexamer. Native PAGE experiments showed that hairpin DNA
does not stabilize hexamers to the same extent as ssDNA, and only about 5% hairpin DNA is bound to the hexamer species on the gel as seen on the autoradiogram (Figure 13A and 13C). If duplex binding does not stabilize hexamers sufficiently, the complexes may be too weak to stay intact on nitrocellulose membranes, which may also explain the low percent of hairpin binding to 4A' seen in the membrane binding assay.

Cross-Linking of Helicase Hexamers Bound to M13 ssDNA.

It is clear now that T7 helicases bind DNA in the form of hexamers, as demonstrated by the membrane binding assays and native PAGE experiments. Previous electron microscopy studies have shown that the hexamer is a ring-shaped structure with a hole in the center that is 25-30 Å in diameter, which is large enough to accommodate single-stranded or double-stranded DNA. Therefore, a cross-linking experiment was designed to test whether the hexameric ring binds DNA through the hole or on the outside. Scheme 1 depicts the experiment performed with circular M13 ssDNA. 4A' or 4B proteins were assembled on the DNA in the presence of MgTMP-PCP, and the subunits were chemically cross-linked with a bifunctional cross-linking agent. If 4A' hexamers assemble around DNA, then cross-linking the subunits should topologically link the hexamer and circular M13 DNA. Testing the complex under denaturing conditions should distinguish between a 4A'•DNA complex formed with DNA outside the ring,
Scheme 1. Cross-linking of 4A' hexamer on M13 ssDNA
Figure 19. Cross-linking of 4A' hexamers around to circular M13 ssDNA and linear ssDNA. Interaction of 4A' protein with ssDNA was examined by protein-protein cross-linking in the presence of ssDNA. 4A' protein (0-30 μM) was assembled on circular M13 DNA (0.07 μM), in the presence of 200 μM MgTMP-PCP, and either incubated at room temperature or cross-linked with DMS, as described in Materials and Methods. The samples were quenched and electrophoresed on a 1% nondenaturing agarose gel containing MgTMP-PCP (panel A), or heat-denatured and electrophoresed on a 1% agarose gel containing SDS (panel B). Lanes 1 and 5 show free M13 DNA; lanes 2-4, and 6-8, show M13 DNA bound to 5, 15, and 30 μM uncross-linked and cross-linked 4A' protein, respectively. A similar experiment was performed with uniformly radiolabeled linear ssDNA. Panel C shows the autoradiogram of samples electrophoresed in a 1% nondenaturing gel containing MgTMP-PCP, and panel D shows samples that were heat-denatured and electrophoresed in a 1% gel containing 0.1% SDS. Lanes 1 and 4, free linear ssDNA; lanes 2 and 3, show ssDNA bound to 15 and 30 μM uncross-linked protein, respectively; lanes 5 and 6 show the same, except with cross-linked protein.
Figure 19
in which case 4A' should dissociate from DNA, and a 4A'•DNA complex with DNA inside the ring, in which case the DNA should be trapped with the ring. Figure 19A shows that under non-denaturing conditions, the electrophoretic mobility of M13 DNA shifts with increasing amounts of uncross-linked or cross-linked 4A' protein, indicating stable interaction between M13 and 4A'. Figure 19B shows the same reactions (as in Figure 19A), heat denatured and analyzed on a gel containing SDS. As expected, the uncross-linked protein dissociates from DNA, and M13 in lanes 2 - 4 has the same mobility as free M13 (lane 1). The cross-linked protein, however, remains trapped on the circular DNA as evidenced by the shifted electrophoretic mobility of M13. Thus, ssDNA binds through the hole in the hexameric ring (Scheme 1). The same experiment was performed also with linear ssDNA. Figure 19C shows the autoradiogram of a native gel analysis of uncross-linked and cross-linked protein bound to radiolabeled linear ssDNA. The linear DNA is gel-shifted by both protein species, similar to circular M13 DNA (Figure 19A). Under denaturing conditions (Figure 19D), both uncross-linked and cross-linked protein hexamers dissociate from DNA. Since the ssDNA is linear, cross-linked protein rings probably slip off the ends during electrophoresis, as would beads on a linear string. The experiment with linear DNA is an important control to show that the cross-linking procedure does not covalently link the protein to DNA. The
same results were seen with 4B protein, demonstrating that hexamers of both T7 helicases bind DNA through the hole in the ring.

Electron Microscopy of Circular M13 ssDNA Bound to 4A' and 4B Proteins.

Gene 4 Proteins Bound to M13 ssDNA.

Gene 4 proteins were assembled on M13mp8 ssDNA in the presence of Mg²⁺-PCP and visualized by negative-staining electron microscopy. The experiments and analysis were carried out by Dr. Egelman and Dr. Yu, at University of Minnesota, and have been published previously (Egelman et al., 1995). Both 4A' and 4B form rings that stack on the entire length of M13 DNA, as shown in Figure 20A (4A') and Figure 20C (4B). About 250 protein rings bind per M13 circle (7229 bases), with a 96-102 Å separation between 4A' rings and a 90 Å separation between 4B rings; each hexamer covers 29 bases of DNA. Interestingly, the rings bind DNA cooperatively even though there appears to be no direct interaction between rings. Cooperativity may arise from changes in DNA structure following binding of one ring leading to further binding of rings in the same area. Figures 20B and 20D show averages of 1000 images of 4A' and 4B, respectively, which reflect side views of the hexameric rings. The hexamer ring, which spans a length of 80 Å, has a distinctly polar shape, with a large-ring domain and a
Figure 20. Electron micrographs of 4A' and 4B proteins assembled on circular M13 ssDNA. Panels A and C show electron micrographs of M13mp8 ssDNA circles covered with 4A' and 4B hexameric rings, respectively. (Bar in panel A = 500 Å). Groups of three adjacent rings were marked off, aligned, and averaged. The averages of 4A' and 4B rings are shown in panels B and D, respectively. (Bars in panels B and D = 100 Å). Electron microscopy was performed as described (Egelman et al., 1995).
Figure 20
small-ring domain. Each monomer is shaped like a lopsided dumbbell, with a large domain connected by a hinge to a smaller domain, which gives rise to the two-tiered appearance of the ring.

Polarity of Hexamer Binding to ssDNA.

Figures 20B and 20D (above) also indicate that the three rings in the frame bind DNA with the same directionality. In order to determine the structural polarity of the rings with respect to the polarity of DNA, linear dsDNA molecules were partially digested with exonuclease III to create 5′ ssDNA tails on both ends of linear dsDNA (shown schematically in Figure 21). 4B rings were assembled on the DNA and visualized by electron microscopy (Figure 21). As expected from the DNA binding studies described earlier, the hexamers bind only to the single-stranded regions of the partially duplex DNA. Image analysis showed that all the hexameric rings bind DNA with the small domain pointed toward the 5′-end (inset in Figure 21).

A three dimensional image of the 4B protein ring has been prepared (Figure 22) from the top and side views obtained by negative staining electron microscopy. A complete structure shows the arrangement of the dumbbell-shaped monomers forming the two-tiered ring structure of the hexamer. The figure also shows a half-ring with a rod
Figure 21. Polarity of 4B rings on ssDNA. φX174 dsDNA was linearized and digested with exonuclease III to create duplex DNA with single-stranded 5' tails as shown in the schematic. Binding of 4B to the DNA, in the presence of MgDPMP-PCP, formed structures with two protein-covered ssDNA tails with a duplex region in between, as shown in the electron micrograph (duplex region indicated by arrow). The polarity of the rings was determined by averaging 800 of the rings on the ssDNA tail. The inset shows the averaged image of the ring, in which the smaller domain is at the 5'-end of the DNA. Preparation of DNA substrate and electron microscopy were carried out as described (Egelman et al., 1995).
Figure 22. Three-dimensional reconstruction of the 4B protein ring. A three dimensional image of the 4B protein ring was constructed as described (Egelman et al., 1995). The hexamer is composed of a small ring stacked on a large ring; it binds DNA through the hole in the center with the small ring facing the 5'-end of the DNA (shown in the half-ring image).
depicting the ssDNA in the hole with the 5'-end toward the small domain of the ring.

UV Cross-linking of 4B Protein to ssDNA.

DNA binding to the helicase was examined further to determine the number of subunits of the ring that interact with DNA. 4B protein was assembled on radiolabeled (dT)$_{20}$ in the presence of MgTMP-PCP and uv cross-linked to the DNA for varying times. The protein species linked to DNA were resolved on an SDS polyacrylamide gel (Figure 23A). At shorter cross-linking times the monomer is predominantly linked to DNA. As time of cross-linking increases, (dT)$_{20}$ gets cross-linked to two, three, or four subunits, simultaneously. Figure 23B shows a plot of the oligomer species cross-linked to DNA versus time of cross-linking. A fit of the cross-linking data (see Materials and Methods) shows that DNA cross-links to one subunit at a rate of 0.3 min$^{-1}$, the second subunit is cross-linked at a rate of 0.1 min$^{-1}$, followed by cross-linking of the third subunit at a rate of 0.1 min$^{-1}$, and so on. Thus, DNA is cross-linked to the first subunit with a 3-fold faster rate compared to cross-linking to the following subunits. These results suggest that DNA binds tightly to one subunit and that the interaction between DNA and the remaining subunits is much weaker.
Figure 23. Photo-cross-linking of 4B protein to ssDNA. Interaction of the ring subunits with DNA was investigated by protein-DNA cross-linking. 4B protein (5 μM) was incubated with MgTMP-PCP (0.5 μM) and 5'-32P(dT)20 DNA (3 μM) and uv cross-linked at 254 nm for varying times as described in Materials and Methods. Panel A shows an image (PhosphorImager; Molecular Dynamics) of the 3-10 % SDS polyacrylamide gradient gel used to resolve the 4B species cross-linked to DNA. The time of cross-linking and the oligomeric state of 4B is indicated. Panel B shows quantitation of each species cross-linked to DNA plotted as % of hexamer cross-linked versus cross-linking time. The solid lines that fit the data are simulated curves for consecutive linking of DNA to one subunit at a time, forming cross-linked 4B species ranging from monomer to tetramer. The simulated curves yield rate constants of 0.3 min⁻¹ for cross-linking to monomer, and 0.1 min⁻¹ for all subsequent cross-linking events that form dimer, trimer and tetramer species.
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Figure 23
Figure 23 (continued)

B

% Hexamer Cross-linked

0 10 20 30 40 50

Time (minutes)

0 1 2 3 4 5 6 7 8

Monomer

Dimer

Trimer

Tetramer
This result was corroborated by electron microscopy experiments performed with 4B protein in the presence of 30-mer ssDNA (Dr. Egelman, and Dr. Yu at University of Minnesota). Top views of the protein were obtained in the absence of DNA and averaged (Figure 24A). Similarly, top views of 4B assembled on 30-mer DNA in the presence of Mg\textsubscript{2}TMP-PCP were obtained and averaged (Figure 24B). Comparison of the two figures shows that there is a considerable increase in density within the central hole in the presence of 30-mer DNA. The original images, with and without DNA, were sorted, based on the strength of their six-fold rotational power. Images with the strongest six-fold power were aligned and averaged as shown in Figure 24C (without DNA) and 24D (with DNA). Now the density in the hole appears clearly one sided, perhaps in between two subunits. The dark patches show regions where statistical differences in the density between the two images in the absence and in the presence of DNA are greater than 4\sigma. Both uv cross-linking and electron microscopy data show that single-stranded DNA binds assymetrically to either one or two subunits of the hexameric ring.
Figure 24. Top views of 4B rings in the absence and presence of ssDNA. Electron microscopy, image averaging, and image analysis techniques were used to determine the effect of ssDNA on hexameric rings of 4B helicase (Yu et al., 1996). Panel A shows an average of 999 4B rings. Panel B shows an average of 999 4B rings in the presence of 30-mer ssDNA. Panel C shows an average of 50 rings of 4B protein that were selected for strong six-fold power, and Panel D shows an average of 50 4B rings bound to 30-mer ssDNA, which were selected for strong six-fold power. In the presence of ssDNA there is an increase in density within the hole of the ring, which appears distinctly off-centered. The dark marks in panel D indicate areas where the difference in density between rings with and without DNA is greater than 4σ.
dTTP and dTMP-PCP Binding Facilitate T7 Helicase Hexamerization and DNA Binding.

T7 gene 4 proteins form stable ring-shaped hexamers in the presence of nucleotide ligands and single-stranded DNA. The current study shows that hexameric 4A' protein binds ssDNA with high affinity in the presence of Mg-dTMP-PCP. In the absence of nucleotides, the protein does not bind DNA, and in the presence of dTDP, only about 20% ssDNA binding is detectable. Previous studies have shown that dTMP-PCP is a suitable analog of dTTP by measuring its ability to promote helicase hexamer formation (Patel & Hingorani, 1993; Chapter II). Oligomeric properties of the helicases are the same in the presence of dTTP or dTMP-PCP. In this study, the ability of dTMP-PCP to promote or stabilize DNA binding has been compared with that of dTTP. As mentioned earlier, DNA binds to the helicase with high affinity/stability in the presence of Mg-dTMP-PCP. In the presence of dTTP, however, the complex is less stable and only about 40% DNA binding can be detected. The helicase•DNA complex may exhibit lower stability with dTTP because of dTTP hydrolysis (to dTDP) occurring under the conditions of the binding assay (DNA binds weakly to the helicase in the presence of dTDP). Nevertheless, both dTTP and dTMP-PCP facilitate DNA binding to a greater extent than
dTDP. These results indicate that interaction of helicase with DNA is modulated by nucleotide ligand binding and hydrolysis such that affinity for DNA is higher in the presence of dTTP and comparatively lower in the presence of dTDP. Such modulation provides a "switch", which may be the basis for a mechanism of helicase movement on DNA coupled to NTP binding and hydrolysis.

4A' and 4B proteins form hexamers in the presence of nucleotides and ssDNA (Patel & Hingorani, 1993; Chapter II). Single-stranded DNA binds mainly to the hexameric form of the T7 helicases as shown by nitrocellulose membrane binding assays and native PAGE. On the native gel various 4A' oligomers can be resolved, but upon addition of ssDNA, all smaller oligomers disappear and the hexamer species becomes predominant. This phenomenon suggests that the protein exists as a mixture of oligomers, but assembles into hexamers on the DNA. Formation of DNA-binding hexamers appears to be a common property among several known helicases. The SV40 large T antigen helicase assembles on DNA as hexamers or double hexamers (Dean et al., 1992) and E. coli DnaB protein (Bujalowski & Jezewska, 1995), and E. coli Rho RNA/DNA helicase (Wang & von Hippel, 1993) also form hexamers that bind DNA (or RNA in the case of Rho).
T7 Helicases Bind ssDNA with High Affinity.

Interaction of T7 helicase with small single-stranded DNAs has been examined in detail by equilibrium binding assays. Each hexamer can bind one strand of DNA with high affinity \( (K_d = 1 \times 10^{-7} \text{ M}) \) and another strand with a 100-fold lower affinity \( (K_d = 1 \times 10^{-5} \text{ M}) \). Thus, there are at least two DNA binding sites on the helicase hexamer. *E. coli* Rho RNA/DNA helicase binds six RNAs, 10-nucleotides in length. Three are bound with high affinity and the other three are bound with lower affinity (Geiselmann et al., 1992; Wang & von Hippel, 1993). In comparison, the T7 helicase binds only two DNAs (10-base to 60-base lengths), but the common feature of high affinity and low affinity sites, although not understood, may be mechanistically significant. It should be noted that the second, low affinity binding site on the helicase is unrelated to the primase DNA-binding site of 4A', because 4B protein, which lacks primase activity and the zinc-finger DNA binding domain, also binds two DNAs with high and low affinities as 4A' protein.

T7 helicase binds at the fork junction to catalyze duplex DNA unwinding. At the fork, the helicase may bind only to ssDNA strands or double-stranded DNA, or both. *E.coli* Rep helicase binds to both ss and ds DNA, and its interactions with both DNAs are modulated by ATP binding and hydrolysis (Das et al., 1980; Arai et al., 1981; Wong & Lohman, 1992; Wong et al., 1992). Similarly, *E.coli* DnaB can
bind both ss and ds DNA, although it has higher affinity for ssDNA in the presence of ATP (Arai & Kornberg, 1981d). DNA binding to SV40 T antigen has been tested directly with a synthetic fork and DNA footprinting assays, and the helicase appears to interact with both ss and ds regions of the fork (SenGupta & Borowiec, 1992; Wessel et al., 1992).

Duplex DNA binding to T7 helicase has been tested in this study; both equilibrium binding assays and native PAGE show that dsDNA binds very weakly to the helicase as relative to ssDNA, and no duplex DNA binding can be detected by electron microscopy. The weak interaction (in nitrocellulose membrane binding assays) is detectable only in the presence of MgTMP-PCP, and no binding occurs in the absence of nucleotide or in the presence of dTDP. Double-stranded DNA can be competed off with ssDNA (at concentrations where ssDNA binds only to the high affinity site); thus, the high affinity site favors ssDNA over dsDNA. But, an experiment in which 4A' is titrated with increasing amounts of hp shows that at high concentrations duplex DNA binds to hexamers simultaneously with ssDNA (Figure 18A). Thus, duplex DNA may interact with the second, low affinity DNA binding site on the hexamer. It is not clear, however, whether simultaneous binding of dsDNA and ssDNA to 4A' is any more or less favorable than binding of two ssDNAs.
Helicase Hexamers Form Rings that Assemble Around ssDNA.

The T7 helicase hexamer is ring-shaped with a hole in the center of the ring that is 25-30 Å in diameter. In the presence of nucleotide triphosphate, the hexamer assembles on ssDNA so as to completely encircle the DNA within the central hole. Electron microscopy studies demonstrate that the hexameric ring does not bind around double-stranded DNA, although details of the interaction at the fork junction itself cannot be visualized. The hexameric ring has a two-tiered structure with a small-ring and a large-ring domain. The entire structure has a height of about 80 Å and an outer diameter of about 130 Å. 4A' and 4B proteins appear to have DNA binding sites about 30 bases in length, as detected from nuclease protection assays and equilibrium DNA binding. Electron micrographs also show that the hexamer covers about 30-bases of ssDNA. Therefore, the DNA binding site of the helicase spans 30 bases or less on the inside of the hexameric ring. UV cross-linking studies and image analysis of electron micrographs show that ssDNA preferentially binds to one or two subunits of the hexamer. All the above results can be incorporated into a model that describes translocation of the helicase on DNA, which is essential for understanding duplex DNA unwinding (Scheme 2).
DNA Binding to T7 Helicases Modulated by dTTP Binding and Hydrolysis: A Model for Helicase Translocation on ssDNA.

T7 helicases bind DNA in the presence of dTTP and dissociate from DNA upon dTTP hydrolysis to dTDP. If all subunits were to bind and release DNA coupled with dTTP binding and hydrolysis, the continuous dissociation and DNA-rebinding events would prohibit processive movement of the helicase on DNA. On the other hand, if the hexamer binds DNA via only one or two subunits at a given time (as observed for T7 helicase), then, as shown in Scheme 2, the cycle of dTTP binding and hydrolysis can allow each unit to bind and release DNA in a consecutive manner, effecting movement on a long DNA substrate without complete dissociation. The ring-shape of the hexamer also provides topological stability to the complex which most certainly enhances processivity.

This model demands some means of providing directionality to the translocation of T7 helicases which appear to unwind duplex DNA only in the 5'-3' direction (Tabor & Richardson, 1981; Matson et al., 1983). One clear determinant of directionality is the polarity with which the helicase hexamer binds ssDNA, with the small ring of the hexamer always facing the 5'-end of the DNA. *E.coli* RuvB protein, which has the same gross three-dimensional structure as T7 helicase (small ring connected to a large ring; Stasiak et al., 1994), also unwinds DNA in the 5'-3' direction (Tsaneva et al., 1993). Another means for imposing directionality may be interaction with the duplex DNA at the
Scheme 2. Translocation of hexameric helicase on ssDNA (A)
fork junction. Because T7 helicase hexamer encircles one strand of ssDNA, it is difficult to imagine how the same DNA binding site (on the inside of the ring) can simultaneously interact with the duplex DNA region of the fork. However, it is entirely possible that the helicase binds duplex DNA on the outer surface of the ring, which directs movement in one direction only. If such an interaction needs to be weak or transient, it may explain the results observed for duplex DNA binding in the equilibrium binding assays and native PAGE.

Translocation on ssDNA can lead to duplex DNA unwinding in a "passive" or an "active" manner. To effect passive unwinding, the helicase may simply track on ssDNA and take advantage of thermal breathing at the fork-junction to move onto exposed single-stranded regions, thus unwinding duplex DNA. In an active unwinding mechanism, in addition to tracking on DNA, the helicase may employ other interactions (e.g. with the second ssDNA strand or the dsDNA at the fork-junction) to cause melting of hydrogen bonds and unwind duplex DNA.

Comparison of 4A' and 4B with Other Hexameric Helicases.

Details of structure and DNA binding properties are available for only a few other helicases. *E.coli* DnaB forms a hexameric ring (outer diameter = 140 Å; inner diameter = 40 Å) which, in contrast to T7 helicases, appears to have
equal propensity for 3-fold or 6-fold symmetry (San Martin et al., 1995; Yu et al., 1996). Although side views of DnaB are not yet available, from the top views it appears that each subunit has a lopsided dumbbell-shape similar to that of T7 helicases, indicating that the DnaB ring also has a two-tiered structure. Earlier reports, based on proteolysis patterns, have also indicated that the DnaB monomer unit has two discrete domains, one larger than the other (Nakayama et al., 1984). Recently, it has also been shown that the DNA binding site of DnaB is approximately 20 bases, and ssDNA binds to one subunit with high affinity in the presence of AMP-PNP, the nonhydrolyzable analog of ATP (Bujalowski & Jezewska, 1995). It is not known whether DNA binds through the hole in the DnaB ring, and details of the mechanism of unwinding are not understood for either enzyme, but based on the common features between T7 gene 4 proteins and E. coli DnaB, it is likely that these helicases share a common mechanism for DNA unwinding.

Electron microscopy studies of SV40 large T antigen show that this helicase forms double hexameric rings that may assemble around duplex DNA (Mastrangelo et al., 1989; 1994). The T antigen appears to be different from T7 gene 4 proteins in that it interacts stably with both single-stranded and double-stranded regions of forked DNA. There are conflicting reports on whether the helicase interacts with both ssDNA strands at the fork, and if it binds DNA
through the ring, or on the outside (SenGupta & Boroweic, 1992; Wessel et al., 1992). It should be noted that SV40 large T antigen has multiple functions. It is essential for initiation complex formation at the SV40 origin of DNA replication and it interacts with replication proteins such as DNA polymerase and ssDNA binding protein (review: Fanning & Knippers, 1992; Dean et al., 1987). T antigen also binds tumor supressor proteins, p53 and retinoblastoma gene product, to promote cellular transformation (Weinberg, 1991). It is possible that the requirements imposed by various functions are the cause for the differences in DNA binding properties of T antigen compared to T7 helicases.

*E. coli* RuvB helicase also shares striking structural similarity with 4A and 4B proteins. The hexamer has a two-tiered ring-shaped structure with a small domain and a large domain. However, like SV40 large T antigen, RuvB forms stable double hexamers that bind around double-stranded DNA in the presence of ATP (Stasiak et al., 1994). Moreover, RuvB requires RuvA protein in order to bind DNA and catalyze duplex DNA unwinding (Tsaneva et al., 1993). The interactions between substrate and helicase in RuvB-catalyzed DNA unwinding may also vary from those of T7 DNA helicases because the primary function of RuvB involves branch migration at Holliday junctions during DNA recombination (review: West, 1994).
Models for Translocation of Helicase on DNA.

Specific models for helicase-catalyzed translocation and unwinding have been proposed for *E.coli* Rep helicase and *E.coli* Rho transcription termination factor, which is an RNA/DNA helicase. The model discussed here for T7 hexameric helicases is quite different from that envisioned for *E.coli* Rep helicase. A rolling type mechanism has been proposed for the dimeric helicase, in which ATP promotes helicase binding to ssDNA and dsDNA simultaneously and ATP hydrolysis facilitates unwinding. Following dissociation of ADP, Rep remains bound to ssDNA via one subunit of the dimer, and the next ATP binding event allows Rep to bind the next length of dsDNA to continue the cycle (Wong & Lohman, 1992). Both models are again different from that proposed for *E. coli* Rho RNA/DNA helicase-catalyzed unwinding, where it appears that all six subunits of the hexamer bind RNA and it wraps around the outside of the hexameric ring. The subunits bind RNA with alternating high and low affinities, coupled to ATP binding and hydrolysis, in order to effect translocation (Geiselmann et al., 1993). Although the specific steps proposed for the unwinding pathways of the helicases are different, the basic theme which involves DNA or RNA "bind-release" steps coupled to NTP binding and hydrolysis, is common to these enzymes. It may be possible that helicase hexamers function as trimers of dimeric units whose mechanism is comparable to that of dimeric helicases. A better understanding of the mechanism is necessary, however,
before the two classes of enzymes can be compared. As observed earlier, the helicases discussed here appear to have different primary functions. T7 helicases are essential for DNA replication, Rep protein is important for DNA repair, and Rho is a transcription termination factor; and these differences may account for variations in the unwinding mechanism.
CHAPTER IV
INTERACTIONS OF BACTERIOPHAGE T7 HELICASES WITH NUCLEOTIDE LIGANDS

PART A: CHARACTERIZATION OF THE NUCLEOTIDE BINDING SITE

ABSTRACT

Bacteriophage T7 helicase/primase protein, 4A, has a conserved, glycine-rich Walker A sequence (G/AXXGXGKT/S) that is thought to be involved in nucleotide binding. In this study, the lysine residue in the Walker A sequence was mutated to an alanine to understand its importance in helicase and primase functions. The mutant protein, 4A'/K318A, exhibited lower dTTPase activity compared to 4A'. The $K_m$ value was 3 to 5-fold higher, and the $k_{cat}$ was about 100-fold lower than that of 4A'. The mutant protein assembled into hexamers and bound DNA in the presence of MgdpTTP, but it was incapable of DNA binding in the presence of MgdpTMP-PCP. The primase activity of 4A'/K318A was only 1.5 to 2.5-fold lower than 4A'; however, the mutant did not have any detectable DNA unwinding activity. Mixing of 4A' and 4A'/K318A resulted in formation of heterooligomers that had lower DNA-stimulated dTTPase activity and helicase
activity, but increased rates of DNA-independent dTTPase activity. Loss of DNA-stimulated dTTPase activity was dependent on mixing time and 4A'/K318A concentration, and was detectable only when the two proteins were mixed in the absence of nucleotide and DNA. Interestingly, the mixed hexamers bound DNA in the presence of MgTP-PcP, even though 4A'/K318A did not bind DNA under the same conditions. Thus, a few active subunits per helicase hexamer are sufficient for stable interaction with DNA. A fully active hexamer is required for DNA-stimulated dTTPase activity and helicase activity.

INTRODUCTION

DNA helicases catalyze nucleotide triphosphate hydrolysis that is coupled to translocation on DNA and duplex DNA unwinding. Bacteriophage T7 gene 4 helicases hydrolyze a number of nucleotides including ATP, dATP, dGTP, and dTTP with varying efficiency (Patel et al., 1992). dTTP is used preferentially by T7 proteins for both primase and helicase activities (Matson & Richardson, 1983). Previous studies have shown that nucleotide binding is necessary for hexamer formation and DNA binding. Furthermore, DNA binding is modulated by NTP binding and hydrolysis.

Nucleotide binding proteins contain a conserved glycine-rich sequence (Walker A sequence), G/AXGXGKT/S,
that appears to be essential for nucleotide binding (Walker et al., 1982). This sequence is present in T7 gene 4 proteins and is conserved among all known helicases (Gorbalenya & Koonin, 1993). High-resolution structures of nucleotide binding proteins such as the F₁-ATPase (Abrahams et al., 1994), elongation factor Tu (Jurnak, 1985; La Cour et al., 1985; Kjeldgaard & Nyborg, 1992), ras p21 protein (Tong et al., 1991), RecA protein (Story & Steitz, 1992), adenylate kinase (Muller & Schulz, 1992), and elongation factor G (Czworkowski et al., 1994) show that motif A forms a flexible loop that lies between a β-strand and α-helix. Amino acids in the loop interact with the phosphate moiety on the nucleotide. For example, the lysine residue in ras p21 motif A interacts with β,γ-phosphates of a GTP analog (Bourne et al., 1991). Mutation of an analogous lysine residue in the RAD3 helicase resulted in reduced NTPase activity (Sung et al., 1988).

In this study, lysine-318 in the putative nucleotide binding sequence of 4A', was mutated to an alanine to investigate the importance of this conserved residue for dTTPase, helicase, and primase activities of 4A'. The mutant protein, 4A'/K318A, was purified and its biochemical properties were investigated. The mutant was also mixed with 4A' protein to study both heterooligomer formation and its effect on 4A' wild-type activities.
MATERIALS AND METHODS

Nucleotides, DNA, and Other Reagents.

All dNTPs and rNTPs were purchased from Sigma Chemicals Co., and dTMP-PCP was purchased from Amersham Life Science Inc. Radiolabeled nucleotides [α-³²P]dTTP (3000 Ci/mmol), [γ-³²P]ATP (4000 Ci/mmol), [α-³²P]dCTP (3000 Ci/mmol), and [α-³²P]CTP (3000 Ci/mmol) were purchased from ICN Biomedicals. Synthetic 17-merF, 30-mer, and 60-mer single-stranded oligodeoxynucleotides (5'-60-merF, 3'-60-mer, and 60-mer.h), and M13 ssDNA were prepared and purified as described in Chapter III. The fluorescent cross-linker SAED (sulfo-N-succinimidyl [[2-(7-azido-4-methylcoumarin-3-acetamido)ethyl]dithio]propionate) was purchased from Pierce. Immobilon-P transfer membrane was purchased from Millipore, and PEI-cellulose TLC plates were purchased from EM Separations Technology. Sephadex G-25 was purchased from Sigma Chemicals Co.

Enzymes.

4A' and 4B proteins were purified as described (Patel et al., 1992; Hingorani & Patel, 1996). 4A'/K318A was prepared by changing the lysine-318 codon, AAG, to GCG (alanine) using Kunkel's method of site-directed mutagenesis (Ng, 1993; Patel et al., 1994; Kunkel et al., 1991). The mutant protein was overexpressed and purified as described
for 4A' and 4B proteins. T7 gene 5 exo' protein and thioredoxin were purified as described (Patel et al., 1991). Chicken egg albumin and β-galactosidase were purchased from Sigma Chemicals Co.

Measurement of dTTPase Activity.

DNA-dependent dTTPase activity of both 4A' and 4A'/K318A proteins was measured, at 22 °C and 37 °C, by monitoring hydrolysis of [α-32P]dTTP to [α-32P]dTDP + P. Samples (25-μl total volume) contained 4A' or 4A'/K318A proteins (0.5-5 μM), which were preincubated with 100 μM dTTP and M13 DNA (50 nM), in dTTPase buffer A (50 mM Tris-acetate, pH 7.5, 50 mM sodium acetate, 1 mM DTT, 0.1 mg/ml BSA, and 10 mM magnesium acetate), for 5-10 min. Reactions were initiated with dTTP (0.1 to 8 mM) + [α-32P]dTTP. 5 μl aliquots were removed at intervals between 1 to 5 min (4A' protein) or 2 to 60 min (4A'/K318A protein) and quenched with an equal volume of 0.5 M EDTA, pH 8.0.

The quenched solutions were analyzed by spotting 1 μl aliquots on a poly(ethylenimine)-cellulose thin-layer chromatography plate (PEI-cellulose TLC), which was developed in 0.3 M potassium phosphate buffer, pH 3.4. [α-32P]dTTP and [α-32P]dTDP were quantitated on a Betagen Betascope 603. Molar amounts of dTDP were plotted versus
reaction time to determine initial rates of dTDP formation. The rates were plotted versus dTTP concentrations and a hyperbolic fit of the data (KaleidaGraph Software) yielded the steady-state $K_m$ and $k_{cat}$ (maximal rate/protein monomer concentration) values. Errors were determined from at least 3 separate experiments, and have been reported as standard errors of the mean.

Effect of Mixing Time on dTTPase Activity of 4A'/K318A and 4A' Protein Heterooligomers.

The effect of mixing time on the dTTPase activity of 4A'/K318A-4A' heterooligomers was investigated by mixing the proteins, a), in the absence of ligands, b), after preincubating each protein with Mg:dTTP, and c), after preincubating each protein with Mg:dTTP and M13 ssDNA. 4A' and 4A'/K318A proteins (4 μM each) were preincubated separately in binding buffer A without magnesium (50 mM Tris-acetate, pH 7.5, 5 mM sodium acetate, 1 mM DTT), in buffer with 10 mM magnesium acetate and 80 μM dTTP, and in buffer containing magnesium acetate (10 mM), dTTP (80 μM) and M13 ssDNA (50 nM). Equal volumes of the protein solutions (10 μl) were mixed and incubated at 22 °C for varying times (0.5 to 30 min). After each mixing time, dTTPase reaction was initiated by addition of an equal volume (20 μl) of a mixture containing dTTP (5 mM final
concentration), [\(\alpha^{32}\text{P}\)]dTTTP (10 \(\mu\)Ci per assay), and M13 ssDNA (50 nM final concentration). The reactions were continued and quenched at times between 1 to 4 min and analyzed as described above. dTTPase rates of the mixed proteins were normalized to the dTTPase rate of 4A' (measured in the absence of mutant protein) and plotted versus mixing times.

dTTPase Activity of 4A' and 4B in the Presence of Increasing Concentrations of 4A'/K318A.

4A' (or 4B) and 4A'/K318A proteins were mixed in the absence of ligands, and dTTPase activity of the heterooligomers was measured, a), in the absence of DNA, b), in the presence of 30-mer ssDNA, and c), in the presence of M13 ssDNA. 4A', or 4B (1 \(\mu\)M), was incubated with 4A'/K318A (0-10 \(\mu\)M) in dTTPase buffer without magnesium for 15 min (22°C). This was followed by addition of dTTTP (80 \(\mu\)M) and magnesium acetate (10 mM) without DNA, with 30-mer (10 \(\mu\)M), or with M13 ssDNA (50 nM). After further incubation for 10 min, the reaction was initiated by addition of dTTTP (5 mM) and [\(\alpha^{32}\text{P}\)]dTTTP (10 \(\mu\)Ci). Aliquots were removed and quenched with EDTA (0.25 M final concentration) after time intervals ranging from 2 to 60 min (in the absence of DNA), and from 2 to 10 min (in the presence of 30-mer or M13 DNA). dTTPase rates were determined as described above and plotted versus 4A'/K318A concentration.
A control assay was performed in which 4A' (1 μM) and 4A'/K318A (0-2 μM) were each allowed to form stable hexamers by preincubation (15 min) with magnesium acetate (10 mM), dTTP (80 μM) and M13 DNA (50 nM) prior to mixing. Following mixing and additional incubation for 5 min, the reactions were initiated, and dTTPase activity was measured as described above.

Equilibrium DNA Binding.

DNA binding to 4A' and 4A'/K318A proteins was measured as described (Hingorani & Patel, 1993; Chapter III). 4A' or 4A'/K318A proteins (0-30 μM) were mixed with 5'-32P 30-mer (1 μM), in the presence of 1 mM dTMP-PCP or 5 mM dTTP, in binding buffer A. The samples were incubated for 20 min (with dTMP-PCP) or 1 min (with dTTP) prior to filtration through the NC-DEAE membrane assembly. Radioactivity on the membranes was quantitated, and the fraction of total DNA bound to protein was calculated and plotted versus protein concentration.

Fluoresence-based binding assays were also used to quantitate the interaction between the proteins and DNA. A fluorescent oligodeoxynucleotide (17-merF) was incubated with increasing concentrations of protein (4A' or 4A'/K318A) in binding buffer A (filtered through a 0.2 μm filter) and fluorescence quenching was measured as described earlier.
(Chapter III). Maximal quenching, obtained from DNA binding
to 4A' in the presence of MgdTMP-PCP, was assigned a value
of 100; DNA binding under other conditions was quantitated
and plotted as percent fraction of maximum quenching versus
protein concentration.

Equilibrium DNA Binding to Heterooligomers of 4A' and
4A'/K318A.

Mixed oligomers of 4A' and 4A'/K318A were assayed for
DNA binding at constant DNA and increasing protein
concentrations. The proteins were mixed in 1:1, 1:2, and 1:3
ratios, in binding buffer A without magnesium, and
preincubated for 15 min prior to use in the binding assay.
Increasing concentrations of the mixed protein (0-30 μM)
were incubated with 1 μM 5'–32P 30-mer DNA (15 min), in the
presence of 1 mM dTMP-PCP and 10 mM magnesium acetate, and
DNA binding was measured using the NC-DEAE membrane assay
described above. The same assay was also performed with 4A'
and 4A'/K318A proteins that were preincubated with MgdTMP-
PCP prior to mixing.

Primase Activity of 4A' and 4A'/K318A Proteins.

The primase activity of 4A' and 4A'/K318A proteins was
assayed by measuring kinetics of RNA primer synthesis. 4A'
or the mutant protein (1 μM) were incubated (1-2 min) with
500 μM ATP, 500 μM CTP, [α-32P]CTP (10 μCi), and 6 mM dTTP in dTTPase buffer (22 °C). The reaction was initiated by addition of M13 template DNA (50 nM) and aliquots were removed at varying times (0-30 min) and quenched with equal volumes of EDTA (200 mM) plus denaturing dye solution (95 % formamide and 0.05 % bromophenol blue). The samples were electrophoresed on a 25 % polyacrylamide (TBE)/3 M urea sequencing gel (Bio-Rad, 35 x 43 cm, 0.2-mm thickness), at 100 W. Electrophoresis was stopped after the bromophenol blue dye had migrated half-way through the gel. Unreacted CTP and the RNA products were quantitated on the Betascope, and total RNA products were plotted versus time of reaction.

Helicase Activity.

DNA unwinding activity of the mutant was measured by the primer-displacement assay and also by the RNA-primed DNA synthesis assay. The primer-displacement assay was performed as described (Patel et al., 1992), by analyzing 4A' (0.5 μM) or 4A'/K318A protein (2.5 μM)-catalyzed displacement of a 5'-32P 60-base DNA strand (60-mer) from an M13•60-mer DNA complex (25 nM) in the presence of dTTP (5 mM). Free, radiolabeled 60-mer was resolved on a 12 % native polyacrylamide gel and visualized on the Betascope.

The RNA-primed DNA synthesis assay was performed to measure both primase and helicase activities of 4A' and the
mutant protein. 4A' (1 μM) or 4A'/K318A (1 μM) was preincubated for 2-4 min with 1 μM T7 DNA polymerase (1:20 ratio of exo' gene 5 protein and thioredoxin), 1 mM each of dATP, dCTP, dGTP, ATP and CTP, 6 mM dTTP, and [α-32P]dCTP (10 μCi) in dTTPase buffer. Reactions were initiated by addition of M13 ssDNA (50 nM) and quenched with EDTA, pH 8.0 (200 μM final concentration), after intervals ranging from 5 to 60 min. A control reaction was performed minus 4A' protein to measure gene 4 protein-independent DNA synthesis in the reaction.

Products of DNA synthesis were analyzed both by DE81-filter binding assays and by denaturing agarose gel electrophoresis. 5 μl aliquots of the quenched reaction were spotted on DE81 filter paper circles (Whatman). The filters were air-dried, washed gently (four washes) with 0.3 M ammonium formate buffer, pH 8.0 (to remove unreacted [α-32P]dCTP), and rinsed in methanol. The filters were air-dried again, and radioactivity on washed as well as unwashed filters was measured by scintillation counting. The molar amount of radiolabeled dCMP incorporated into DNA was calculated and plotted versus reaction time. For analysis by denaturing gel electrophoresis, 5 μl aliquots of the reaction were mixed with alkaline gel loading dye (50 mM NaOH, 1 mM EDTA, 3 % Ficoll 400, and 0.05 % bromophenol
blue) and loaded on a 0.6 % alkaline agarose gel (15 x 30 cm) prepared in alkaline buffer (50 mM NaOH and 1 mM EDTA). Electrophoresis was carried out in alkaline buffer for 10 h (4 °C, 400 mA constant current), and DNA products were analyzed on a Betascope.

Primase and helicase activities of mixed oligomers of 4A' and 4A'/K318A were also analyzed by the above assay. A constant amount of 4A' protein (0.2 μM) was mixed with 4A'/K318A (0-2 μM), in binding buffer A without magnesium, and incubated for 15 min at 22 °C. The protein mix was added to buffer containing T7 polymerase, dNTPs, rNTPs, and magnesium acetate (see above). DNA synthesis was initiated by adding 50 nM M13 ssDNA and analyzed as described above.

Reversible Cross-Linking of Proteins using SAED.

Protein-protein cross-linking was performed to test formation of mixed oligomers of 4A' and 4A'/K318A. All steps in the experiment were carried out in the dark. A stock solution (150 μl) of fluorescent 4A'/K318A mutant protein was prepared by cross-linking 100 μM protein with 100 μM SAED for 1 h (22 °C) in buffer containing 40 mM HEPES, pH 8.0, and 20 mM sodium acetate. Unreacted SAED was removed by gel-filtration through a 2 ml Sephadex G-25 column (the column was prepared by connecting two 1 ml tuberculin-syringe barrels, blocking the lower outlet with glass wool,
and loading the column with sephadex G-25 equilibrated overnight in HEPES buffer). Two drop fractions of the eluate were collected, protein content was assayed using the BioRad protein assay dye concentrate, and the peak protein-containing fractions were pooled. Fluorescent 4A'/K318A protein (20 μM) was mixed with 5 or 10 μM 4B, 10 μM chicken egg albumin, and 10 μM β-galactosidase, in separate reactions, and incubated at 22 °C for 10 min. Magnesium acetate (10 mM) and dTMP-PCP (1 mM) were added to the reactions, which were incubated for 5 min prior to photo-cross-linking by irradiation at 366 nm (UVGL-25 Mineralight lamp at a distance of 2 cm for 1 h at 4 °C). Following irradiation, the initial disulfide link was cleaved by incubating the reaction mix with 100 mM DTT for 20 min. The samples were mixed with SDS gel loading dye (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2 % SDS, 0.1 % bromophenol blue, and 10 % glycerol) and electrophoresed on a 9 % SDS-polyacrylamide gel (Laemmli, 1970) at 20 mA constant current. The proteins were blotted onto an Immobilon-P membrane using a Janssen SemiDry Electroblotter (electroblotting performed as recommended in the manual provided by the manufacturer). The membrane was air-dried and the fluorescent proteins were visualized by excitation at 366 nm and photographed.
RESULTS

Preparation of the K318A Mutant of 4A' Protein.

Amino acid residues in the conserved Walker motif A interact with the phosphate moiety of nucleotide ligands as shown for elongation factor-Tu (Figure 25; adapted from Berchtold et al., 1993). Motif A in gene 4 proteins has a conserved lysine residue (318) which may interact with the phosphate groups of nucleotide ligands (Gorbalenya & Koonin, 1993). Site-directed mutagenesis was used to substitute lysine-318 with a smaller, neutral amino acid, alanine. The mutant protein, 4A'/K318A, was overexpressed and purified, and its biochemical properties were tested in vitro to investigate the importance of the lysine residue and the conserved nucleotide-binding loop. 4A'/K318A was prepared, purified, and partly characterized by Winnie Ng as part of her Senior Honors Thesis project at The Ohio State University (Ng, 1993; Patel et al., 1994).

dTTPase Activity of the 4A'/K318A Protein.

Steady-state parameters of the dTTPase activities of 4A'/K318A and 4A' proteins were measured at different temperatures: 22 °C and 37 °C. In the absence of ssDNA, the mutant protein hydrolyzed dTTP with a rate constant lower than 0.001 s⁻¹ at 22 °C (k_cat for 4A' = 0.02 s⁻¹). dTTPase
Figure 25. Schematic diagram showing the hydrogen-bonding interactions between the conserved nucleotide-binding motif A of Elongation factor-Tu (T. thermophilus) and nucleotide triphosphate. Analogous interactions can be expected between the conserved motif A in T7 helicase (G-317, K-318, S-319) and its nucleotide substrate.
Table 2: Steady-state kinetic parameters of M13 ssDNA-stimulated dTTPase activity of 4A' and 4A'/K318A proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Temperature (°C)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A'</td>
<td>37</td>
<td>16.5 ± 1.0</td>
<td>1.7 ± 0.3</td>
<td>9.7</td>
</tr>
<tr>
<td>4A'</td>
<td>22</td>
<td>2.8 ± 0.24</td>
<td>0.89 ± 0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>4A'/K318A</td>
<td>37</td>
<td>0.19 ± 0.04</td>
<td>4.2 ± 0.8</td>
<td>0.045</td>
</tr>
<tr>
<td>4A'/K318A</td>
<td>22</td>
<td>0.04 ± 0.01</td>
<td>4.3 ± 0.03</td>
<td>0.01</td>
</tr>
</tbody>
</table>
activity was also measured in the presence of M13 ssDNA. Table 2 shows the steady-state $k_{cat}$, $K_m$, and $k_{cat}/K_m$ values for 4A' and 4A'/K318A-catalyzed dTTP hydrolysis measured at 22 °C and 37 °C. The mutant protein has 5-fold and 3-fold higher $K_m$ values than 4A', at 22 °C and 37 °C, respectively. The $k_{cat}$ is about 80-fold lower than that of 4A' at both temperatures, and the $k_{cat}/K_m$ ratio of 4A'/K318A is 200 to 300-fold lower than that of 4A'.

dTTPase activities were measured at increasing concentrations of ssDNA to check whether DNA concentration was the limiting factor in the reaction. DNA-stimulated dTTPase rates increased up to 50 nM DNA, and remained constant at higher DNA concentrations. All assays have been performed with 50 nM M13 ssDNA to measure the maximum rates of dTTP hydrolysis. dTTPase activity was also measured for varying 4A'/K318A concentrations to test for protein concentration-dependent activation. Both $k_{cat}$ and $K_m$ values were constant over a wide range of protein concentrations tested (0.5 to 4 μM).

DNA Binding Properties of 4A'/K318A Mutant Protein.

The ability of the mutant protein to bind ssDNA was measured by equilibrium membrane binding assays as well as fluorescence-based DNA binding assays. Figure 26A shows results of an NC-DEAE membrane binding experiment performed
Figure 26. Equilibrium binding of 4A' and 4A'/K318A proteins to ssDNA. Equilibrium DNA binding assays were performed at 22 °C using NC-DEAE filters or fluorescent DNA as described in Materials and Methods. A, shows the titration of a constant amount of 5'-32P 30-mer (1 μM) with increasing concentrations of 4A' in the presence of 1 mM Mg2TMP-PCP (●), and with increasing concentrations of 4A'/K318A in the presence of 1 mM Mg2TMP-PCP (■) and 5 mM Mg2TTP (♦). The fraction of total DNA bound is plotted versus protein monomer concentration. B, shows a similar assay performed by measuring fluorescence quenching of 17-merF DNA (0.3 μM), which was titrated with increasing amounts of 4A' (0-3 μM) in the presence of 0.5 mM Mg2TMP-PCP (●) and 2 mM Mg2TTP (♦), and with 4A'/K318A (0-3 μM) in the presence of 0.5 mM Mg2TMP-PCP (□) and 2 mM Mg2TTP (○). The isotherm shows percent of maximal fluorescence quenching plotted versus protein monomer concentration.
Figure 26

% Fluorescence quenching

[B]_{bound} / [B]_{total}

[Protein] μM

0 5 10 15 20 25 30

0 0.2 0.4 0.6 0.8 1
with a constant amount of 30-mer DNA and increasing amounts of 4A' and 4A'/K318A protein. 4A' binds 30-mer DNA with high affinity in the presence of MgdTMP-PCP; the mutant protein, however, does not appear to bind DNA under the same conditions. The mutant protein does bind up to 60% DNA in the presence of MgdTTP.

Similar results were obtained from titration of the fluorescent DNA (Figure 26B). In this assay, only about 30% DNA is bound by 4A'/K318A in the presence of MgdTTP, which is still more than the amount of DNA bound in the presence of MgdTMP-PCP. The 4A'/K318A-DNA complex appears quite stable in the presence of MgdTTP, but much weaker in the presence of MgdTMP-PCP.

Oligomeric Properties of the Mutant Protein.

HPLC gel-filtration experiments were performed to examine oligomerization of 4A'/K318A mutant protein under various conditions. The elution profiles of 4A'/K318A protein have been published previously (Ng, 1993). Low concentrations (5 μM) of the mutant protein formed monomer or dimer species in the absence of nucleotide ligands. As protein concentration was increased, the peak broadened and eluted faster, indicating the presence of a mixture of dimers and higher oligomers. In the presence of MgdTTP, the mutant protein (at low concentration) formed a mixture of dimers and hexamers, and as protein concentration was
increased, predominantly hexamers were formed (as seen for
4A' protein with Mg\textsubscript{2}TTP and Mg\textsubscript{2}TMP-PCP). About 8 to 10-fold
higher concentrations of mutant protein were necessary for
stable hexamer formation, relative to 4A' protein. The
requirement for higher 4A'/K318A concentration may be due to
subsaturating amount of Mg\textsubscript{2}TTP in the experiment (K\textsubscript{m} of
4A'/K318A for dTTP is about 5 mM). The presence of dTTP +
ssDNA further stabilized 4A'/K318A hexamers showing that
oligomeric properties of the mutant protein are similar to
those of 4A'.

Primase Activity of the Mutant Protein.

The 4A' helicase/primase protein requires dTTP in order
to recognize primase-binding sites on DNA and synthesize
complementary RNA primers (5'-pppACCC, 5'-pppACCA, 5'-
pppACAC). It is not known whether dTTP hydrolysis is
required for primase activity. Because 4A'/K318A is
defective in dTTPase activity, but binds stably to ssDNA in
the presence of dTTP, the mutant was used to examine the
role of dTTP binding and hydrolysis in primase activity. The
primase activity of 4A' and the mutant proteins was measured
by monitoring incorporation of radiolabeled CTP into RNA
primers synthesized on M13 ssDNA template.

Figure 27 shows the products of RNA primer synthesis
activity of 4A' (panel A) and 4A'/K318A proteins (panel B)
resolved by gel-electrophoresis. The mutant protein is
Figure 27. Steady-state primase activity of 4A' and 4A'/K318A proteins. Panels A and B show the RNA primers synthesized by 4A' and 4A'/K318A proteins, respectively (resolved on a 25% polyacrylamide (TBE)/3 M urea gel). Lanes 1-10 show products of reactions that were quenched at 1, 2, 4, 8, 10, 15, 20, 25, 30, and 0 minutes. The radiolabeled RNA species, pppAC, pppACC/A, pppACC/AC/A, and unreacted [α-32P]CTP, are indicated on the gel. Panel C shows quantitation of total RNA (all species), which were synthesized by 4A'(●) and 4A'/K318A (▲) with steady-state rate constants of 0.055 s⁻¹ and 0.037 s⁻¹, respectively.
Figure 27
Figure 27 (continued)
active as a primase and the level of primer synthesis appears to be comparable to that of the wild-type 4A' protein. The dimer, pppAC, is the major product in both cases, and the only major differences are that 4A' preferentially synthesizes one trimer over the other (pppACA or pppACC), and only two of three possible tetramers are detectable in the 4A' reaction (pppACCC, pppACCA and pppACAC). Quantitation of total RNA in the reaction shows that 4A' synthesizes RNA with an initial rate constant of 0.055 s\(^{-1}\), which is about 1.5-fold higher than the rate constant of the mutant protein, 0.037 s\(^{-1}\) (panel C). There is an initial lag in 4A'/K318A-catalyzed RNA synthesis; however, over time the total amount of RNA reaches the level of RNA synthesized by 4A' protein. Rate of RNA synthesis is finally limited by depletion of dTTP in both reactions. Primase activity of the mutant was tested over a range of protein concentrations (0.01 \(\mu\)M to 1 \(\mu\)M). At all concentrations, primase activity of the mutant was only about 2-fold lower than that of 4A'. Thus, it appears that dTTP hydrolysis may not be required for primase activity.

Primase and Helicase Activities of 4A' and 4A'/K318A Proteins.

The mutant protein exhibits normal primase activity; therefore, the RNA-primed DNA synthesis assay can be used to examine its helicase activity. The assay is based on
synthesis of small RNA primers on circular M13 ssDNA template. These primers are extended by T7 DNA polymerase, which synthesizes the complementary strand of DNA. The longest DNA product is 7 kb, the size of the template DNA. If an active helicase is present in the reaction, it unwinds the newly synthesized duplex DNA, allowing the polymerase to synthesize longer, rolling circle DNA products (Scheme 3). Figure 28A shows an autoradiogram of an alkaline agarose gel used to resolve DNA products synthesized in the presence of 4A' and 4A'/K318A activity. A control reaction shows DNA synthesized in the absence of any gene 4 proteins. DNA products (7 kb or shorter), which are facilitated by primase activity, are found in both 4A' and 4A'/K318A-containing reactions (DNA products in the control reaction are likely due to contaminating primers from the M13 DNA preparation that are extended by the polymerase). DNA products above 7 kb (upto 20 kb and longer), which are facilitated by helicase activity (Scheme 3), are detectable only in the reaction containing 4A' protein. The absence of long DNA products in the 4A'/K318A-containing reaction indicates that the mutant lacks helicase activity.

The DNA products facilitated by primase activity were quantitated by filter binding assay, and the rate of DNA synthesis was determined from the molar amount of radiolabeled dCMP incorporated into DNA in the presence of 4A' and 4A'/K318A proteins, as shown in Figure 28B.
Scheme 3. RNA-primed DNA synthesis assay
Figure 28. RNA-primed DNA synthesis assay of 4A' and 4A'/K318A proteins. Panel A shows the products of RNA-primed DNA synthesis facilitated by 4A' and 4A'/K318A proteins (1 μM), and DNA products synthesized in a control reaction with no gene 4 protein (resolved on a 0.6 % alkaline agarose gel). Lanes 1-6 show products of reactions quenched after 5, 10, 15, 20, 30, and 60 minutes of reaction. The sizes of marker DNAs are indicated on the gel. Panel B shows quantitation of total DNA synthesis by DE81 filter binding assays (see Materials and Methods); the molar amount of dCMP incorporated into DNA (after correction for DNA products in the control reaction) is plotted versus reaction time for 4A' (●) and 4A'/K318A (□). The rate constants for DNA synthesis facilitated by 4A' and 4A'/K318A are 0.2 s⁻¹ and 0.08 s⁻¹, respectively.
<table>
<thead>
<tr>
<th>4A'</th>
<th>4A’/K318A</th>
<th>control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1 μM)</td>
<td>(1 μM)</td>
<td></td>
</tr>
<tr>
<td>1  2  3  4  5  6</td>
<td>1  2  3  4  5  6</td>
<td>1  2  3  4  5  6</td>
</tr>
</tbody>
</table>

>21 kb

7 kb

Figure 28
Figure 28 (continued)

B

![Graph showing the incorporation of dCMP over time (in minutes). The graph plots dCMP incorporated (in µM) against time (in minutes). The data points are marked with black circles and white squares.](image)

```latex
\begin{figure}
\centering
\includegraphics[width=\textwidth]{graph.png}
\caption{Graph showing the incorporation of dCMP over time (in minutes).}
\end{figure}
```
(corrected for DNA synthesized in the control reaction). The rate of DNA synthesis with 4A' protein is 0.2 s⁻¹, which is about 2.5-fold higher than the rate of synthesis in the presence of 4A'/K318A, which is 0.08 s⁻¹. This relationship remains constant when tested over a wide range of protein concentrations (0.05 to 1 μM), and also agrees well with the results of the direct primer synthesis assays, which showed that the mutant protein has only 1.5 to 2-fold lower primase activity compared to 4A'.

The mutant protein does not exhibit DNA unwinding activity when examined by the primase-dependent helicase assay. This result was confirmed by a direct primer-displacement assay which showed that 4A' efficiently unwinds a primer annealed to M13 ssDNA but the mutant is unable to unwind the primer even at 5-fold higher concentration than 4A' (Figure 29).

Heterooligomer Formation of 4B and 4A'/K318A Proteins.

A reversible cross-linking experiment was used to investigate heterooligomer formation between the wild-type and mutant proteins. The assay requires separation of the two proteins by mass; therefore, the cross-linking experiment was performed with 4A'/K318A (63 kDa) and 4B (56 kDa) proteins. 4A'/K318A was covalently modified with the sulfo-N-succinimidyl ester group of SAED, a fluorescent cross-linking agent, via formation of amide linkages.
Figure 29. Primer-displacement assay for helicase activity of 4A' and 4A'/K318A proteins. Unwinding activity of both 4A' (0.5 μM) and 4A'/K318A (2.5 μM) proteins was examined by helicase-catalyzed displacement of a 5'-32P 60-mer from an M13·60-mer complex as described in Materials and Methods. Lane 1 on the gel shows heat-denatured DNA. Lanes 2-6 and 7-11 show reactions of 4A' and 4A'/K318A, respectively, quenched after 5, 10, 15, 20, and 30 minutes.
between the linker and lysine residues in the protein. The fluorescent mutant protein was mixed with 4B and photo-cross-linked. As explained in Scheme 4, the cross-linker attached to a 4A'/K318A subunit can link with a neighbouring subunit by a light-catalyzed reaction. Following cross-linking, a disulfide link on the fluorescent linker was reduced with high concentration of DTT, resulting in transfer of the fluorescent label from a 4A'/K318A subunit to an associated subunit.

Figure 30 shows the fluorescent proteins resolved on an SDS-polyacrylamide gel. In the absence of other proteins, 4A'/K318A protein cross-links to itself (lane 1). When 4A'/K318A is mixed with 4B protein, the fluorescent tag is transferred from the mutant to 4B protein (lanes 2 and 3). Lanes 4 and 5 show results of control experiments performed with fluorescent 4A'/K318A mixed with β-galactosidase and chicken egg albumin, respectively. A very small amount of the fluorescent tag is transferred to these control proteins, indicating that transfer does not occur efficiently without specific association between proteins, and also that there is no significant amount of free SAED cross-linker in the reaction. Thus, 4B and 4A'/K318A form heterooligomers, and because 4A' and 4B proteins have similar properties, it can be derived that 4A' and 4A'/K318A proteins also form mixed oligomers.
Scheme 4. SAED-mediated cross-linking of heterooligomers
Figure 30. Reversible cross-linking of 4A'/K318A to 4B protein using SAED. 4A'/K318A was chemically cross-linked to SAED, and free SAED was separated from the protein by gel-filtration. Fluorescent 4A'/K318A protein (20 μM) was mixed with 4B and the control proteins, chicken egg albumin and β-galactosidase, allowed to form stable hexamers and photoscross-linked. The linker was cleaved and the fluorescent proteins were resolved on a 9 % SDS-polyacrylamide gel and photographed. Lane 1 shows 4A'/K318A protein cross-linked to itself; lanes 2 and 3 show 4A'/K318A cross-linked to 5 and 10 μM 4B protein, respectively; lanes 4 and 5 show cross-linking of 4A'/K318A to 10 μM β-galactosidase (MW: 116,250) and chicken egg albumin (MW: 45,000), respectively.
Helicase Activity of 4A' and 4A'/K318A Heterooligomers.

The helicase activity of heterooligomers of 4A' and 4A'/K318A was examined by the RNA-primed DNA synthesis assay. Figure 31 shows the DNA products synthesized in the presence of a constant amount of 4A' mixed with increasing concentrations of the mutant protein. 4A'-catalyzed unwinding facilitated synthesis of rolling-circle DNA products (> 7 kb), as expected. When increasing amounts of 4A'/K318A protein were added to the reaction and allowed to mix with 4A', there was a substantial reduction in DNA unwinding activity. In the presence of 5-fold higher mutant protein, 4A' helicase activity became undetectable. M13 single-stranded DNA and dTTP were present in excess in the reactions to avoid reduction in helicase activity due to limiting substrate. The mutant protein had a dominant effect on 4A' helicase activity, indicating that heterooligomers containing a few dTTPase-defective subunits cannot unwind DNA.

Effect of Incubation Time on dTTPase Activity of Mixed 4A' and 4A'/K318A Proteins.

The effect of mixing of 4A'/K318A and 4A' on DNA-stimulated dTTPase activity was investigated to understand the loss of unwinding activity upon heterooligomer formation. An experiment was designed to examine the DNA-stimulated dTTPase activity of mixed oligomers formed under
Figure 31. RNA-primed DNA synthesis activity of 4A' in the presence of increasing concentrations of 4A'/K318A. RNA-primed DNA synthesis products of 4A' (0.2 μM), in the presence of increasing amounts of 4A'/K318A (0-2 μM), were resolved on 0.6 % alkaline agarose gels. The two proteins were mixed in the absence of ligands and assayed as described in Materials and Methods. The five lanes in each experiment correspond to reactions quenched after 5, 10, 20, 30, and 60 min. The sizes of marker DNAs and concentrations of 4A'/K318A protein are indicated on the gel.
various conditions, and to test if the effect of mixing was time dependent. Equal amounts of 4A' and 4A'/K318A proteins were mixed for increasing times in the absence and in the presence of ligands (MgdTTP and DNA), followed by measurement of dTTPase rates. Figure 32 shows that when the proteins were each incubated in the absence of ligands and then mixed, there was a time dependent loss of about 50 to 60 % of 4A' dTTPase activity. When the proteins were preincubated with MgdTTP prior to mixing, only about 10 to 20 % decrease in dTTPase activity was detectable. Most strikingly, when the proteins were preincubated with both MgdTTP and M13 ssDNA prior to mixing, no decrease in activity occurred even after 30 minutes of mixing.

The rapid decrease in dTTPase activity of 4A' ($t_{1/2} = 20$ s), when mixed with 4A'/K318A in the absence of ligands, can be explained by formation of heterooligomers that are not as active, or inactive compared to 4A' protein. This result is consistent with the fact that both 4A' and 4A'/K318A exist as a mixture of oligomers and unstable hexamers, in the absence of ligands (gel-filtration experiments: Patel & Hingorani, 1993; Chapter II), and can easily form mixed hexamers. The residual dTTPase activity (~40 %) may be due to pure 4A' hexamers, and some partially active mixed hexamers. 4A' and the mutant protein form stable hexamers in the presence of MgdTTP and DNA, which explains the relatively small decrease in dTTPase activity when the
Figure 32. Time-dependent decrease in dTTPase activity of 4A' mixed with 4A'/K318A. The two proteins 4A' and 4A'/K318A (1 μM) were preincubated separately under different conditions, and the DNA-stimulated dTTPase activity of the mixed proteins was assayed as described in Materials and Methods. The plot shows dTTPase activity of the mixed proteins with each protein preincubated in the absence of ligands (●), in the presence of Mg[dTTP] (▼), and in the presence of Mg[dTTP] and M13 ssDNA (♦), before mixing. dTTPase rates were scaled to the rate of 4A'-catalyzed dTTP hydrolysis and percent of maximal dTTPase activity was plotted versus mixing time.
proteins were preincubated with MgδTTP, and no loss in activity when the proteins were preincubated with MgδTTP and DNA. Under these conditions, subunits in stable hexamers exchange very slowly, and mixing does not occur in the time scale of the assay.

dTTPase Activity of Heterooligomers in the Presence of Single-Stranded M13 DNA.

The ability of 4A'/K318A to inhibit 4A' dTTPase activity was investigated further by titrating a constant amount of 4A' with increasing concentrations of 4A'/K318A protein. In the presence of M13 ssDNA, 4A' hydrolyzes dTTP with a rate constant of 2 to 3 s⁻¹ at 22 °C. When increasing concentrations of 4A'/K318A were added to 4A' and heterooligomer formation was allowed, dTTPase activity of 4A' dropped sharply (Figure 33A). Most of the loss in activity (4 to 5-fold) occurred up to a 1:1 ratio of the two proteins. A low level of dTTPase activity (0.3-0.4 s⁻¹) was still detectable at 10-fold higher 4A'/K318A concentration. The mutant protein itself has very low dTTPase activity (kₐₑᵣ = 0.04 s⁻¹ in the presence of M13 ssDNA) and it does not contribute significantly to the residual activity. A small percent of unmixed 4A' hexamers may be responsible for the low activity, or it may be an intrinsic property of the heterooligomers. A similar experiment was performed with 4B protein (Figure 33A), which showed that on mixing with
Figure 33. dTTPase activity of 4A' and 4B proteins in the presence of increasing concentrations of 4A'/K318A. 4A' or 4B protein (1 μM) was mixed with 4A'/K318A (0-10 μM) in the absence of ligands. The mixed proteins were assayed for dTTPase activity as follows: panel A shows the decrease in dTTPase activity of 4A' (▲) and 4B (♦), when mixed with increasing concentrations of 4A'/K318A and assayed in the presence of 5 mM MgdtTP and M13 ssDNA (the inset in A shows a control experiment in which 4A' and 4A'/K318A were incubated with MgdtTP and DNA prior to mixing and measuring dTTPase activity); panel B shows the decrease in dTTPase activity of 4A' (▲) and 4B (♦), when mixed with increasing concentrations of 4A'/K318A and assayed in the presence of 5 mM MgdtTP and 10 μM 30-mer DNA; panel C shows the increase in dTTPase activity of 4A' (▲) and 4B (♦), when mixed with increasing concentrations of 4A'/K318A and assayed in the presence of 5 mM MgdtTP. dTTPase rate constants ($k_{cat}$) of 4A' and 4B have been plotted versus 4A'/K318A concentration.
Figure 33
4A'/K318A, dTTPase activity of 4B protein decreases much the same as that of 4A'.

It should be noted that decrease in activity is not due to limiting DNA concentrations (if DNA is depleted by high amounts of 4A'/K318A in the reaction), because the same effect was observed at M13 ssDNA concentrations as high as 200 nM. Also, when the two proteins were preincubated with MgdTTP and ssDNA prior to mixing, there was no decrease in dTTPase activity (inset, Figure 33A).

dTTPase Activity of Heterooligomers in the Presence of 30-mer ssDNA.

The effect of 4A'/K318A protein on 30-mer ssDNA-stimulated dTTPase activity of 4A' was investigated by titrating a constant amount of 4A' with increasing concentrations of the mutant protein. Figure 33B shows that 4A' hydrolyzes dTTP with a steady-state rate constant of 0.25 s⁻¹ at 22 °C, in the presence of 30-mer DNA. On mixing with increasing amounts of 4A'/K318A, there is a small decrease in dTTPase activity, but the rate remains fairly constant at 0.15 to 0.2 s⁻¹. Thus, 30-mer ssDNA-stimulated dTTPase activity of the mixed oligomers is not very different from that of 4A' protein. Mixing with 4A'/K318A protein has a similar effect on the 30-mer DNA-stimulated dTTPase activity of 4B protein (Figure 33B).
dTTPase Activity of Heterooligomers in the Absence of DNA.

The effect of 4A'/K318A on DNA-independent dTTPase activity of 4A' was also examined. Figure 9C shows that 4A' hydrolyzes dTTP with a rate constant of 0.01 to 0.013 s⁻¹, in the absence of DNA, at 22 °C. Following addition of increasing amounts of mutant protein, dTTPase activity increases 3 to 4-fold and reaches a maximum of about 0.04 s⁻¹. This increase is not due to the dTTPase activity of the mutant protein, which is lower than 0.001 s⁻¹. It appears that, in the absence of DNA, heterooligomers hydrolyze dTTP at a higher rate than both 4A'/K318A and 4A'. The same is true for 4B protein and 4A'/K318A heterooligomers (Figure 33C; the kcat of the heterooligomers is about 5 to 6-fold higher than that of 4B alone).

Equilibrium DNA Binding Properties of 4A' and 4A'/K318A Heterooligomers.

4A'/K318A mutant protein does not bind DNA in the presence of MgdTMP-PCP. To examine the DNA binding properties of heterooligomers, studies were performed by titrating a constant amount of 30-mer DNA with increasing concentrations of mixed proteins (1:1, 1:2, and 1:3 ratios of 4A' and 4A'/K318A). As shown in Figure 34A, the 30-mer binds 4A' protein with a ratio of 1 DNA per 8 to 10 4A' monomers, and no DNA binding can be detected with 4A'/K318A protein. In contrast, heterooligomers of 4A' and 4A'/K318A
Figure 34. Equilibrium DNA binding by mixtures of 4A' and 4A'/K318A proteins. The two proteins, 4A' and 4A'/K318A, were mixed in ratios of 1:1, 1:2, and 1:3, in the absence of ligands. Panel A shows titration of a constant amount of 5'-32P 30-mer DNA (1 μM) titrated with increasing concentrations of 4A' (■), 4A'/K318A (○), and 1:1 (□), 1:2 (▲), and 1:3 (▲) mixtures of 4A' and 4A'/K318A proteins. DNA binding was measured using nitrocellulose-DEAE membrane binding assays (Materials and Methods), and the fraction of total DNA bound to protein was plotted versus protein concentration. Protein concentrations shown on the X-axis represent micromolar amounts of 4A' or 4A'/K318A alone, or 4A' + 4A'/K318A mixed in various ratios. Panel B shows a similar titration of 5'-32P 30-mer DNA with a 1:1 mixture of 4A' and 4A'/K318A proteins that were preincubated with MgDTMP-PCP prior to mixing. The fraction of total DNA bound was plotted versus 4A' monomer concentration (one half of total protein) and versus total monomer concentration (4A' + 4A'/K318A).
Figure 34
bind DNA to saturation in the presence of MgδTMP-PCP. The interaction between heterooligomers and DNA is weaker compared to 4A' protein; however, the stoichiometry of binding appears similar to that observed with 4A’ protein. Unless heterooligomer formation restores DNA binding ability of mutant protein subunits, these results suggest that all subunits in the hexamer need not be active for stable binding to DNA. This is consistent with recent results that directly show that DNA interacts with one to two subunits per hexamer (Chapter III).

In a control experiment, DNA binding was measured with a mixture of 4A’ and 4A'/K318A (1:1), each preincubated with MgδTMP-PCP prior to mixing. Figure 34B shows that in this case the correct stoichiometry for DNA binding (1 DNA per 6-8 protein monomers) depends solely on 4A’ protein concentration and not on the total protein. In other words, the proteins remained unmixed in the assay and the inactive mutant protein was invisible.

DISCUSSION

Properties of the Mutant 4A'/K318A Protein.

Bacteriophage T7 gene 4 proteins, 4A’ and 4B, contain the conserved nucleotide binding motif A (Walker et al., 1982), which may be essential for nucleotide binding and hydrolysis activity. Substitution of lysine-318 to alanine
yielded a mutant protein with reduced helicase activity compared to 4A'. A systematic, in vitro investigation showed that the mutant, 4A'/K318A, has lower DNA-stimulated dTTPase activity compared to the wild-type protein (both $k_{cat}$ and $K_m$ values are affected by the mutation). 4A'/K318A forms hexamers in the presence of dTTP and ssDNA, much the same as the wild-type protein, and binds DNA stably in the presence of Mg$\text{d}TTP$. Thus, the mutation apparently has a greater effect on NTP hydrolysis than on nucleotide and DNA binding. Interestingly, DNA does not bind to 4A'/K318A in the presence of dTMP-PCP, the nonhydrolyzable analog of dTTP. Direct measurement of nucleotide binding, described in the next chapter, shows that there is no stable interaction between dTMP-PCP and the mutant protein. So, nucleotide binding ability is affected to the extent that the mutant cannot bind dTMP-PCP (which has a longer P-C bond length and a shorter P-C-P angle compared to dTTP) even though this difference in structure can be easily tolerated by 4A' protein.

**Primase Activity of the 4A'/K318A Mutant Protein.**

Primase activity of the mutant is very similar to that of 4A' protein (1.5 to 2.5-fold lower), indicating that dTTP hydrolysis is not required for 4A'-catalyzed synthesis of RNA primers. A recent report by Mendelman and Richardson (1991) indicates that dTMP-PCP supports less than 1 % primer
synthesis relative to dTTP, and concludes that dTTP hydrolysis is necessary for primer synthesis, which contradicts the results of this study. But, an earlier report by the same group stated that dTMP-PCP supports the same level of primer synthesis as dTTP (Matson & Richardson, 1983). In view of the conflicting evidence, the primer synthesis assay was performed with 4A' in the absence of nucleotides, in the presence of dTTP, and in the presence of dTMP-PCP. The results showed that 4A'-catalyzed RNA primer synthesis requires dTTP. At high concentrations of 4A', the amount of RNA formed in the presence of dTMP-PCP is similar to that in the presence of dTTP (Patel et al., 1994). At low protein concentrations, however, optimal primase activity does require dTTP hydrolysis. If 4A protein translocates processively on ssDNA to screen for primase sites (Tabor & Richardson, 1981), dTTPase activity would be essential to facilitate translocation. At high protein concentrations, increased efficiency in random binding to primase sites can counteract the need for processive translocation. These results show that the actual primer-synthesis reaction, catalyzed by 4A protein, does not require dTTP hydrolysis.

**In vivo Characterization of 4A'/K318A Protein.**

Biochemical properties of the 4A'/K318A mutant protein have been tested *in vivo* using complementation assays (K. Griffin & A. Rosenberg, Brookhaven National Laboratory, NY;
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Patel et al., 1994). 4A'/K318A effectively complements a T7 phage defective in primase activity, confirming the results of \textit{in vitro} studies, which predict that mutant protein can provide primase activity \textit{in vivo}. As expected, 4A'/K318A does not support growth of a T7 phage defective in both primase and helicase activities. Thus, both \textit{in vitro} and \textit{in vivo} results demonstrate that mutation of lysine-318 in the nucleotide binding site has a drastic effect on the helicase activity, while the primase activity is not greatly affected. Also, overexpression of the mutant protein is detrimental to the growth of the wild-type phage, suggesting that high concentration of 4A'/K318A has a dominant-lethal effect, and the mutant inhibits activities of the wild-type protein, most likely by heterooligomer formation.

A Double Mutant of 4A' Protein Motif A.

Concurrent with this study, the preparation and characterization of a double mutant of 4A protein was reported (Notarnicola & Richardson, 1993; Mendelman et al., 1993). The mutant protein has glycine-317 and lysine-318 in the conserved sequence changed to valine and methionine, respectively. This mutant (GK-VM) is deficient in NTPase and helicase activity, but unlike 4A'/K318A, it has highly reduced primase activity. The double mutant does not bind DNA in the presence of both dTTP and dTMP-PCP; although, interaction with dTTP has been detected. The difference in
primase activity between the GK-VM mutant and 4A'/K318A may lie in the inability of the mutant to interact stably with DNA. It has not been tested whether loss of DNA binding is a direct consequence of the mutations, or whether the double mutation affects hexamer formation, which is essential for DNA binding. It should be noted that, in a recent publication, the same group has reported that the double mutant was prepared from a gene 4 clone containing a previously undetected mutation, aspartate-485 to glycine (Notarnicola et al., 1995). Characterization of the D485G mutant indicates that it is defective in hexamer formation, and binds DNA at least 10-fold weaker than wild-type protein. It is very likely that differences between the double mutant and 4A'/K318A are simply due to the D485G mutation in the original protein!

Heterooligomer Formation Between 4A' and 4A'/K318A Proteins.

4A' and 4A'/K318A proteins form heterooligomers when mixed as unstable hexamers and other oligomers, as demonstrated directly by a reversible cross-linking experiment. When 4A' and 4A'/K318A proteins are allowed to form stable hexamers prior to mixing, there is no exchange of subunits between the two proteins and heterooligomers are not formed.

Both helicase activity and DNA-stimulated dTTPase activity of 4A' are drastically decreased by heterooligomer
formation with 4A'/K318A, indicating that fully functional hexamers are essential for these two activities. The heterooligomeric effect can be explained if the presence of mutant subunits in the hexamer prevents its processive translocation on DNA. The mutant subunits may either bind DNA tightly for a prolonged time because of lower level of dTTPase activity, or alternatively, the mutant subunits may dissociate more easily from DNA due to their lower affinity for dTTP and DNA. Mutant subunits can thus interfere with processive translocation, and by either mechanism, disrupt both DNA-stimulated dTTPase activity and helicase activity.

The effect of heterooligomer formation on dTTPase activity is not as dramatic in the presence of 30-mer ssDNA. It should be noted that 30-mer DNA stimulates DNA-independent dTTPase rate (\(k_{\text{cat}} = 0.02 \text{ s}^{-1}\)) by about 10-fold (\(k_{\text{cat}} = 0.2 \text{ s}^{-1}\)), while long M13 ssDNA stimulates it about 100-fold (\(k_{\text{cat}} = 2 \text{ s}^{-1}\)). The difference between 30-mer-simulated and ssM13-stimulated dTTPase activity may reflect the fact that there is little translocation on the 30-mer, and steady-state dTTPase rate with the 30-mer may be limited by binding and dissociation from DNA. Thus, 4A' bound to long DNA can translocate and undergo multiple cycles of dTTP hydrolysis without dissociating from DNA; whereas, 4A' bound to a small oligodeoxynucleotide has stimulated dTTPase activity, but it has to completely dissociate from DNA after each hydrolysis cycle. From this point of view, the effect
of heterooligomer formation on 30-mer-stimulated dTTPase activity is understandable. The mixed hexamers can dissociate and rebind DNA with active sites that hydrolyze dTTP, and it is the unsuccessful binding events that lead to a small net decrease in dTTPase activity. One observation that supports this theory is that the heterooligomeric effect reduces ssMl3-stimulated dTTPase rate to the same level (0.2 to 0.3 s⁻¹), indicative of dTTP hydrolysis limited by dissociation and rebinding to DNA.

The effect of heterooligomer formation on DNA-independent dTTPase activity of 4A' is a more curious phenomenon. When 4A' is mixed with 4A'/K318A, the mixed oligomer hydrolyzes dTTP at a higher rate than 4A' alone. There are several possible explanations for this observed effect. First, perhaps the increase in total protein concentration upon addition of the mutant protein favors hexamer formation. If hexamers are the active species catalyzing dTTP hydrolysis, then increasing the number of hexamers can cause a net increase in dTTPase rate. It has been reported earlier that dTTPase activity of gene 4 proteins increases with protein concentration (Bernstein & Richardson, 1988), which supports the idea that stable, high-order oligomers are required for optimal dTTPase activity. In the mixed oligomer assay, however, concentration of 4A' itself is more than adequate for stable hexamer formation in the presence of 5 mM Mg-dTTP (Hingorani
& Patel, 1996; Chapter II). Therefore, the above explanation is most likely not valid in this case.

Formation of mixed oligomers may result in increased dTTPase activity if all subunits in the pure 4A' hexamer do not bind or hydrolyze dTTP simultaneously. If each heterooligomer contains one or more subunits that are as active as in hexamers of 4A' alone, distribution of active subunits into a larger number of hexamers would result in a net increase in dTTP hydrolysis. The fact that DNA binds specifically to only one or two sites on the hexamer may mean that only one or two subunits hydrolyze dTTP at one time. The number of dTTP hydrolyzed per hexamer in a single turnover has not been measured directly yet; therefore, the above theory can be verified only after analysis of the pre-steady-state kinetics of T7 helicase-catalyzed dTTP hydrolysis.

The third, more direct explanation may be that the intrinsic dTTPase activity of 4A' subunits is higher in heterooligomers as compared to pure 4A' hexamers. This is possible if 4A' subunits hydrolyze dTTP by a linked and controlled pathway in the hexamer, and this linkage is lost or uncoupled in a mixed oligomer, leading to increased activity. It is difficult to test this theory directly until more is known about the mechanism of helicase-catalyzed dTTP hydrolysis, and how hydrolysis is coupled to translocation and DNA unwinding.
With the exception of primase activity, both 4A' and 4B helicases appear to have comparable properties, to the extent that heterooligomer formation with a mutant protein affects their dTTPase and helicase activities the same way. Thus, although 4B protein lacks primase activity and the N-terminal 63 amino acids, it very likely has the same mechanism for DNA unwinding as 4A' protein, although it hydrolyzes dTTP at about 2-fold higher rates than 4A' protein.

DNA binding studies with heterooligomers of 4A' and 4A'/K318A proteins confirm earlier results that T7 helicase hexamers bind DNA via only one or two subunits (Chapter III; Egelman et al., 1996). The results in this study are consistent with the model for helicase-translocation on DNA, shown in Scheme 2 of Chapter III. DNA binds to the hexamer via one or two subunits, and movement occurs by passage of DNA from one site on the hexamer to another, coupled to dTTP hydrolysis. During translocation of a heterooligomer on DNA, as the DNA strand is bound and released consecutively by units in the ring, an encounter with an inactive subunit can halt translocation and dTTPase activity and disrupt duplex DNA unwinding.
CHAPTER IV
INTERACTIONS OF BACTERIOPHAGE T7 HELICASES WITH NUCLEOTIDE
LIGANDS

PART B: COOPERATIVE NUCLEOTIDE BINDING EFFECTS HEXAMER
FORMATION AND DNA BINDING

ABSTRACT

Equilibrium interactions of T7 helicases and nucleotide ligands (dTTP, dTMP-PCP, dTDP, ATP and ATPγS) have been examined using membrane binding assays. Nucleotide binding studies show that the six potential binding sites on the hexamer are nonequivalent, both in the absence and presence of DNA. The nucleotides bind with high affinity to three sites (dTTP: \(K_d = 5 \times 10^{-6}\) M; dTMP-PCP: \(K_d = 6 \times 10^{-7}\) M; dTDP: \(K_d = 4 \times 10^{-6}\) M; ATP: \(K_d = 3 \times 10^{-5}\) M; ATPγS: \(K_d = 2 \times 10^{-6}\) M). The hexamer exhibits strong negative cooperativity for further nucleotide binding and interaction with the remaining sites on the hexamer is undetectable even at very high concentrations of protein and ligand. Nucleotide binding within the high-affinity sites shows a positive cooperativity that is sensitive to protein concentration. This effect results from ligand binding-linked
oligomerization of the T7 helicases, wherein both nucleotide binding and the oligomeric equilibria change as a function of protein and nucleotide concentration. A study of DNA binding shows that 1-2 NTPs bound per hexamer are sufficient for stoichiometric interaction between the helicase and DNA. Nucleotide binding-linked oligomer assembly is a dynamic process by which ring-shaped hexamers form around DNA with one to three NTPs bound to each hexamer.

This study also examines the preferred use of dTTP by the T7 helicases by comparing it with ATP, which is more commonly used as a source of energy. ATP and its analogs stabilize hexamer formation, bind to three sites on the hexamers, and facilitate DNA binding to the helicase. Nevertheless, DNA unwinding with ATP is at least 100-fold slower than with dTTP. It appears that there is a subtle difference between the use of ATP and dTTP by the helicase, which may involve coupling of NTP hydrolysis to DNA unwinding.

INTRODUCTION

Bacteriophage T7 gene 4 proteins are DNA helicases that unwind duplex DNA ahead of the polymerase during phage DNA replication. Both helicases, 4A and 4B, form hexameric rings that bind around single-stranded DNA and use energy from NTP binding and hydrolysis to effect translocation and
unwinding. It is not understood yet what drives hexamer formation and DNA binding, and how helicases use nucleotide binding and hydrolysis to effect DNA unwinding. Helicases studied to date show some common features that provide important clues about their mechanism of action.

Oligomerization is one important property shared among helicases; all known helicases either form hexamers (SV40 T antigen: Mastrangelo et al., 1989; E.coli RuvB: Stasiak et al., 1994; phage T4 gene 41 helicase: Dong et al., 1995; E.coli DnaB: Reha-Krantz & Hurwitz, 1987a; Bujalowski et al., 1994; phage T7 gene 4 helicases: Patel & Hingorani, 1993) or dimers (E.coli Rep: Wong & Lohman, 1992; E.coli Helicase II: Runyon et al., 1993). Oligomerization provides helicases with multiple DNA and nucleotide binding sites, which may be required for stable association with DNA during translocation and unwinding.

Nucleotide binding is another essential feature shared among helicases. Nucleotide ligands effect hexamer formation (SV40 T antigen: Mastrangelo et al., 1989, 1994; T7 helicases: Patel & Hingorani, 1993; T4 gene 41 helicase: Dong et al., 1995), and modulate 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). Direct measurements of nucleotide binding to helicases have been performed with
E. coli Rho (Stitt, 1988; Geiselmann & von Hippel, 1992) and E. coli DnaB (Arai & Kornberg, 1981b; Biswas et al., 1986; Bujalowski & Klonowska, 1993). These hexameric helicases have six potential nucleotide binding sites that are nonequivalent and can be clearly distinguished as three high-affinity and three low-affinity sites. This negative cooperativity has not been directly investigated, but it appears to be an important property, and it may play an important regulatory role in coordination of NTP binding and hydrolysis among the subunits of the hexamer.

This study examines equilibrium nucleotide binding to bacteriophage T7 helicases and its linkage to hexamer formation and DNA binding. The results indicate that 3 nucleotides bind per 4A’ or 4B hexamer with high affinity, and nucleotide binding is coupled to hexamer formation. Also, 1 to 2 NTPs bound per hexamer are sufficient for tight interaction between the helicase and DNA.

T7 helicases hydrolyze a number of NTPs such as dTTP, ATP, dATP, and GTP. Both 4A’ and 4B proteins have the lowest Kₘ for dTTP, and prefer to utilize dTTP for primase and helicase activity (Matson et al., 1983; Matson & Richardson, 1983, 1985; Patel et al., 1992). Other known helicases, however, use ATP to power their DNA unwinding activity. T7 gene 4 proteins form hexamers in the presence of ATP and bind DNA in the presence of AMP-PCP (Hingorani & Patel, 1996; Chapter II & III). This study shows that interaction
of ATP and its analogs with 4A' and 4B proteins is similar to that of dTTP. The rate of helicase-catalyzed DNA unwinding, however, is significantly lower with ATP, compared to unwinding with dTTP.

MATERIALS AND METHODS

Nucleotides, DNA, Enzymes and Other Reagents.

dTMP-PCP and dAMP-PCP were purchased from Amersham Life Science Inc. [α-32P]dTTP (3000 Ci/mM), [α-32P]ATP (3000 Ci/mM), and [γ-35S]ATP (1000 Ci/mM) were purchased from ICN Biochemicals Inc. Methylene diphosphonic 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 and 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. Oligodeoxynucleotides used in the synthesis of radiolabeled dTMP-PCP, binding assays, and unwinding assays were synthesized at the Biochemical Instrument Center at The Ohio State University. The DNAs were purified by denaturing polyacrylamide gel electrophoresis (25-mer, 30-mer, 36-mer: 18% polyacrylamide/7M urea; 5-60-mer and 3-60-merF: 12%
polyacrylamide/7M urea), electroeluted from the gel (Elutrap, Schleicher and Schuell Inc.), ethanol precipitated with sodium acetate and reconstituted in distilled water. DNA concentrations were determined from absorbance measurements at 260 nm in TE buffer/7 M urea using the calculated extinction coefficients of 25-mer (249,040 M$^{-1}$ cm$^{-1}$), 30-mer (305,010 M$^{-1}$ cm$^{-1}$), 36-mer (377,000 M$^{-1}$ cm$^{-1}$), 5-60-mer (655,780 M$^{-1}$ cm$^{-1}$), and 3-60-merF (629,510 M$^{-1}$ cm$^{-1}$). 4A' and 4B proteins were purified as described earlier (Chapter II). Purified HIV-1 reverse transcriptase used for synthesis of radiolabeled dTMP-PCP was a gift from Dr. Karen Anderson (Yale University).

Preparation of Radiolabeled Nucleotides:

Synthesis of [α-$^{32}$P]dTMP-PCP.

[α-$^{32}$P]dTMP-PCP was prepared by HIV-1 reverse transcriptase-catalyzed pyrophosphorolysis of [α-$^{32}$P]dTMP containing 26/36-mer DNA. The 25-mer and 36-mer complementary DNAs were mixed (2 μM each) in nucleotide synthesis (NS) buffer (50 mM Tris-acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, and 0.1 mg/ml BSA) to form the partially duplex DNA. dTTP (20 μM), [α-$^{32}$P]dTTP (40 μCi) and 0.3 μM reverse transcriptase were added to the DNA (final volume = 100 μl). The mixture was
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incubated at 37 °C for 15 minutes to incorporate [α-32P]dTMP at the 3'-end of the 25-mer. The solution was spun through a 0.5 ml Biogel P-30 column to remove unreacted dTTP. The filtrate was mixed with 100 µl NS buffer containing a standard nonradioactive sample of dTMP-PCP (visualized under uv light, 254 nm) containing high specific activity of [α-32P]dTMP-PCP, was then treated by reverse transcriptase, which was incubated at 37 °C for 2 hours to allow [α-32P]dTMP-PCP synthesis. Following incubation, the solution was loaded onto a 1 ml DEAE-Sephadex column, which was prepared by loading DEAE-Sephadex A-50 equilibrated over-night in water into a tuberculin syringe. Sephadex column, which was prepared by loading DEAE-Sephadex A-50 into a tuberculin syringe (equilibrated overnight in water) into a tuberculin syringe. Following incubation, the solution was loaded onto a 1 ml DEAE-Sephadex column, which was prepared by loading DEAE-Sephadex A-50 into a tuberculin syringe (equilibrated overnight in water) into a tuberculin syringe. The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4). The column was washed with 1 ml water, and [α-32P]dTMP-PCP was eluted with 1 M TEAB (1 M Triethanolamine, pH 7.4).
vacuum. Purified [α-32P]dTMP-PCP was reconstituted in water and stored at -70 °C.

Reverse transcriptase (RT) was chosen as the catalyst for [α-32P]dTMP-PCP synthesis because it uses P-C-P as a substrate more efficiently than other DNA polymerases that were tested. RT-catalyzed pyrophosphorolysis transfers about 40-50 % of [α-32P]dTMP from the partially duplex DNA to P-C-P. Purification of [α-32P]dTMP-PCP by anion exchange chromatography in a disposable syringe column is convenient because it allows confinement of radioactivity and easy recovery of most of the 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.

Synthesis of [α-32P]dTDP.

[α-32P]dTDP was prepared by 4A’-catalyzed hydrolysis of [α-32P]dTTP. The reaction (20 μl) contained [α-32P]dTTP (30 μCi), 0.05 μM single-stranded M13mp18 DNA, and 0.2 μM 4A’ in nucleotide synthesis buffer. The mixture was incubated at 37 °C for 2 hours, followed by anion exchange chromatography to separate [α-32P]dTDP from [α-32P]dTTP and ssM13 DNA. The sample was loaded onto 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 M - 1 M TEAB, pH 8.0. Fractions (~ 60 μl) of the eluate were collected and 1 μl aliquots were analyzed by thin-layer chromatography (PEI-cellulose TLC plate, developed in 0.3 M potassium phosphate buffer, pH 3.4) to identify the nucleotide in each fraction. Initial fractions of pure [α-\(^{32}\)P]dTDP were combined and extracted with phenol/chloroform (1:1 mixture of phenol and chloroform, equilibrated with 0.1 M Tris-Cl, pH 7.6) to remove any contaminating 4A' protein. The solution was cycled several times through methanol washes and drying under vacuum. [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 glass microanalysis filter assembly (Fisher Scientific). dTTP binding was measured both at 22 °C and 4 °C. Assays at 4 °C were performed on ice, and filtration was carried out using chilled nitrocellulose membranes and membrane wash buffer B (50 mM Tris-acetate, pH 7.5, 40 mM sodium acetate, and 10 mM magnesium acetate). Nitrocellulose membrane
circles (25 mm; 0.2 µm) were washed in 0.5 N NaOH (5 min), rinsed with water, and equilibrated in membrane wash buffer B for 2-3 hours prior to use. Assays were performed both in the absence and in the presence of 30-mer DNA (5 µM). 4A' or 4B (5, 20 µM) were added to samples (15 µl) containing 0-150 µM dTTP + [α-32P]dTTP (0.05 µCi) in binding buffer B (50 mM Tris-acetate, pH 7.5, 40 mM sodium acetate, 10 mM magnesium acetate, and 10% glycerol). 10 µl volumes were filtered through the membrane assembly within 30-35 s of adding the protein. The membranes were washed before and after filtration with 0.5 ml of membrane wash buffer. One µl aliquot of each sample was spotted on a separate nitrocellulose membrane to quantitate the total amount of dTTP in the sample. Radioactivity on the membranes was quantitated either on a Betascope 603 blot analyzer (Betagen), or on a PhosphorImager (Molecular Dynamics), or by liquid scintillation counting. Nonspecific binding of dTTP to the nitrocellulose membranes was measured by replacing protein with buffer and was corrected in all the titrations reported. The molar fraction of dTTP bound to 4A' protein was calculated and plotted versus dTTP concentration.
Equilibrium Binding of dTMP-PCP to 4A' Protein.

dTMP-PCP binding to 4A' was measured both at constant dTMP-PCP and at constant protein concentrations. [α-\(^{32}\)P]dTMP-PCP was titrated with increasing 4A' protein in the absence and in the presence of 30-mer DNA (3.3 μM) using the nitrocellulose membrane assay described above. Samples (15 μl) were prepared in binding buffer B containing 10 μM dTMP-PCP + [α-\(^{32}\)P]dTMP-PCP and increasing amounts of 4A' protein (0-35 μM). The above mixture was incubated for 10-15 minutes at 22 °C prior to filtration through the nitrocellulose membrane assembly. The membranes were washed before and after filtration with 0.5 ml membrane wash buffer B, and radioactivity on the membranes was quantitated as described earlier. The molar amount of dTMP-PCP bound to 4A' protein was determined and plotted versus 4A' concentration.

Equilibrium binding at constant protein and increasing [α-\(^{32}\)P]dTMP-PCP concentrations (0-50 μM) was performed with 4A' (0.1, 0.5, 5, 10 and 20 μM) and 4B (20 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). The molar amount of dTMP-PCP bound to 4A' protein was determined and plotted versus total or free dTMP-PCP concentration. Nonspecific dTMP-PCP binding to nitrocellulose was corrected in all titrations reported.
Equilibrium Binding of dTMP-PCP Measured by Quenching of Protein Fluorescence.

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 1 ml buffer F (50 mM Tris-acetate, pH 7.5, 50 mM sodium acetate, 3 mM magnesium acetate and 10 % glycerol; filtered through 0.2μm filter) in a quartz cuvette. The solution was incubated for 2 min following each addition, and nucleotide binding was measured by monitoring quenching of tryptophan fluorescence (λ<sub>ex</sub>=291 nm; λ<sub>em</sub>=335 nm). Fluorescence intensity at each dTMP-PCP concentration was corrected for inner filter effect as described (Lohman and Mascotti, 1992); photobleaching and dilution effects were found to be negligible. The fraction of fluorescence quenching detected was calculated and plotted versus total dTMP-PCP concentration.

Competitive Binding of dTMP-PCP and dTDP to 4A' Protein.

Equilibrium binding of [α-32P]dTDP to 4A' was measured first in the absence of dTMP-PCP. A constant amount of 4A' (20 μM) was titrated with dTDP (0-200 μM) + [α-32P]dTDP in binding buffer B 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 (0-200 μM) + [α-³²P]dTDP. The same
experiment was performed with [α-³²P]dTMP-PCP and
nonradiolabeled dTDP. All titrations were quantitated by
measuring 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 (0-200 μM) + [α-³²P]ATP were mixed in binding
buffer B and filtered through nitrocellulose membranes
within 30-40 s as described for dTTP binding. ATPγS binding
was measured similarly by titrating 4A' (0.2 μM) with ATPγS
(0-25 μM) + [γ-³⁵S]ATP. The molar amount of nucleotide bound
to 4A' was determined and plotted versus total nucleotide
concentration.
ssDNA Binding 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 B containing 4A' (17 μM with dTMP-PCP, 12 μM with ATPγS, AMP-PCP and dAMP-PCP), 5'-32P 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 mixture was incubated at 22 °C for 20 min and DNA binding was measured using the nitrocellulose-DEAE membrane assembly as described (Chapter III; Hingorani and Patel; 1993).

Equilibrium binding of 5'-32P 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 and DEAE membranes gave a measure of protein-bound DNA and free DNA concentrations, respectively. The fraction of DNA bound per 4A' hexamer was determined and plotted versus the nucleotide concentration.

Curve Fitting.

Equilibrium nucleotide binding isotherms were fit to a hyperbola or to the quadratic solution for 1:1 binding of ligand to a macromolecule shown below:
\[ [ML] = \frac{1}{2} \left\{ (K_d + L_t + M_t) - \left[ (K_d + L_t + M_t)^2 / 4 - (M_t L_t)^{0.5} \right] \right\} \] (4)

where \( K_d \) is the dissociation constant, \( L_t \) is total ligand concentration (dTTP, dTMP-PCP, dTDP, ATP, and ATP\( \gamma \)S), \( M_t \) is total protein concentration (monomer) and \( ML \) is the molar amount of ligand bound to protein.

The equilibrium binding isotherms were fit also to the Hill equation (Equation 5) to estimate cooperativity in ligand binding.

\[ Y_L = \frac{[L]^n}{K + [L]^n} \] (5)

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

Fluorescence-Based Stopped-Flow Unwinding Assay.

DNA unwinding rate of 4A' protein was measured in the presence of dTTP or ATP, using a fluorescein-tagged, synthetic-fork substrate. 5'-60-merF ssDNA (1 \( \mu M \)) was mixed with 3'-60-mer ssDNA (1 \( \mu M \)) in double-distilled water.
(filtered through 0.2 μm filter) containing 100 mM NaCl. The solution was heated at 95 °C for 5 min and cooled overnight to form the forked DNA substrate (33 bases of fluorescein-tagged 5′-60-merF ssDNA complement 33 bases of 3′-60-mer ssDNA). Helicase assays were carried out on a KinTek SF-2001 stopped-flow (KinTek Inc., University Park, PA) equipped with a 75 W Xe lamp. Rate of unwinding was measured by monitoring increase in fluorescence (λ<sub>ex</sub>=494 nm) at wavelengths >530nm using a cut-on filter (Oriel Corporation) and slit-widths of 1-2 mm. 4A' (20 μM) in SF buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 3 mM MgCl, and 10 % glycerol; filtered through 0.2 μm filter) was rapidly mixed in the stopped-flow with the fork (5 nM concentration in SF buffer containing 500 μM dTTP or 2 mM ATP). Three to five traces were averaged for each unwinding experiment and fit to an exponential term, which yielded rate of unwinding. Control experiments were carried out with the forked DNA, in the absence of protein, to measure bleaching of fluorescence over the time course of the assay.
Equilibrium Binding of MgDTTP.

Bacteriophage T7 helicases hydrolyze nucleotide substrates such as dTTP, dATP, ATP, etc. with varying specificities. Of these nucleotides, dTTP is hydrolyzed with the lowest $K_m$ value and is thought to be the ligand of choice of the T7 gene helicases (Patel et al., 1992). Nitrocellulose membrane binding assays have been used in this study to directly measure binding affinities of various nucleotides for 4A' and 4B, and to examine their effects on oligomeric and DNA binding properties of the helicases.

The assays were performed at constant protein and increasing ligand concentrations. Figure 35A shows the titration of 5 μM 4A' protein with MgDTTP, performed at 4 °C to minimize dTTP hydrolysis during the experiment ($k_{cat} = 0.03$ s$^{-1}$ at 22 °C, and hydrolysis is undetectable at 4 °C up to two hours). The data fit to a quadratic equation (Eqn. 4) with a $K_a$ of $4.9 \pm 0.5 \times 10^{-6}$ M. Binding appears to saturate with dTTP bound to only half the molar amount of 4A' monomers present in the reaction. The experiment was performed at a higher protein concentration (> $K_a$) to measure the stoichiometry of dTTP binding, and to determine whether the remaining nucleotide binding sites on the hexamer can be occupied at high protein concentrations. Titration of 20 μM
Figure 35. Equilibrium binding of MgdTTP to 4A'. dTTP binding to 4A' protein was measured at 4 °C using nitrocellulose membrane binding assays as described in Materials and Methods. A, shows binding of \( [\alpha-^{32}\text{P}]dTTP \) to 5 \( \mu\text{M} \) 4A' at increasing dTTP concentrations (0-80 \( \mu\text{M} \)). dTTP binding fits to a quadratic equation (Eqn. 4) with a \( K_d \) of 4.9 x 10^{-6} \text{M}. B, shows the curve for a similar titration performed at 20 \( \mu\text{M} \) 4A' and increasing dTTP (0-40 \( \mu\text{M} \)), 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 per hexamer. The inset in B shows the data at 20 \( \mu\text{M} \) 4A' fit to the Hill equation with a coefficient of 1.73 ± 0.15.
Hill coefficient = 1.8

Figure 35
4A' with dTTP also shows that binding saturates with 3 dTTP per 4A' hexamer (Figure 35B). A similar experiment, carried out at 22 °C to test for temperature effects on nucleotide binding, yielded identical results. Lack of binding to the remaining three sites on the hexamer, even at high concentrations of dTTP (200 µM) and protein (20 µM), suggests that these sites do not bind dTTP or the interaction is very weak ($K_d > 1 \times 10^{-4} \text{ M}$).

At 20 µM protein concentration, which is well above the $K_d$ for dTTP binding (5 µM), the binding isotherm should be stoichiometric, and the inflection point in the isotherm should provide the stoichiometry of dTTP binding. But, the inflection point in the binding curve in Figure 35B does not correspond to a stoichiometry of 3 dTTP per 4A' hexamer, and the curve does not fit well to a quadratic equation. The curve appears shifted toward higher dTTP concentrations and fits to the Hill equation (Eqn. 5) with a Hill coefficient of 1.7 (inset in Figure 35B). The Hill coefficient ($> 1$) indicates that dTTP binds 4A' with positive cooperativity. This effect is related to the high protein concentration in the assay, because at 5 µM 4A' the isotherm fits the Hill equation with a coefficient of 1.1. These data show that 4A' protein exhibits both positive and negative cooperativity in dTTP binding. Extreme negative cooperativity is responsible for lack of nucleotide binding to 3 sites, and at high
protein concentrations, 3 nucleotide ligands bind with positive cooperativity to the high-affinity sites.

Equilibrium Binding of MgTTTP in the Presence of ssDNA.

Nucleotide binding modulates interaction of 4A' with ssDNA. In order to examine the effect of ssDNA on nucleotide binding, binding assays were carried out with 4A and dTTP in the presence of ssDNA. Figures 36A and 36B show dTTP binding to 5 µM and 20 µM 4A', respectively, in the presence of enough 30-mer to saturate DNA binding (> one 30-mer per hexamer). The experiment was performed at 4 °C to minimize dTTP hydrolysis (k_{cat} = 0.0017 s^{-1} at 4 °C in the presence of 30-mer ssDNA). A maximum of 3 dTTP bind per hexamer at both protein concentrations, and the isotherms fit to the Hill equation with coefficients of 1.8 (5 µM 4A') and 1.3 (20 µM 4A'). The presence of ssDNA does not appear to have significant effects on dTTP binding to 4A', both the number of dTTP bound per hexamer, and positive cooperativity.

Equilibrium Binding of MgTMP-PCP.

dTMP-PCP binding to 4A' was measured directly with [α-32P]dTMP-PCP, prepared via a novel enzyme-catalyzed reaction (Scheme 5), and nitrocellulose membrane binding assays. Equilibrium binding was measured with the nonhydrolyzable analog to allow measurements at 22 °C without complications
Figure 36. 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. A, shows binding of [α-32P]dTTP to 5 μM 4A' in the presence of 30-mer DNA; 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 at three dTTP per 4A' hexamer as shown on the second Y-axis.
Figure 3A

Fractional Saturation vs. [dTTP]_{free} µM

Figure 3B

Fractional Saturation vs. [dTTP]_{free} µM

Figure 36
Scheme 5. HIV reverse transcriptase-catalyzed synthesis of $[\alpha^{-32}\text{P}]d\text{TMP-PCP}$
from dTTP hydrolysis. Figure 37 shows a constant amount of dTMP-PCP titrated with 4A' in the absence and in the presence of ssDNA. Both binding curves appear stoichiometric, and concentration of 4A' at the inflection point indicates that about two 4A' monomers (15 μM) are required to bind one dTTP (8 μM). This is consistent with the results shown in Figure 35 where 3 dTTP bind one hexamer with high affinity. In the absence of DNA, there is a slight sigmoidal character to the dTTP binding curve at lower 4A' concentrations, which may be related to the changes in 4A'-oligomeric states with changing protein concentration. This effect complicates the binding isotherms; therefore, dTMP-PCP binding has been studied in greater detail at constant protein and increasing ligand concentrations.

Figures 38A and 38B show dTMP-PCP binding to 20 μM 4A' and 20 μM 4B, respectively. Binding saturates at 3-4 dTMP-PCP per 4A' or 4B hexamer. No additional binding can be detected even at dTMP-PCP concentrations as high as 200 μM. The binding isotherms fit to the Hill equation with coefficients of 2.5 and 2.3 for 4A' and 4B, respectively. In the presence of ssDNA, no more than 4 dTMP-PCP bind per hexamer with Hill coefficients of 2.5 and 3.1 for 4A' and 4B, respectively (Figure 41A and 41B). The effects of negative and positive cooperativity appear to be the same for both dTTP and dTMP-PCP.
Figure 37. Equilibrium binding of Mg\textsuperscript{2+}dTMP-PCP to 4A' at constant nucleotide concentration. Binding of [\textalpha\textsuperscript{32P}dTMP-PCP (10 \textmu M) to increasing 4A' (0-35 \textmu M) was measured at 22 °C as described in Materials and Methods. (●), shows dTMP-PCP binding to 4A' in the absence of DNA, and (▲), shows dTMP-PCP binding in the presence of 3.3 \textmu M 30-mer ssDNA. The inflection points of the isotherms provide a stoichiometry of one dTMP-PCP bound to two 4A' monomers.
Figure 38. Equilibrium binding of Mg\textsc{dTMP-PCP} to gene 4 proteins at constant protein concentration. [\alpha-\textsuperscript{32}P]\textsc{dTMP-PCP} binding to 4A' and 4B proteins was measured at 22 °C using nitrocellulose membrane binding assays. A, shows binding of [\alpha-\textsuperscript{32}P]\textsc{dTMP-PCP} to 4A' (20 \mu M) at increasing \textsc{dTMP-PCP} concentrations (0-40 \mu M); B, shows a similar titration performed under the same conditions with 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 \textsc{dTMP-PCP} molecules bound per hexamer.
Figure 38
Oligomerization of gene 4 proteins is facilitated by the presence of nucleotide ligands, and hexamer stability is dependent on nucleotide concentration (Chapter II). Any corresponding dependence of nucleotide binding on protein concentration was investigated by measuring dTMP-PCP binding at varying 4A' protein concentrations. The results are summarized in Table 3. At 0.1 µM 4A', a maximum of 3 dTMP-PCP bound per hexamer; dTMP-PCP binding fit to a hyperbola with a $K_d$ of $2.2 \pm 0.3 \times 10^{-6}$ M and to the Hill equation with a coefficient of 1.4. As 4A' concentration was raised, the binding isotherms showed increasing sigmoidicity. At the highest protein concentration tested (20 µM), a maximum of 3 to 4 dTMP-PCP molecules bound the hexamer, and the Hill coefficient increased to 2.5. These results show that positive cooperativity increases with protein concentrations, as can be expected from nucleotide binding linked to oligomerization of gene 4 proteins. In this system, oligomeric species of 4A' bind nucleotides with differing affinities. As oligomer equilibrium shifts with protein and nucleotide concentrations, there is a net change in nucleotide binding affinity, resulting in distinctive changes in cooperativity. Accordingly, a simple isotherm with one $K_d$ value cannot be used to describe the binding isotherms, especially at higher protein concentrations (Table 3).
Table 3: Stoichiometry, $K_d$, and Hill coefficient values for dTMP-PCP binding to 4A' protein

<table>
<thead>
<tr>
<th>4A' (μM)</th>
<th>dTMP-PCP per Hexamer</th>
<th>$K_d$ (10^{-6} M)</th>
<th>Hill coefficient</th>
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<tr>
<td>0.1</td>
<td>2.9</td>
<td>2.23 ± 0.31</td>
<td>1.36 ± 0.09</td>
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<td>0.5</td>
<td>2.4</td>
<td>0.69 ± 0.14</td>
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The negative cooperativity, observed for both dTTP and dTMP-PCP binding, suggests that the remaining 3 sites on the hexamer do not bind nucleotides, or perhaps the interaction is weak and the ligands dissociate during filtration through the nitrocellulose membranes. In order to address this concern, dTMP-PCP binding has been measured by a fluorescence-based assay. As shown in Figure 39A, dTMP-PCP binding to 4A' causes about 10% decrease in the intrinsic tryptophan fluorescence of 4A' protein. This signal was monitored in a titration of a constant amount of 4A' with increasing dTMP-PCP (Figure 39B). The effect of ligand binding linked to oligomerization causes a shift in the isotherm; therefore, the inflection point cannot be used to determine the molar amount of nucleotide bound to 4A' (Lohman & Mascotti, 1992). However, the fluorescence-based binding curve can be compared with an isotherm obtained by measuring binding of radiolabeled dTMP-PCP to 4A' under the same conditions. The two curves overlap at all concentrations of dTMP-PCP up to saturation of binding, indicating that the same molar amounts of dTMP-PCP are bound per hexamer in both assays.

Other control experiments were performed to test for any artifacts that may be responsible for the observed half-site binding. For example, three sites on the hexamer may be occupied by tightly-bound nucleotides (copurified during protein preparation), and be unavailable for interaction
Figure 39. dTMP-PCP binding measured by change in intrinsic protein fluorescence. Panel A shows fluorescence excitation spectra of 4A' with (•) and without (x) Mg-dTMP-PCP ($\lambda_{ex} = 291$ nm). Panel B, 4A' protein (10 μM) was titrated with dTMP-PCP (0-40 μM) and the resulting quenching of protein fluorescence was measured as described in Materials and Methods. Fluorescence quenching was normalized to 1 and plotted versus total dTMP-PCP concentration (□). For comparison, [α-32P]dTMP-PCP binding to 4A' was measured under exactly the same conditions by the membrane binding assay, and the number of dTMP-PCP bound per hexamer was plotted versus total dTMP-PCP concentration (●).
Figure 39
with nucleotide ligands. This hypothesis was tested by treating 4A' with EDTA (5 mM) for 1 to 2 hours, to sequester magnesium ions and promote dissociation of tightly-bound nucleotides, prior to measurement of dTMP-PCP binding. EDTA-treated 4A' still bound only 3 dTMP-PCP per hexamer with high affinity. Binding was also tested over a prolonged time period to test for slow binding, however, no additional dTMP-PCP bound to the hexamer even after incubation for 3 hours.

Interaction of MgdTDP with 4A' Protein.

During dTTP hydrolysis, gene 4 proteins may exist in intermediate states with dTTP and dTDP bound to different subunits within one hexamer. Therefore, it is possible that the observed half-site binding is due to the presence of only nucleotide triphosphate in the reaction. An equilibrium binding assay was performed to measure dTDP binding to 4A' protein in the absence and presence of dTMP-PCP. [α-32P]dTDP was prepared by 4A'-catalyzed hydrolysis of [α-32P]dTTP for use in the membrane binding assay. Figure 40A shows dTDP binding to a constant amount of 4A' protein. The isotherm fits to a quadratic equation with a $K_d$ of $4.17 \pm 0.97 \times 10^{-6}$ M, and a maximum of 3 dTDP bind per 4A' hexamer. Both dTTP and dTDP bind with similar affinity, suggesting that removal of a phosphate group does not significantly affect
Figure 40. Equilibrium binding of Mg\text{dTDP} to 4A' protein in the absence and in the presence of d\text{TMP-PCP}. d\text{TDP} binding to 4A' was measured at 22 °C using [\alpha-\text{\textsuperscript{32}P}]d\text{TDP} and nitrocellulose membrane binding assays. A, shows [\alpha-\text{\textsuperscript{32}P}]d\text{TDP} binding to 4A' (20 \muM) at increasing d\text{TDP} concentrations (0–200 \muM). Three d\text{TDP} bind per 4A' hexamer and the isotherm fits to a quadratic equation with a K_d of 4.17 ± 0.97 x 10^{-6} M. B, shows binding of [\alpha-\text{\textsuperscript{32}P}]d\text{TDP} and [\alpha-\text{\textsuperscript{32}P}]d\text{TMP-PCP} to 4A'(20 \muM) at increasing d\text{TDP} concentrations (0–200 \muM). (▲), shows [\alpha-\text{\textsuperscript{32}P}]d\text{TDP} binding to 4A' in the presence of 20 \muM nonradiolabeled d\text{TMP-PCP} and (●), shows [\alpha-\text{\textsuperscript{32}P}]d\text{TMP-PCP} (20 \muM) binding to 4A' at increasing concentrations of nonradiolabeled d\text{TDP}. In the presence of d\text{TMP-PCP}, d\text{TDP} binds 4A' with an apparent K_d of 76.6 ± 6.8 x 10^{-6} M.
Figure 40
interaction of nucleotides with 4A'. dTDP binding is sensitive to magnesium ion concentration, however, and dTDP binding is 6-fold weaker at 3 mM Mg\(^2+\) than at 10 mM Mg\(^2+\). This effect may be a result of lower stability of the Mg\(_2\)dTDP complex as compared to Mg\(_2\)dTTP.

A constant amount of 4A' and dTMP-PCP was titrated with increasing concentrations of dTDP in order to determine if more than 3 nucleotide binding sites on 4A' are occupied in the presence of both dTDP and dTMP-PCP. In the presence of dTMP-PCP (Figure 40B), dTDP binds to 4A' with an apparent $K_d$ of $76 \pm 6.8 \mu M$, indicating that dTDP binding to 4A' is 18-fold weaker in the presence of dTMP-PCP. Furthermore, the amount of dTMP-PCP bound to 4A' decreases with increasing dTDP concentration, and no more than 4 nucleotides bind per hexamer at all dTDP concentrations tested. dTDP and dTMP-PCP apparently compete for the same sites on the hexamer, and the presence of both ligands does not affect the negative cooperativity in nucleotide binding to 4A'.

Titration of 4A'-ssDNA Complex with dTMP-PCP.

It is clear now that 4A' and 4B proteins form hexamers that bind ssDNA in the presence of NTPs, and stable hexamers bind up to three nucleotide ligands with high affinity. A DNA binding experiment was designed in order to confirm that three NTPs per hexamer support DNA binding, and to determine the minimum number of nucleotides per hexamer required for
Figure 41. DNA binding to 4A' and 4B hexamers measured 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. Correspondingly, [α-32P]dTMP-PCP binding to 4A' was measured under the same conditions using the nitrocellulose binding assays. A, shows binding of radiolabeled 30-mer DNA (4 μM) to 4A' (17 μM) at increasing dTMP-PCP concentrations (○), as well as binding of [α-32P]dTMP-PCP to 4A' in the presence of 4 μM nonradiolabeled 30-mer (■). The isotherms show that complete 30-mer DNA binding (one 30-mer per hexamer) occurs with a minimum two dTMP-PCP molecules bound per 4A' hexamer. B, shows the results of identical assays of DNA binding (○) and dTMP-PCP binding (■) with 4B protein (20 μM). DNA binding to 4B saturates with one dTMP-PCP molecule bound per hexamer.
stable hexamer formation and ssDNA binding. Equilibrium binding of a radiolabeled 30-mer ssDNA (to 4A' and 4B proteins) was measured as a function of dTMP-PCP concentration. Figures 41A and 41B show DNA binding to 17 μM 4A' and 20 μM 4B protein, respectively, measured by the NC-DEAE membrane binding assay (Chapter III). As dTMP-PCP concentration increases more DNA binds to the protein; the inflection points of the binding isotherms provide the minimum concentration of dTMP-PCP required to saturate DNA binding. Figures 41A and 41B also show complementary experiments in which dTMP-PCP binding to 4A' and 4B was measured under the same conditions. Comparison of the two isotherms shows that DNA binding to 4A' (one 30-mer per hexamer) saturates with an average of 2 dTMP-PCP molecules bound per hexamer (Figure 41A). Even more striking is the result that complete DNA binding to 4B occurs with an average of 1 dTMP-PCP bound per hexamer (Figure 41B). These experiments are highly informative because the results suggest that 4A' and 4B proteins assemble into hexamers with only 1 to 2 dTMP-PCP bound per hexamer, and these hexamers are competent for DNA binding. The results are consistent with an earlier finding that mixed hexamers of 4A' and 4A'/K318A (a mutant defective in dTMP-PCP-facilitated DNA binding) bind ssDNA with the same stoichiometry as hexamers of 4A' alone (Patel et al., 1994; Chapter IV, Part A).
Helicase Activity of 4A' in the Presence of dTTP and ATP.

Studies in the literature indicate that bacteriophage T7 helicases prefer to utilize dTTP as the energy source for duplex DNA unwinding. T7 helicases hydrolyze dTTP with the lowest $K_m$ value compared to several other nucleotides (Patel et al., 1992; Matson & Richardson, 1983). A report by Matson and Richardson (1985; Matson et al., 1983) notes that dTTP promotes optimal DNA binding and unwinding by gene 4 proteins, but no quantitative data have been published. Most well-studied helicases such as *E. coli* DnaB (Reha-Krantz & Hurwitz, 1978b; Arai & Kornberg, 1981a-c; Lebowitz & McMacken, 1986), *E. coli* Rep (Yarranton & Gefter, 1979; Wong & Lohman, 1992), and *E. coli* transcription terminator Rho (Lowery & Richardson, 1977a,b) use ATP hydrolysis to facilitate unwinding. Therefore, it is curious that gene 4 proteins prefer dTTP as a substrate and energy source. In order to confirm earlier reports that T7 helicases prefer dTTP for unwinding, and to get a quantitative measure of the difference between dTTP and ATP, T7-catalyzed DNA unwinding was measured in the presence of each nucleotide. A small, fluorescein-labeled, synthetic-fork substrate was prepared by annealing two 60-base oligodeoxynucleotides with 33-base complementary regions and 27-base single-stranded tails. A schematic in Figure 42A depicts the synthetic fork with fluorescein within the duplex region, and the hexameric helicase bound to the 5'-tail. Unwinding of the fork results
Figure 42. 4A'-catalyzed DNA unwinding in the presence of dTTP and ATP. DNA unwinding was measured in a stopped-flow by monitoring increase in fluorescence ($\lambda_{\text{ex}}=494$ nm; $\lambda_{\text{em}}>530$ nm) of a fluorescein-tagged fork DNA as described in Materials and Methods. A, 4A' (2 µM) was mixed rapidly with 5 nM fork + 500 µM dTTP at 18 °C, and the signal was monitored for 80 s. The smooth curve shows the best fit of one exponential term to the data and yields an unwinding rate of 0.12 s$^{-1}$ or 4 bp/s. B, shows a similar assay performed with 2 mM ATP, which yields an exponential rate of 0.0013 s$^{-1}$ or 0.04 bp/s.
$y = m_1 \cdot \exp(-m_2 \cdot m_0) + m_3$

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Figure 42
in a fluorescence change of about 10% (\(\lambda_{em} = 494\ \text{nm}, \ \lambda_{ex} > 530\ \text{nm}\)). DNA unwinding was initiated by rapidly mixing 4A' with the fork and NTP in a stopped-flow instrument (18 °C), and the fluorescence signal was monitored continuously for 100-800 s. Figure 42A shows the increase in fluorescence upon 4A'-catalyzed unwinding of the fork, in the presence of dTTP. The curve was fit to a single exponential term which yielded a rate of 0.12 s\(^{-1}\), or 0.12 x 33 base pairs = 4 bp/s. That the change in fluorescence corresponds to the actual unwinding was confirmed from a detailed study performed by Peter Ahnert in the laboratory (the same fork substrate was radiolabeled and unwinding was measured by quantitating the fork and the single-stranded products, separated by polyacrylamide gel electrophoresis). The fluorescence-based assay was then used to measure 4A'-catalyzed unwinding in the presence of ATP, which yielded a rate of 0.04 bp/s (Figure 42B). Thus, DNA unwinding rate in the presence of dTTP is at least 100-fold faster than in the presence of ATP, even though 4A' hydrolyzes ATP at least 5-fold faster than dTTP (Patel et al., 1992).

Interaction of 4A' with ATP and ATP\(_{yS}\).

The difference in unwinding rates was investigated further by comparing equilibrium interaction of ATP. As shown in Figure 43A, the \(K_d\) of the 4A'•ATP complex is 2.9 x
Figure 43. 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. A, shows [α-32P]ATP binding 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 x 10^{-6} M and binding saturates with a maximum of 3 ATP per hexamer. B, shows binding of [γ-35S]ATP to 4A' (0.2 μM) at increasing ATPγS (0-25 μM). The isotherm fits to a quadratic equation with a K_d of 5 x 10^{-6} M and saturates at 3 ATPγS bound per 4A' hexamer.
Figure 43

A

ATP bound per hexamer

[ATP$_{\text{bound}}$] μM

[ATP]$_{\text{total}}$ μM

B

[ATP$_\gamma$S] bound per hexamer

[ATP$_\gamma$S]$_{\text{total}}$ μM

[ATP$_\gamma$S] bound μM

Figure 43
10^-7 M, which is 6-fold weaker than the K_d for the 4A'•dTTP complex (5 μM). Figure 43B shows that ATPγS (nonhydrolyzable analog) binds with a K_d of 1.7 x 10^-6 M, which is 2 to 3-fold weaker than the K_d of the 4A'•dTMP-PCP complex. Binding saturates with a maximum of 3 ATP or ATPγS bound per 4A' hexamer - as seen with dTTP. At high concentrations of 4A' (20 μM) a fourth ATPγS molecule binds to the hexamer, similar to dTMP-PCP. It should be noted that 4A' protein also contains a primase site that initiates RNA primer synthesis with ATP; therefore, it is possible that ATP and ATPγS bind to the primase NTP-binding sites on the hexamer rather than the helicase NTP-binding sites. Competitive binding of dTMP-PCP and ATPγS showed, however, that both ligands compete for the same high-affinity sites on the helicase hexamer. Thus, even though ATP binds to the same sites, and in a similar fashion as dTTP or dTMP-PCP, it is unable to facilitate optimal rates of 4A'-catalyzed DNA unwinding.

Effect of ATP Analogs on ssDNA Binding to 4A'.

The preferential use of dTTP over ATP in 4A'-catalyzed unwinding was studied further by measuring the ability of ATP analogs to support stable DNA binding to 4A' protein. Equilibrium binding of ssDNA to 4A' was titrated with increasing concentrations of nucleotides. As shown in Figure
Figure 44. 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'-'32P 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 DNA binding is undetectable in the presence of ATPγS.
44, DNA binds tightly to 4A' protein in the presence of AMP-PCP and dAMP-PCP. The binding isotherm is sigmoidal in the presence of AMP-PCP, perhaps because of weak affinity between 4A' and AMP-PCP. In the presence of dAMP-PCP, however, the DNA binding isotherm appears identical to that in the presence of dTMP-PCP (Figure 41A). Interestingly, no DNA binding is detectable in the presence of ATPγS, even at concentrations as high as 1 mM.

DISCUSSION

Negative Cooperativity.

DNA helicases catalyze duplex DNA unwinding with energy derived from nucleotide binding and hydrolysis. This study examines interactions of various nucleotides with bacteriophage T7 helicases, 4A' and 4B, to better understand and quantitate the parameters and effects of nucleotide binding. Nitrocellulose membrane binding assays and fluorescence-based assays showed that nucleotides bind gene 4 proteins with both positive and negative cooperativity. Only three nucleotides bind per helicase hexamer, indicating that the remaining sites bind nucleotide very weakly (at least 50-fold weaker than tight-binding nucleotides), or remain unoccupied. This negative cooperativity is a property of all the nucleotides tested, including dTTP, ATP, dTDP, and the nonhydrolyzable analogs of ATP and dTTP. The
negative cooperativity or half-site binding is not an artifact caused by the presence of only nucleotide triphosphate or diphosphate in the assays. When tested with a mixture of dTDP and dTMP-PCP, 4A' does not bind more than a total of 4 nucleotides per hexamer, and the two nucleotides compete for the same high-affinity binding sites. Single-stranded DNA has no discernable effect on the negative cooperativity. Furthermore, the same, apparent half-site binding is observed at low protein concentrations at which 4A' is predominantly in dimeric state. Thus, negative cooperativity in nucleotide binding exists in both dimeric and hexameric forms of gene 4 proteins.

Similar negative cooperativity in nucleotide binding has been observed with other hexameric helicases; although, the effect is not as striking as in the case of T7 helicases. *E.coli* DnaB helicase has three high-affinity and three low-affinity ATP binding sites, which are reportedly sensitive to temperature. At 0 °C, all six ATP bind to the hexamer with similar affinity, rendering cooperativity almost undetectable (Arai & Kornberg, 1981b; Bujalowski & Klonowska, 1993). This is not the case with gene 4 proteins, because binding isotherms at 4 °C and 22 °C are identical and show only 3 dTTP bound per hexamer. *E.coli* Rho transcription termination protein, studied by Stitt (1988), was found to have only 3 tight ATP binding sites. A later report showed that at high concentrations of Rho and ATP,
additional ATP bind to the hexamer with 20 to 30-fold lower affinity compared to the tight binding sites (Geiselmann & von Hippel, 1992).

The mechanistic significance of negative cooperativity in hexameric helicases remains unclear at the present time. The extreme negative cooperativity or 'half-site reactivity', has been seen for several other enzymes, including aspartate carbamoyltransferase (Suter & Rosenbusch, 1976; Seydoux et al., 1974). In such a case, while a hexamer of identical subunits may be favored structurally, NTP binding and hydrolysis may be required of only three subunits, while the other three subunits remain functionally silent. Alternatively, a different interaction of nucleotides with the remaining three subunits may serve another purpose, perhaps of a structural or regulatory nature. Such a situation exists in the F$_i$-ATPase hexamer where biochemical studies and high-resolution structure determination have shown that out of 6 ATP binding sites, only 3 are catalytically active (Abrahams et al., 1994). Once the molecular mechanism of the coupling between nucleotide binding/hydrolysis and DNA unwinding is better understood, the reason for negative cooperativity may become clear.
Ligand-linked Oligomerization.

Previous studies of 4A' and 4B oligomerization have shown that nucleotide ligands promote hexamer formation. It is also clear that oligomerization of 4A' is sensitive to nucleotide ligand concentration (Chapter II). Reciprocally, nucleotide binding is sensitive to protein concentration. Three nucleotides bind per hexamer with a positive cooperativity that increases with protein concentration. If hexamers bind nucleotides with a higher affinity than monomers or dimers, positive cooperativity may be a manifestation of ligand-linked oligomerization (Scheme 6). A diagnostic test for cooperativity resulting from ligand-induced oligomerization is the dependence of the magnitude of cooperativity on protein concentration. At low and high protein concentrations, where the protein exists predominantly as monomers/dimers and hexamers, respectively, there should be no cooperativity in nucleotide binding (Wong & Lohman, 1995). This study shows that at low 4A' concentrations, dTMP-PCP binding isotherms are hyperbolic, and at higher protein concentrations the isotherms do not fit to a hyperbola with the same $K_d$ value (0.6 μM). Stable hexamer formation, in the absence of nucleotides, requires more than 100 μM 4A' protein (Patel & Hingorani, 1993; Chapter II). Therefore, it is not feasible to test whether cooperativity disappears once 4A' exists purely as hexamers;
it is possible that 4A' hexamers bind nucleotides with an intrinsic positive cooperativity.

A model of the ligand binding-linked oligomerization process has been depicted in Scheme 6. At low protein concentrations, gene 4 proteins exist as stable monomers/dimers in equilibrium. In the presence of nucleotide ligands, 'half-filled' dimers are formed (one nucleotide per dimer). For simplicity the scheme shows that 3 dimers associate to form hexamers via pathways 1-4. Considering the propensity of gene 4 proteins for 'head-to-tail' interactions, it is more likely that hexamers are formed via a number of intermediate species, including trimers, tetramers, etc. As explained earlier, under conditions where pathways 1 or 4 are predominant (high and low protein concentrations, respectively), no cooperativity should be detectable (dissociation constant dominated by nucleotide binding affinity of only one protein species). At intermediate protein concentrations, positive cooperativity can occur if hexamers are formed via pathways 2 and 3, i.e., hexamers with less than three NTP per hexamer are formed. Tighter binding of nucleotide ligands to the empty sites on the partially-filled hexamers results in positive cooperativity. 4A' concentration (20 μM), at which significant positive cooperativity in nucleotide binding has been observed, is neither low nor high enough for pathways 1 or 4 (Chapter II: HPLC gel-filtration of 4A' and 4B in the
Scheme 6. Helicase oligomerization linked to nucleotide binding
absence of nucleotide ligands). Evidence supporting this model of hexamer assembly, i.e., formation of partially-filled hexamers of 4A' and 4B proteins has been obtained from DNA and nucleotide binding assays. It was shown previously that DNA binds stably only to the hexamer species (Chapter III; Hingorani & Patel, 1993). Therefore, measurement of DNA binding to gene 4 proteins, at increasing dTMP-PCP concentrations, provided the amount of hexamers present at each dTMP-PCP concentration. Comparison of dTMP-PCP binding and DNA binding, under identical conditions, confirmed the presence of partially-filled hexamers, formed with an average of 1 to 2 nucleotides bound per hexamer.

Thus, in addition to modulating ssDNA binding to the helicase, nucleotide ligands modulate formation of active hexamers via ligand binding-linked oligomerization. NTP binding very likely causes changes in contact regions between subunits of oligomers that favor formation of stable hexamers. Nucleotide binding can change intersubunit contacts simply by binding at the interface of two subunits, as is the case for F₁-ATPase (Abrahams et al., 1994), and many other allosteric proteins that form oligomers (Traut, 1994; Neet, 1995). Binding at the interface of two subunits can also provide a physical explanation for negative cooperativity or half-site binding to the helicase dimers, and the fourth nucleotide is bound only at high protein concentrations, when protein forms hexamers.
Negative cooperativity need not be an inherent property of the hexamer, but may be induced by nucleotide binding itself. Nucleotide binding to one unit can affect the affinity of the adjacent subunit for a nucleotide ligand. Nucleotide induced conformational changes and communication through subunits are cornerstones of all proposed mechanisms of helicase activity. For example, a model for translocation and helicase activities of *E.coli* Rho proposes a conformational ‘switch’ of subunits, which is linked to ATP hydrolysis (Geiselmann et al., 1993). The switching promotes alternating high and low affinity RNA binding, thereby, effecting translocation. In general, dynamic changes in nucleotide binding affinity among subunits of the hexamer, which are coupled to cycles of NTP binding, hydrolysis, and product dissociation, result in changing affinity of each unit for DNA. Accordingly, the helicase can cycle through conformational states that effect translocation and processive DNA unwinding. Scheme 7 depicts such a pathway of unwinding, assuming that the three tightly bound NTPs are hydrolyzed consecutively in a process linked to translocation. Various forms of this alternating ‘DNA bind-release’ mechanism have been envisioned for other helicases (Lohman, 1992; 1993). Results from the titration of 4A•ssDNA complexes with dTMP-PCP demonstrate that the helicase binds stably to ssDNA with only 1-2 NTPs bound per hexamer. In addition, previous studies with mixed hexamers
Scheme 7. Translocation of the hexameric helicase on ssDNA (B)

$T = d\text{TP}$

$D = d\text{TDP} + P_i$
of active and inactive subunits have shown that the hexamer can bind ssDNA via 1-2 active subunits (Chapter IV, Part A; Patel et al., 1994). Therefore, when one or a few of the six subunits hydrolyze dTTP, resulting in low affinity for DNA, the helicase binds another length of DNA via a dTTP-filled unit and moves on DNA through sequential interactions, perhaps catalyzing DNA unwinding in this manner (Scheme 7).

ATP Utilization by T7 Gene 4 Helicases.

Most known helicases use ATP as the nucleotide substrate to catalyze DNA unwinding. Bacteriophage T7 gene 4 helicases are unusual in their preference for dTTP over ATP. 4A' helicase unwinds DNA with at least a 100-fold slower rate in the presence of ATP as compared to dTTP. Interactions of ATP with T7 helicases have been examined in order to understand the basis for the ineffectiveness of ATP. 4A' forms stable hexamers in the presence of ATP and ATPγS, although higher concentrations of ATP are required, compared to dTTP. ATPγS binding to 4A' is comparable to that of dTMP-PCP, and ATP binding is only about 6-fold weaker compared to dTTP. ATP hydrolysis is stimulated by DNA (Patel et al., 1992), which indicates that 4A' binds DNA in the presence of ATP. A study of DNA binding to 4A', in the presence of ATP analogs, showed that AMP-PCP and dAMP-PCP
support stable DNA binding, although DNA binding is undetectable in the presence of ATPγS.

ATP and dTTP differ mainly in their affinity for 4A', which may affect stable hexamer formation and DNA binding at low ATP concentrations. These differences can be overcome simply by increasing ATP concentration, which is comparable to in vivo conditions where ATP is more abundant than dTTP. High ATP concentrations, however, do not increase the rate of ATP-facilitated DNA unwinding. There are subtle differences in DNA binding to the helicase in the presence of ATP analogs. For example, although ATPγS binds to 4A', it does not support interaction between 4A' and DNA. This may indicate a problem in ATP-induced affinity of T7 helicases for DNA. It is equally likely, however, that the problem is limited to ATPγS, because AMP-PCP does promote DNA binding, although its own interaction with 4A' is rather weak. Interestingly enough, dAMP-PCP-facilitated DNA binding appears identical to dTMP-PCP-facilitated binding, and T7 helicases use dATP quite efficiently to catalyze DNA unwinding (Patel et al., 1992). A direct interpretation is that the missing 2'-hydroxyl group on the ribose moiety may be an important feature, specific to T7 helicases. The difference in ATP and dTTP may lie in their ability to promote specific interactions between helicase and DNA, or
it may be a more subtle difference within the coupling of NTP hydrolysis to DNA unwinding.
CHAPTER V
KINETIC MECHANISM OF HELICASE-CATALYZED dTTP HYDROLYSIS

ABSTRACT

Bacteriophage T7 gene 4 DNA helicases catalyze duplex DNA unwinding in a reaction coupled to dTTP binding and hydrolysis. The T7 helicase, 4A', assembles into its active, hexameric form upon binding to dTTP. Nucleotide binding studies have shown that three dTTP molecules bind per hexamer at equilibrium, and one to two dTTP per hexamer are sufficient for stable DNA binding. What is not clear, however, is the kinetic mechanism of dTTP binding and hydrolysis, which facilitates helicase translocation on DNA and DNA unwinding. This study investigates the pre-steady-state kinetics of the DNA-independent dTTPase activity of 4A', as a first step to understanding how dTTPase activity is coupled to DNA unwinding. The first turnover of dTTP hydrolysis has been examined by acid-quench and pulse-chase experiments using a rapid chemical quench-flow instrument. Based on the results, a minimal kinetic mechanism of dTTP binding and hydrolysis has been proposed:
Dm and H stand for 4A' dimer and hexamer, respectively, T is dTTP, D is dTDP + P_i, and the numbers (in superscript) denote the number of the nucleotide binding site on the hexamer, e.g., HT^i denotes dTTP bound to the first nucleotide-binding site on the hexamer. In this mechanism, the hexamer binds and hydrolyzes one dTTP per turnover. Furthermore, three sites on the hexamer cycle through dTTP binding and hydrolysis, one at a time. The apparent bimolecular dTTP binding rate (k_{on}) is 7-9 x 10^4 M^{-1}s^{-1}. 4A'-catalyzed dTTP hydrolysis rate (k_h) saturates at 0.7 s^{-1}, and the steady-state limiting turnover rate (k_{diss}; product dissociation) is 0.06 to 0.07 s^{-1}. This study provides the first kinetic mechanism of dTTP hydrolysis for a hexameric helicase, which shows how dTTP hydrolysis is tightly coordinated between the subunits of the hexamer, such that three sites on the hexamer bind and hydrolyze dTTP, one
after another. In view of recent evidence that ssDNA binds specifically to one subunit at a time, cycling of dTTP hydrolysis among sites can be directly related to cycling of DNA among the sites, which results in translocation of the hexameric helicase on ssDNA.

INTRODUCTION

DNA helicases are enzymes that unwind duplex DNA during the processes of DNA replication, recombination, repair, and conjugation. Helicases bind at the junction of forked DNA and, using energy derived from NTP binding and hydrolysis, disrupt hydrogen bonds that stabilize duplex DNA. DNA helicases are found in various organisms, and in all known helicases NTP hydrolysis is coupled to unwinding activity (reviews: Lohman, 1992, 1993; Matson, 1991; Matson and Kaiser-Rogers, 1990).

Bacteriophage T7 gene 4 encodes two hexameric helicases, 4A and 4B, which are essential for DNA phage DNA replication. These enzymes translocate over long lengths of DNA, processively, to catalyze DNA unwinding. Thus far, DNA binding studies have shown that 4A' and 4B hexamers interact stably with ssDNA (but not with dsDNA), and ssDNA binds tightly to one site on the hexamer (Chapter III). It has been demonstrated also that dTTP binding and hydrolysis modulate interactions of the T7 helicase with ssDNA (Chapter
Based on these results, we proposed a model wherein helicase "walks" along the ssDNA lattice, with its subunits cycling through 'high-affinity' and 'low-affinity' DNA binding states to effect translocation (Scheme 7, Chapter IV, Part B). There is ample evidence to suggest that NTP binding and hydrolysis are coupled to conformational changes that drive helicase movement along DNA (Hill & Tsuchiya, 1981; Lohman, 1992, 1993), but molecular details of how NTPase activity is coupled to translocation and DNA unwinding have yet to be understood.

Solution of a complete mechanism of helicase activity requires detailed information about the kinetics of dTTP binding and hydrolysis, as well as kinetics of DNA binding and duplex DNA unwinding. In this study, pre-steady-state kinetic techniques have been used to delineate the kinetic pathway of dTTP hydrolysis catalyzed by 4A' protein. Previous equilibrium nucleotide binding studies have shown that three dTTP bind per hexamer. It is not known whether the nucleotides are hydrolyzed simultaneously or sequentially, and if there is any coordination in dTTP hydrolysis by the hexamer subunits. We have begun to address these questions by measuring 4A'-catalyzed dTTP hydrolysis in a single turnover, in the absence of ssDNA. The results provide the basis for future investigations of 4A' dTTPase activity in the presence of DNA, necessary to understand the
coupling of NTP hydrolysis to helicase-catalyzed DNA unwinding.

MATERIALS AND METHODS

Nucleotides and Other Reagents.

dTTP was purchased from Sigma Chemicals Co. and [α-32P]dTTP (3000 Ci/mmol) was purchased from ICN Biomedicals. PEI-cellulose TLC plates were purchased EM Separations Technology. 4A' protein was purified as described (Chapter II).

Steady-State Kinetics of dTTP Hydrolysis.

Steady-state kinetics of 4A' dTTPase activity were measured at 18 °C by monitoring hydrolysis of [α-32P]dTTP to [α-32P]dTDP + P, as described in Chapter IV, Part A. 4A' protein (0.2 μM) was equilibrated at 18 °C for 5 min in dTTPase buffer B (50 mM Tris-acetate, pH 7.5, 40 mM sodium acetate, 1 mM DTT, and 10 % glycerol) plus 10 mM magnesium acetate. Reactions were initiated by addition of dTTP (0-60 μM) + [α-32P]dTTP, and quenched with EDTA after varying time intervals (0-50 min, depending on dTTP concentration). The products were analyzed by PEI-cellulose TLC and quantitated as described (Chapter IV, Part A). Initial rates of dTDP formation were determined from plots of molar amounts of
dTDP versus time of reaction. Steady-state $k_{cat}$ and $K_m$ values were determined from hyperbolic fit of the plot of dTTPase rates versus dTTP concentration.

Pre-Steady-State Kinetics of dTTP Hydrolysis.

Pre-steady-state kinetics of dTTP hydrolysis were measured using a rapid chemical quench-flow instrument (KinTek Corporation, State College, PA). 4A' (6, 12, 20, and 30 μM final concentration) was prepared in dTTPase buffer B containing 1 mM magnesium acetate (typical sample volume: 300-350 μl). The nucleotide solution containing dTTP (50-600 μM final concentration) and [α-32P]dTTP (20 μCi) was prepared in dTTPase buffer B plus 19 mM magnesium acetate. The experiments were carried out by loading the enzyme solution (15 μl) from one syringe and nucleotide solution (15 μl) from a separate syringe on the instrument, and rapidly mixing the two for 5 msec to several seconds. The reactions were quenched with 1 M HCl from a third syringe after mixing. Chloroform (100 μl) was added to denature the enzyme, after which the reactions were neutralized with base (0.25 M Tris-base, 1 M NaOH) within a minute of quenching. The product, [α-32P]dTDP, was analyzed by TLC, quantitated on a PhosphorImager (Molecular Dynamics) and plotted versus time of reaction, as described above for steady-state assays. In each assay a zero point was measured without
enzyme to quantitate the background dTDP. All experiments were performed at 18 °C (temperature maintained by a water-jacket around the reactant loops) and the solutions loaded into the reactant loops, i.e., ddH$_2$O, HCl, and dTTPase buffer B, were filtered through a 0.2 μm filter.

**Kinetics of dTTP Binding: Pulse-Chase Experiments.**

Pulse-chase experiments were performed at 18 °C in the rapid chemical quench-flow instrument. A' (6, 20 μM) and dTTP (50-600μM) solutions were prepared in dTTPase buffer B + 1 mM or 19 mM magnesium acetate, respectively, as described above. The reactions were initiated by rapidly mixing the two solutions and after varying time intervals (0.005-80 s), the reactions were chased with an excess of nonradiolabeled dTTP (10 mM final concentration). After a chase period of 30 s at 18 °C (6-10 x t$^{1/2}$ for dTTP hydrolysis), the reactions were quenched with 50 μl 1 N HCl. Chloroform (100 μl) was added to denature the enzyme and the solution was neutralized with base (0.25 M Tris-base, 1 M NaOH). The reactions were also chased by addition of 0.5 M EDTA instead of dTTP, and in this case quenching of the solution was not necessary; the products were directly analyzed by TLC. Both methods of chase yielded identical results.
Data Analysis:

Curve Fitting.

Kinetic curves were generated by plotting molar amounts of dTDP versus time of reaction. The burst kinetics were fit to an exponential term (or a sum of exponential terms; n) describing the burst phase, followed by a linear term describing the steady-state turnover rate as shown below:

\[ A_t = \left( A_0 \left[ 1 - \exp(-k_{obs} t) \right] \right) + kt + C \]  

(6)

where \( A_t \) is the amount of product formed at time \( t \), \( A_0 \) is the burst amplitude, \( k_{obs} \) is the observed burst rate, \( k \) is the rate of the linear phase, and \( C \) is the intercept on the Y-axis. The data were fit by nonlinear regression analysis using the KaleidaGraph data analysis and graphics program (Synergy Software, Reading, PA). The \( K_d \) curves from both pre-steady-state and steady-state experiments were fit to a hyperbola using the same software.

Simulation of Kinetic Mechanisms.

The acid-quench and pulse-chase results were modeled using the HopKINSIM kinetic simulation program (developed by Daniel Wachsstock, Johns Hopkins University, based on the KINSIM program developed by Carl Frieden and Bruce Barshop; Barshop et al., 1983). The simulated curves (constrained by measured kinetic constants) were compared with the kinetic
data to develop a mechanism for 4A'-catalyzed dTTP hydrolysis (Scheme 8).

RESULTS

Steady-State Kinetics of dTTP Hydrolysis.

T7 gene 4 helicases, 4A' and 4B, use dTTP binding and hydrolysis to power duplex DNA unwinding. At 22 °C the $k_{cat}$ for dTTP hydrolysis is 0.02 to 0.03 s⁻¹, in the absence of DNA, and the $K_m$ is $8.5 \pm 1.2 \times 10^{-6}$ M. At 18 °C the $k_{cat}$, shown in Figure 45, is $0.012 \pm 0.001$ s⁻¹, and the $K_m$ is equal to $2.5 \pm 0.6 \times 10^{-6}$ M.

Pre-Steady-State Kinetics of dTTP Hydrolysis by 4A' Helicase.

Pre-steady-state kinetics of dTTP hydrolysis were measured using the rapid chemical quench-flow instrument. 4A' was mixed rapidly with $[^{32}\text{P}]dTTP$, the reaction was quenched in msec to 60 s time intervals, and formation of $[^{32}\text{P}]dTDP$ was measured over time. High concentrations of enzyme were used in these experiments to measure accurately the first turnover.

Figure 46A shows the time course of an experiment performed with 20 μM 4A' and 100 μM dTTP. The pre-steady-state kinetics are biphasic, with a fast phase followed by a
Figure 45. Steady-state kinetics of 4A'-catalyzed dTTP hydrolysis. Steady-state dTTPase activity of 4A' protein was measured at 18 °C. 4A' (0.2 μM) was mixed with [α-32P]dTTP (0-60 μM) and the reaction was quenched with EDTA after varying times. The product, [α-32P]dTDP, was analyzed by TLC as described in Materials and Methods. Initial rates of dTTP hydrolysis (at various dTTP concentrations) were determined as slopes from plots of molar amount of dTDP formed versus time. Initial rates were plotted versus dTTP concentration and fit to a hyperbola with a $k_{cat}$ equal to 0.012 s⁻¹ (maximum rate / 4A' monomer concentration) or 0.072 (maximum rate / 4A' hexamer concentration) and $K_m$ equal to 2.5 ± 0.6 x 10⁻⁶ M.
slow linear phase. The data were fit to an equation describing an exponential burst phase followed by a linear steady-state rate (Eqn. 6, Materials and Methods) (review: Johnson, 1992). The burst shows that the product, dTDP, accumulates on the active site of the enzyme at a rate of $0.25 \pm 0.03 \text{ s}^{-1}$. The amplitude of the burst ($2.8 \pm 0.2 \text{ M}$) is about one-sixth of 4A' (monomer) concentration in the assay, suggesting that only one site per hexamer hydrolyzes dTTP in one turnover. The linear rate constant, determined from the slope of the linear phase divided by 4A' monomer concentration, equals $0.0092 \text{ s}^{-1} (0.183 \text{ M s}^{-1}/20 \text{ M 4A'})$, indicating a slow step after hydrolysis that limits steady-state turnover. The linear rate is comparable to the $k_{\text{cat}}$ for steady-state dTTP hydrolysis ($0.01 \text{ s}^{-1}$, Figure 45). Note that if only one dTTP is hydrolyzed per hexamer in one turnover, the steady-state rate constant should be $0.183 \text{ M s}^{-1}/3.3 \text{ M} (4\text{A'} \text{ hexamer})$, which is $0.056 \text{ s}^{-1}$.

The acid-quench experiment was performed at higher dTTP concentrations to measure the maximum burst rate (Table 4). The burst rate increases with dTTP concentration in a hyperbolic manner (Figure 46B) providing an apparent $K_d$ of about $2 \times 10^{-4} \text{ M}$ and a maximum rate constant of $0.73 \pm 0.05 \text{ s}^{-1}$. The burst amplitude is independent of dTTP concentration, confirming that only one site per hexamer hydrolyzes dTTP in the first turnover (Figure 46A, Table 4).
Figure 46. Pre-steady-state acid-quench kinetics of 4A’-catalyzed dTTP hydrolysis. 4A’ (20 μM in dTTPase buffer B + 1 mM MgOAc) was rapidly mixed with dTTP (50-600 μM) + [α-32P]dTTP at 18 °C to initiate the reaction. The reactions were quenched with 1 M HCl, treated with chloroform, and neutralized with base. The products were analyzed by TLC as described in the Materials and Methods. Panel A shows the pre-steady-state burst kinetics with 100 μM (●) and 600 μM (□) dTTP. The data fit to a burst equation (Eqn. 6) with burst rate constants equal to 0.26 s⁻¹ (at 100 μM dTTP), and 0.53 s⁻¹ (at 600 μM dTTP); the amplitude and the steady-state rate constant were 2.8-3.2 μM and 0.05 s⁻¹, respectively, for both curves. Panel B shows the dTTP concentration dependence of the pre-steady-state burst rate. The burst rate constants were plotted versus dTTP concentration and the data fit to a hyperbola with a Kₘ of 200 ± 40 μM, and a maximum hydrolysis rate equal to 0.73 s⁻¹.
Figure 46
Table 4: Parameters of pre-steady-state acid-quench kinetics of dTTP hydrolysis

<table>
<thead>
<tr>
<th>dTTP (μM)</th>
<th>Amplitude (μM)</th>
<th>Burst rate (s⁻¹)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A' monomer (6 μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>1.2 ± 0.1</td>
<td>0.41 ± 0.08</td>
<td>0.048</td>
</tr>
<tr>
<td>4A' monomer (20 μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>3.7 ± 0.5</td>
<td>0.11 ± 0.02</td>
<td>0.049</td>
</tr>
<tr>
<td>100</td>
<td>2.8 ± 0.15</td>
<td>0.26 ± 0.03</td>
<td>0.055</td>
</tr>
<tr>
<td>200</td>
<td>3.7 ± 0.3</td>
<td>0.37 ± 0.08</td>
<td>0.061</td>
</tr>
<tr>
<td>400</td>
<td>3.4 ± 0.16</td>
<td>0.51 ± 0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>600</td>
<td>3.2 ± 0.3</td>
<td>0.53 ± 0.14</td>
<td>0.043</td>
</tr>
<tr>
<td>4A' monomer (30 μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>7.7 ± 0.5</td>
<td>0.2 ± 0.02</td>
<td>0.084</td>
</tr>
</tbody>
</table>
As expected, the steady-state rate constant does not vary significantly with increasing dTTP concentrations (because these experiments were performed at dTTP concentrations above the measured $K_m$, 2.5 μM).

Based on the acid-quench experiments described above, a minimal mechanism for 4A'-catalyzed dTTP hydrolysis can be proposed:

\[
\begin{align*}
&\text{Dm} + T \rightleftharpoons \text{DmT} \rightleftharpoons \text{HT} \rightleftharpoons \text{HD} \rightleftharpoons \text{H} + D \\
&k_{\text{on}} \quad k_{\text{cat}} \quad k_h \quad k_{\text{diss}}
\end{align*}
\]

$k_{\text{on}}$ is the apparent rate of dTTP (T) binding either to the dimer (Dm) or to the hexamer (H), $k_h$ is the rate of dTTP hydrolysis on the enzyme active site, and $k_{\text{diss}}$ is the rate of product dissociation ($D = \text{dTDP}, P_i$). Previous studies have shown that 4A' hexamer binds 3 dTTP with high affinity. From the data shown above, it appears that only one dTTP is hydrolyzed per hexamer in one turnover. In addition, the above kinetics indicate that the steady-state $k_{\text{cat}}$ is largely a measure of recycling of the enzyme, limited perhaps by the dissociation of products from the enzyme.
Kinetics of dTTP Binding to 4A'.

Pulse-chase experiments were performed to investigate the kinetics of dTTP binding to the helicase (k_on in the above scheme). 4A' was mixed with [α-32P]dTTP in a rapid chemical quench-flow instrument, and the reactions were chased with excess nonradiolabeled dTTP instead of quenching with acid. During the chase period, any 'tightly-bound' substrate, i.e., substrate that does not dissociate from the enzyme, can undergo hydrolysis and be measured as product. This experiment provides a measurement of the active enzyme•substrate complex. Figure 47 A shows the time course of such an experiment performed with 20 μM 4A' and 200 μM dTTP. The observed burst kinetics contain two pre-steady-state exponentials followed by a relatively slow linear steady-state phase. The data were fit to an equation composed of the sum of two exponentials and a linear term (Eqn. 6, Materials and Methods). The amplitude of the first exponential corresponds to one site per hexamer, which appears to bind dTTP at a rate of 13.3 ± 3 s⁻¹ (see inset to Figure 47 A for expanded initial burst phase). The amplitude of the second phase corresponds to a second site per hexamer, which appears to bind dTTP at a much slower rate of 0.12 s⁻¹. The linear steady-state rate constant is 0.05 s⁻¹, same as the steady-state k_cat (0.07 s⁻¹).
Figure 47. Pre-steady-state pulse-chase kinetics of dTTP hydrolysis. Panel A: 4A' (20 µM) was mixed with dTTP (200 µM) at 18 °C to initiate the reaction. The reactions were chased either with excess dTTP (10 mM) (●), or EDTA (□). 30 s after addition of excess dTTP, the reactions were quenched with HCl and neutralized, and the products were analyzed by TLC. EDTA-quenched reactions were analyzed without further treatment. The resulting kinetics were fit to the sum of two exponentials and a linear phase (Eqn. 6). Both dTTP-chase and EDTA-chase curves exhibit a fast exponential phase (one site per hexamer) with a rate of ~ 14 s⁻¹, a slow exponential phase (one site per hexamer) with a rate of 0.12-0.19 s⁻¹, and a steady-state rate constant equal to 0.05 s⁻¹ (Table 5). The inset in Panel A shows the time course up to 2 s. Panel B shows the same reaction performed with 100 µM (●) and 600 µM (◆) dTTP. The burst rates increase with dTTP concentration (Table 5), however, the amplitudes, the rate of the second exponential, and the steady-state rate remain unchanged. Panel C shows the linear dependence of the first-phase burst rate on dTTP concentration, which yields an apparent $k_{\text{on}}$ equal to $7.4 \times 10^5$ M⁻¹ s⁻¹. Panel D shows the pulse-chase experiment performed at 6 µM (●), 12 µM (□), and 20 µM (◆) 4A' concentrations. The burst rate constants and steady-state rate constants are listed in Table 5.
Figure 47 (continued)

C

\[ y = -0.66573 + 0.073998x \quad R = 0.9766 \]

D

Sites per hexamer vs. Time (sec)
The chase experiment was also performed with EDTA as the chase instead of excess nonradiolabeled dTTP. EDTA can be used as a chase because it chelates Mg\(^{2+}\) in the reaction and prevents rebinding of radiolabeled dTTP once it has dissociated from the enzyme; thus chasing the enzyme·dTTP complex. Figure 47 A shows that EDTA is an effective chase, because the kinetics of the two experiments (chased with EDTA and with dTTP) are the same, within experimental error. It appears that EDTA does not strip Mg\(^{2+}\) from the 4A'·dTTP complex or otherwise affect stability of the complex, because both dTTP-chase and EDTA-chase experiments yield the same amplitudes for the burst phases. Since EDTA effectively mimics the effect of excess unlabeled dTTP as chase, it has been used in the pulse-chase experiments.

The pulse-chase experiments were performed at increasing dTTP concentrations to investigate substrate binding kinetics. dTTP concentration dependence of the rates of the first burst phase is linear, indicating a bimolecular binding reaction (Table 5). The slope of the line is equal to 7.4 \(x\) 10\(^{-4}\) M\(^{-1}\) s\(^{-1}\), which is the apparent \(k_{on}\) for dTTP binding to one site on the helicase hexamer (Figure 47C). The rate of the second phase, however, is independent of dTTP concentration (Table 5). The most direct explanation for this result is that 'tight-binding' of dTTP to the second site (on the hexamer) is limited by a step that is independent of dTTP concentration.
Table 5: Parameters of pre-steady-state pulse-chase kinetics of dTTP hydrolysis

<table>
<thead>
<tr>
<th>dTTP (μM)</th>
<th>Amplitude 1 (μM)</th>
<th>Burst rate 1 (s⁻¹)</th>
<th>Amplitude 2 (μM)</th>
<th>Burst rate 2 (s⁻¹)</th>
<th>k_{cat} (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A' monomer (20 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 0.2</td>
<td>10 ± 1</td>
<td>2 ± 0.2</td>
<td>0.23 ± 0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4 ± 0.3</td>
<td>8 ± 0.8</td>
<td>2.4 ± 0.2</td>
<td>0.24 ± 0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>200&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 0.3</td>
<td>13.3 ± 3</td>
<td>3.3 ± 0.3</td>
<td>0.12 ± 0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>200&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6 ± 0.2</td>
<td>14.3 ± 1.6</td>
<td>2.6 ± 0.3</td>
<td>0.19 ± 0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>300</td>
<td>4.8 ± 0.5</td>
<td>26.9 ± 6</td>
<td>4.1 ± 0.6</td>
<td>0.21 ± 0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>400</td>
<td>2.8 ± 0.3</td>
<td>23 ± 2</td>
<td>1.8 ± 0.9</td>
<td>0.18 ± 0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>500</td>
<td>3.1 ± 0.2</td>
<td>38.2 ± 5</td>
<td>3.4 ± 0.4</td>
<td>0.19 ± 0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>4A' monomer (6 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.1 ± 0.05</td>
<td>4.9 ± 0.6</td>
<td>1.2 ± 0.1</td>
<td>0.08 ± 0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>100</td>
<td>0.93 ± 0.1</td>
<td>6.5 ± 2</td>
<td>1.2 ± 0.2</td>
<td>0.12 ± 0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup> dTTP chase;  <sup>b</sup> EDTA chase.
Pulse-chase experiments were also performed at different concentrations of 4A' (6 and 12 μM monomer concentration) to investigate any effects of varying protein concentration on the kinetics of dTTP binding. The time course in Figure 47D shows that at 6 μM 4A', dTTP binds to one site on the hexamer with a bimolecular rate constant of $1 \times 10^5$ M$^{-1}$ s$^{-1}$ and to the second site on the hexamer with a rate of 0.1 s$^{-1}$, which is independent of dTTP concentration (Table 5). Similar results were obtained at 12 and 20 μM 4A', with slight variations within experimental error.

DISCUSSION

Steady-State Kinetics of T7 Helicase-Catalyzed dTTP Hydrolysis.

This study investigates the kinetics of the DNA-independent dTTPase activity of 4A'. Steady-state measurements show that the turnover rate constant ($k_{cat}$) for dTTP hydrolysis catalyzed by 4A' is $0.07 \pm 0.003$ s$^{-1}$. In general, $k_{cat}$ is a measure of the slowest step or a combination of slow steps in the reaction pathway, and can be limited by substrate binding, chemistry, or product dissociation. Thus, steady-state kinetic parameters do not provide any information about specific steps in the pathway of helicase-catalyzed dTTP hydrolysis.
Pre-steady-state kinetics of DNA-independent dTTP hydrolysis by 4A' were measured for the following reasons: a) to measure the rate constants for the individual steps in dTTP hydrolysis; b) to determine the rate-limiting step in the reaction; c) to determine the number of sites per hexamer that hydrolyze dTTP in one turnover; d) to examine any coordination of dTTP hydrolysis among the subunits (of the hexamer), that may be important for helicase activity; and e) to establish a model for the mechanism of dTTP hydrolysis, on which subsequent studies relating dTTP hydrolysis to DNA unwinding can be based.

Pre-Steady-State Kinetics of T7 Helicase-Catalyzed dTTP Hydrolysis:
Sequential Model for 4A'-Catalyzed dTTP Hydrolysis.

Briefly, pre-steady-state kinetics of dTTP hydrolysis showed that the 4A' hexamer hydrolyzes one dTTP per turnover. Pulse-chase experiments showed that one dTTP binds per hexamer with a bimolecular rate constant of 7-8 x 10^4 M^-1 s^-1 and a second dTTP molecule binds to the hexamer at a rate that is slower than or comparable to hydrolysis of dTTP bound to the first site. Based on these kinetic results and all the available information about the hexameric helicase, a sequential mechanism for 4A'-catalyzed dTTP hydrolysis is proposed (Scheme 8A; pictorial depiction in Scheme 8B):
We have shown previously that dTTP binds to the 4A' dimer and induces stable hexamer formation (Scheme 6, Chapter IV, Part B). In this model, the hexamer species is formed with one dTTP bound, which is converted into product. Following hydrolysis, a second dTTP binds to the hexamer (with high affinity), but is hydrolyzed only after dissociation of products from the first site. The same process continues with the third dTTP binding site and cycles back to the first site as shown.

Formation of "HT1" occurs with an apparent $k_{on} = 7 \times 10^4$ M$^{-1}$ s$^{-1}$. Since dTTP binding is linked to oligomerization, most likely, dTTP binds to a monomer or dimer species, which assembles into a hexamer. The measured on rate is much lower than the diffusion-controlled rate constant of $10^8 - 10^9$ M$^{-1}$ s$^{-1}$. The exact reason for this is unclear, but it may be that the apparent $k_{on}$ represents several steps. Consistent with this theory, dTTP dependence of the burst rate in the acid-
Scheme 8B. Kinetic mechanism of 4A'-catalyzed dTTP hydrolysis (sequential mechanism)
quench experiments provided an apparent $K_d$ of 200 $\mu$M, which may represent formation of a weak 4A$'$$\cdot$dTTP complex prior to tight binding of dTTP to the hexamer with a $K_d$ of 5 $\mu$M (equilibrium binding experiments, Chapter IV, Part B).

dTTP binding appears to be a multi-step process which includes hexamer formation and may even be limited by conformational changes necessary for tight enzyme$\cdot$substrate complex formation. Preliminary data from fluorescence-based binding assays (stopped-flow kinetics) confirm the apparent rate of dTTP binding to 4A$'$ as $8 - 9 \times 10^4$ M$^{-1}$ s$^{-1}$ (Experiments performed by Kristen Moore in the laboratory). Similar experiments are being used to determine the rates of helicase hexamer formation and to test for any potential conformational changes during dTTP binding and hexamer assembly.

One dTTP per hexamer is hydrolyzed at a maximum rate of 0.7 s$^{-1}$ in one turnover. This could mean that only one site per hexamer hydrolyzes dTTP over and over again, or that different sites on the hexamer cycle through dTTP hydrolysis, one at a time. The pulse-chase data show that dTTP binds to a second site on the hexamer with its rate limited by a step after the first dTTP binding, but before the end of the first turnover (defined as recycling of enzyme). This is depicted in the model (Scheme 8A and 8B) as second dTTP binding to the hexamer after hydrolysis of the first dTTP, but before product dissociation. Thus, more than
one dTTP binds per hexamer, but only one dTTP is hydrolyzed at a time. After hydrolysis of the first two dTTP per hexamer, the reaction enters steady-state. Enzyme turnover is limited by a step following hydrolysis, which in this case yields a $k_{cat}$ of 0.05-0.07 s$^{-1}$. This steady-state limiting rate may reflect either product dissociation or some conformational change required for turnover of the enzyme, which will be clarified by future experiments that directly measure the rate of product dissociation from the enzyme.

It is important to test this model by global analysis using kinetic simulation, to make certain that one and only one model explains all the available data. The experimentally determined rate constants were applied to the model described by Scheme 8, which was simulated by the HopKINSIM kinetic simulation program. The simulated curves were compared to data from all the pre-steady-state experiments and the fit was constrained by the measured kinetic constants. Figure 48 shows data from one set of pulse-chase and acid-quench experiments (4A' 20 μM; dTTP 100 μM) compared with the corresponding simulated curves. The curves fit the data with the following intrinsic constants: $k_{on} = 1 \times 10^5$ M$^{-1}$ s$^{-1}$, $k_h = 0.7$ s$^{-1}$, and $k_{diss} = 0.08$ s$^{-1}$ (refer to Tables 4 and 5 for kinetic constants from curve-fitting of the data). The kinetic constants for dTTP binding and hydrolysis are the same, within error, between the
Figure 48. Agreement of the simulated kinetics of dTTP hydrolysis to pre-steady-state acid-quench and pulse-chase results. Kinetic results from acid-quench (□), and pulse-chase experiments (○), performed with 20 μM 4A' and 100 μM dTTP, are compared to simulated kinetic curves (solid lines). The simulated curves were generated by the HopKINSIM program using the sequential mechanism of dTTP hydrolysis shown in Scheme 8A, 8B.
experimental data and the simulated curves. The product
dissociation constant defined in the sequential model, $k_{\text{diss}}$, is also comparable to the $k_{\text{cat}}$ value determined from steady-state experiments ($0.07 \text{ s}^{-1}$ with only one dTTP hydrolyzed per turnover, per hexamer).

The minimal mechanism for sequential dTTP hydrolysis catalyzed by 4A' agrees well with the experimental data from this study. It has been shown earlier that ssDNA binds tightly to one site on the hexamer, and helicase affinity for DNA is modulated by dTTP binding and hydrolysis (high-affinity with dTTP and low-affinity with dTDP) (Chapter III; Hingorani & Patel, 1993). Based on this information a mechanism for helicase translocation has been proposed, in which DNA is bound and released sequentially by sites on the hexamer, resulting in movement of the helicase on ssDNA (depicted in Scheme 9). In summary, this study shows that coordination of dTTP binding and hydrolysis among subunits is an intrinsic property of the hexameric helicase and likely plays an important role in the coupling of dTTPase activity to translocation and DNA unwinding.

Comparison of Translocation and DNA Unwinding Models Proposed for Various Helicases.

This is the first time a model for the molecular mechanism of a hexameric helicase has been proposed that is based on a comprehensive study of its structure, DNA binding
Scheme 9. Translocation of the hexameric helicase on ssDNA (C)

\[ T = d\text{TTP} \]
\[ D = d\text{TDP} + P_i \]
properties, and nucleotidase activity. There are a number of similarities among helicases currently being investigated by various research groups. Most significantly, hexameric helicases assemble into ring-shaped structures (review: Lohman & Bjornson, 1996). Our study of the T7 helicases provides an explanation for the importance of the ring-assembly. According to the proposed model, the helicase ring functions as a rotating "motor" to translocate on DNA, and the ring-shape must provide topological stability to the helicase-DNA complex and aids processivity.

There are, however, a number of apparent differences even among hexameric helicases. For example, different hexameric helicases interact with DNA in different ways: SV40 T antigen (SenGupta & Boroweic, 1992; Wessel et al., 1992) and *E. coli* DnaB (Arai & Kornberg, 1981d) bind both ssDNA and dsDNA, while T7 helicases appear to interact stably only with ssDNA (Chapter III; Hingorani & Patel, 1993). Nevertheless, the sequential mechanism of translocation, involving coordinated NTP binding and hydrolysis can be generally applicable to any helicase that functions as a multisubunit assembly and uses NTPase activity to modulate DNA interactions.

*E. coli* Rep Helicase.

One other helicase, whose mechanism of unwinding has been extensively studied, is the dimeric *E. coli* Rep
helicase. Wong & Lohman (1992) have proposed an active, "rolling" mechanism for Rep-catalyzed DNA unwinding. Briefly, each subunit of the Rep dimer alternates between binding to ssDNA and dsDNA (controlled by ATP binding and hydrolysis), and the enzyme unwinds DNA by binding to and actively melting successive lengths of duplex DNA (Wong & Lohman, 1992; review: Lohman & Bjornson, 1996). It has been suggested, that the T7 helicases may function as a trimer of 'Rep-like' dimers (Lohman & Bjornson, 1996). Our current information, however, indicates there are more differences than similarities between hexameric and dimeric helicases. For example, during unwinding each subunit of a Rep dimer can bind either ssDNA or dsDNA to form Rep dimers that are 'half-saturated' (one ss or dsDNA strand per dimer) or 'fully-saturated' (two ss or dsDNA strands per dimer). With the T7 helicases, on the other hand, ssDNA binds tightly to one site on the hexamer and interaction of duplex DNA is very weak compared to the high affinity binding of ssDNA. For now, it appears that dimeric and hexameric helicases may have different mechanisms for translocation and unwinding DNA; however, further details of the mechanism of T7 helicases and other hexameric helicases are necessary before any model can be chosen or rejected.
E. coli Rho Transcription Termination Factor.

Some theoretical models have been proposed to explain translocation of helicases on DNA. *E. coli* Rho transcription termination factor is a hexameric ring-shaped protein that catalyzes unwinding of DNA–RNA hybrid duplexes, coupled to ATPase activity. Each subunit in the Rho hexamer binds ssRNA and ATP; three with high affinity, and three with much lower affinity (Stitt, 1988; Geiselman & von Hippel, 1992; Geiselmann et al., 1992; Wang & von Hippel, 1993). In the model, a dimer is the functional unit of the hexamer. It is proposed that ATP hydrolysis drives release of the 5’-end of ssRNA from one dimer, which then binds the 3’-end of ssRNA to yield 5’ to 3’ translocation, and this cycle is repeated with the next dimer unit and so on (Geiselmann et al., 1993). A completely different model has been proposed by Platt (1994), in which the high-affinity sites keep Rho tethered to its initial binding site on the RNA, while the low affinity sites cycle RNA binding and release to effect translocation.

To date there are no details available about the kinetics of Rho-catalyzed ATP hydrolysis and its coupling to unwinding activity. Based on the available information, there appear to be significant differences between the T7 DNA helicase and Rho DNA–RNA helicase; nevertheless, the coupling of NTPase activity to translocation may be similar to that proposed for 4A’ protein, and Rho subunits may.
sequentially bind and release RNA as they bind and hydrolyze ATP, one unit at a time.

Whether helicases translocate on DNA (or RNA) with all or a few subunits contacting ssDNA, or whether they contact both ss and dsDNA, they have one common feature: they are motor proteins that convert energy from NTP binding and hydrolysis to facilitate conformational changes that drive DNA unwinding. One important goal of studies of DNA unwinding enzymes will be to determine the "trigger" or the "power stroke" that translates chemical energy into mechanical energy. Similar questions are being asked for the classical motor proteins such as kinesin (Gilbert et al., 1995, Hackney, 1994) and dynein (Johnson, 1985), and these proteins and helicases may well have analogous mechanisms for motor activity.

Another protein that shares some interesting features with the hexameric helicases is the $F_1$-ATPase from the ATP synthase complex. The $F_1$ portion of the synthase complex has the catalytic sites for ATP synthesis, and it functions as an ATPase when detached from the complex. The $F_1$-ATPase is a ring-shaped hexamer of alternating $\alpha$- and $\beta$-subunits, which have similar tertiary folds (reviews: Walker et al., 1990; Penefsky & Cross, 1991). All six subunits bind ATP, but only three of the six subunits have catalytic activity. Several
years ago, a "binding-change" mechanism was proposed for ATP synthesis by the synthase complex, which suggested that the three sites on the F\textsubscript{i} portion cycle sequentially between "substrate-binding", "ATP-forming", and "product-release" states, and the transition between different catalytic states is achieved by rotation of the subunits relative to the rest of the synthase complex (Cross, 1981; Boyer, 1993). A recent high-resolution structure of the bovine mitochondrial F\textsubscript{i}-ATPase supports this mechanism by showing that the three catalytic subunits, in the hexamer, simultaneously exist in three different states, empty, bound to AMP-PCP, or bound to ADP (Abrahams et al., 1994).

Rotary Motors

Although their functions are radically different, the F\textsubscript{i}-ATPase and the T7 helicase both form ring-shaped hexamers, bind and hydrolyze (or synthsize) NTP in a sequential manner, and may undergo conformational changes linked to NTPase activity. These properties suggest a common functional motif, a "rotary motor", that may be utilized for different functions. Related to this theme, it is interesting that the portal proteins of bacteriophage are also ring-shaped oligomers with associated ATPase activity (Donate, et al., 1988). Portal proteins are essential components of DNA packaging systems that drive DNA translocation from one prokaryotic cell compartment to
another (Black, 1988; Bazinet & King, 1985). It has been speculated that portal proteins are rotating motors, and several models have been proposed to explain their translocase activity (Doering et al., 1994; Peskin et al., 1994). The common structural and biochemical features of these proteins are intriguing because they suggest the existence of a modular rotary motor that can be tinkered with or built upon, to yield protein machinery with varying functions.

Summary.

The results of this study have led to a model for a sequential mechanism of dTTP binding and hydrolysis catalyzed by 4A' helicase. An immediate question that arises is whether there is similar coordination between the subunits in the presence of ssDNA. This issue can be addressed by measuring kinetics of dTTP binding and hydrolysis in the presence of ssDNA, on which the helicase can translocate over long distances (e.g., M13 ssDNA). It is also necessary to determine how the helicase interacts with a DNA fork-junction, and whether translocation on ssDNA via the sequential dTTPase mechanism is sufficient to unwind duplex DNA, or if other interactions (e.g., with the other ssDNA strand at the fork) are necessary for helicase activity. Quantitation of the kinetics and energetics of helicase-catalyzed dTTPase activity in the presence of its natural DNA substrate will provide the information necessary
to fully understand the mechanism of NTP hydrolysis coupled to DNA unwinding, and to solve the mechanism of unwinding itself.
APPENDIX A

ABBREVIATIONS AND SYMBOLS

Nucleotides:
AMP-PCP; adenosine 5'-(β,γ-methylenetriphosphate)
dAMP-PCP; 2'-deoxyadenosine 5'-(β,γ-methylenetriphosphate)
ATP; adenosine 5'-triphosphate
ATPγS; adenosine 5'-0-(3-thiotriphosphate)
CTP; cytidine 5'-triphosphate
dCTP; deoxycytidine 5'-triphosphate
NTP; nucleotide 5'-triphosphate
NDP; nucleotide 5'-diphosphate
dTDP; deoxythymidine 5'-diphosphate
dTMP-PCP; deoxythymidine 5'-(β,γ-methylenetriphosphate)
dTTP; deoxythymidine 5'-triphosphate
DNA; deoxyribonucleic acid
hp; hairpin
RNA; ribonucleic acid
Symbols:
Å; angstrom
Ci; curie
°C; degree celsius
$k_{cat}$; steady-state catalytic rate constant
$K_d$; dissociation constant
kDa; kilodalton
M; molar
mCi; millicurie
mg; milligram
ml; milliliter
mm; millimeter
μm; micrometer
mM; millimolar
μM; micromolar
μl; microliter
msec; millisecond
nM; nanomolar
nm; nanometer

Miscellaneous:
BSA; bovine serum albumin
CoCl$_2$; cobalt chloride
DEAE; diethylaminoethyl
DMS; dimethyl suberimidate
ds; double-stranded

DTT; dithiothreitol

EDTA; ethylenediamine-N,N',N'-tetraacetic acid

F; fluorescein

h; hours

HEPES; N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HPLC; high-pressure liquid chromatography

IPTG; isopropyl β-D-thiogalactopyranoside

KCl; potassium chloride

min; minutes

MgCl₂; magnesium chloride

MgOAc; magnesium acetate

NaOAc; sodium acetate

NaCl; sodium chloride

NaOH; sodium hydroxide

NC; nitrocellulose

NTPase; nucleotidase

PAGE; polyacrylamide gel electrophoresis

PEI; poly(ethylenimine)

P-C-P; methylenediphosphonic acid, trisodium salt

RT; reverse transcriptase

s; seconds

SAED; sulfo-N-succinimidyl[2-(7-azido-4-methylcoumarin-3-acetamido)ethyl]dithio]propionate

SDS; sodium dodecyl sulfate
ss; single-stranded
TdT; terminal deoxynucleotidyltransferase
TEA; triethanolamine
TEAB; triethanolamine bicarbonate
TLC; thin-layer chromatography
Tris-HCl; tris(hydroxymethyl)aminomethane hydrochloride
uv; ultraviolet
### APPENDIX B

#### BUFFERS

<table>
<thead>
<tr>
<th>Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline buffer:</td>
<td>$50 \text{ mM NaOH, 1 mM EDTA}$</td>
</tr>
<tr>
<td>Alkaline gel-loading dye:</td>
<td>$50 \text{ mM NaOH, 1 mM EDTA, 3 %}$ and $\text{Ficoll 400, 0.05 % bromophenol blue}$</td>
</tr>
<tr>
<td>Binding buffer A:</td>
<td>$50 \text{ mM Tris-acetate, pH 7.5, 10 mM MgOAc, 5 mM NaOAc, 1 mM DTT}$</td>
</tr>
<tr>
<td>Binding buffer B:</td>
<td>$50 \text{ mM Tris-acetate, pH 7.5, 10 mM MgOAc, 40 mM NaOAc, 10 % glycerol}$</td>
</tr>
<tr>
<td>Buffer B:</td>
<td>$20 \text{ mM potassium phosphate, pH 7.2, 0.1 mM EDTA, 1 mM DTT, 10 % glycerol}$</td>
</tr>
<tr>
<td>Buffer F:</td>
<td>$50 \text{ mM Tris-acetate, pH 7.5, 3 mM MgOAc, 50 mM NaOAc, 10 % glycerol}$</td>
</tr>
<tr>
<td>Denaturing dye solution:</td>
<td>$95 % \text{ formamide, 0.05 % bromophenol blue}$</td>
</tr>
</tbody>
</table>
| **DNA-tailing buffer** (Boehringer Mannheim): | 250 mM potassium cacodylate,  
25 mM Tris-HCl, 0.25 mg/ml  
BSA, pH 6.6 (25 °C) |
| **dTTPase buffer A:** | 50 mM Tris-acetate, pH 7.5, 10  
mM MgOAc, 50 mM NaOAc, 1 mM  
DTT, 0.1 mg/ml BSA |
| **dTTPase buffer B:** | 50 mM Tris-acetate, pH 7.5, 40  
mM NaOAc, 1 mM DTT, 10%  
glycerol |
| **Kinase buffer:** | 70 mM Tris-HCl, pH 7.6, 10 mM  
MgCl₂, 100 mM KCl, 1 mM 2- 
mercaptoethanol |
| **Membrane wash buffer A:** | 50 mM Tris-acetate, pH 7.5, 10  
mM MgOAc, 5 mM NaOAc |
| **Membrane wash buffer B:** | 50 mM Tris-acetate, 10 mM  
MgOAc, 40 mM NaOAc |
| **Native gel-loading dye:** | 0.25 % bromophenol blue, 15%  
Ficoll |
| **Nucleotide synthesis buffer:** | 50 mM Tris-acetate, pH 7.5, 10  
mM MgOAc, 50 mM NaOAc, 1 mM  
DTT, 0.1 mg/ml BSA |
| **SDS gel-loading dye:** | 50 mM Tris-HCl, pH 6.8, 100 mM  
DTT, 2% SDS, 0.1%  
bromophenol blue, 10%  
glycerol |
Sequencing gel-loading buffer: 98 % deionized formamide, 10 mM EDTA, pH 8.0, 0.25 % xylene cyanol FF, 0.025 % bromophenol blue

SF buffer: 50 mM Tris-HCl, pH 7.5, 3 mM MgCl₂, 50 mM NaCl, 10 % glycerol

Standard elution buffer: 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA

TAE buffer: 40 mM Tris-acetate, pH 7.5, 1 mM EDTA, pH 8.0

TBE buffer: 45 mM Tris-borate, pH 7.5, 1 mM EDTA, pH 8.0

TE buffer: 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0

TEA buffer: 50 mM TEA, 10 mM MgCl₂, 10 mM NaCl, 1 mM DTT, pH adjusted to 8.2 with 1 N HCl

TEAB buffer: 1 M triethanolamine in H₂O, pH adjusted to 8.0 with CO₂

Tris-glycine buffer: 25 mM Tris-base, 200 mM glycine
APPENDIX C

OLIGODEOXYNUCLEOTIDE SEQUENCES

10-mer; 5′-ATAGT GTCAC
17-merF; 5′-AAATT AATAC GACTC AC F
25-mer; 5′-GCCTC GCAGC CGTCC AACCA ACTCA
26-mer; 5′-AAATT AATAC GACTC ACTAT AGGGA G
30-mer; 5′-AGCTT GCATC ATAGT GTCAC CTGTT ACGTT
36-mer; 5′-CGGAG CGTCG GCAGG TTGGT TGAGT AGGTC TTGTT T
40-merA; 5′-AAATT AATAC GACTC ACTAT AGGGA GACCA CAACG GTTTC
40-merB; 5′-GAAAC CGTTG TGGTC TCCCT ATAGT GAGTC GTATT AATTT hp; 5′-GAATT CGCCA GTGTC ATGCG TTT CGCAT GACAC TGGCG AATTC
60-mer; 5′-AATTC GAAAT CATGG TCATA GCTGT TTCCT CATGA CGATT ACCTG AACCA TCCTG ACTCT
5′-60-merF; 5′-GCCTC AATAC CAGGG TCAGG TTCGT TAGAG CGGAT TACTA F ACTAC ATTAG AATTC AGAC
3′-60-mer; 5′-GTCTG AATTC TAATG TAGTA TAGTA ATCCG CTCAT TGCTT GTATG GTCAC CATAA CTCTG

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APPENDIX D

CHEMICAL STRUCTURES

dTMP-PCP:

SAED:
REFERENCES


Ng, W. M. (1993) Senior Thesis (The Ohio State University, Columbus, OH).


