Stoichiometry and DNA Unwinding by the Bacteriophage T4 41:59 Helicase*

Kevin D. Raney‡, Theodore E. Carver, and Stephen J. Benkovic§

From the Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania 16802

The bacteriophage T4 41 protein is a replicative helicase that forms a hexamer in the presence of ATP and associates with the T4 59 protein. The stoichiometry of the 41:59 helicase complex and its mechanism for DNA unwinding have been investigated using steady-state and single-turnover kinetics. A partial duplex DNA fork containing two regions of single-stranded DNA (ssDNA) of 30 nucleotides each, and 30 base pairs served as the substrate. 59 was found to increase the steady-state unwinding rate of the substrate by 200-fold over the rate of 41 alone. Maximum unwinding occurred when 59 and 41 were equimolar, revealing a 1:1 stoichiometry for the complex. Varying 41 while holding 59 constant resulted in sigmoidal kinetics suggesting strong cooperativity for formation of the 41 hexamer and providing a lower limit for hexamer assembly of 65 nM. Substrates were prepared that contained a biotin-streptavidin block in either the leading or lagging strand of the duplex region of the substrate. The first order rate constant for unwinding was reduced only when the block was placed in the lagging strand of the DNA fork, indicating that the helicase interacts primarily with the lagging DNA strand.

Bacteriophage T4 DNA replication is facilitated by the phage-encoded 41 helicase. 41 is a single-stranded DNA (ssDNA) stimulated nucleotide triphosphatase with particularly high affinity for ATP and GTP (Morrical et al., 1995; Liu and Alberts, 1981). Interaction of 41 with the T4 61 protein (primase) makes up the T4 primosome that is responsible for unwinding dsDNA for leading strand DNA synthesis as well as priming of the lagging strand for lagging strand DNA synthesis (Hinton and Nossal, 1987; Liu and Alberts, 1981; Burke et al., 1985). 41 assembles into a hexamer upon binding ATP or GTP, and this oligomer is believed to be the active form of the enzyme (Dong et al., 1995).

A third protein, the product of the T4 59 gene, has recently been shown to enhance the ability of 41 to perform its function as a helicase. 59 stimulates the DNA independent and DNA dependent ATPase activity of 41 (Morrical et al., 1994). 41 activity is inhibited when T4 32 protein binds to ssDNA. In the presence of 59, the activity of 41 as an ATPase and a helicase is restored, suggesting that 59 enables 41 to load onto ssDNA that is coated with 32 (Barry and Alberts, 1994; Morrical et al., 1994; Yonesaki, 1994; Tarumi and Yonesaki, 1995). 59 has been proposed to be essential during late stages of T4 DNA recombinational-dependent replication, a process which is also dependent on the presence of 41 (Kreuzer and Morrical, 1993).

Other helicases also require an accessory protein to attain high levels of activity. The Escherichia coli replicative helicase, DNA B, interacts with DNA C to bind to DNA at the replication origin (Wahl et al., 1989), and the E. coli RuvB helicase interacts with RuvA to carry out its activities in recombination and repair (Tsaneva et al., 1993). The DNA B helicase has been shown to exist as hexamer (Reha-Krantz and Hurwitz, 1978; Bujalowski et al., 1994) as has RuvB (Stasiak et al., 1994), and there may be a similar mechanism of action for these helicase enzymes and their respective accessory proteins.

In this report, a DNA unwinding assay using rapid kinetic chemical quench-flow techniques has been used to investigate the mechanism of the 41 helicase and its interaction with 59. Single-turnover and steady-state kinetic analyses have provided the rate of DNA unwinding of oligonucleotide substrates by 41 in the presence and absence of 59 as well as the stoichiometry and limiting concentration for complex formation. The mechanism of unwinding has been probed using partially hybridized oligonucleotide substrates containing altered topology or steric blocks within the duplex region of the DNA fork. The results of these studies are discussed in context of recent electron microscopy studies of other hexameric helicases that suggest that DNA passes through the hole of the hexamer (Stasiak et al., 1994; Egelmann et al., 1995).

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were purchased from Operon Technologies (Alameda, CA) and purified by preparative gel electrophoresis. The concentration of oligonucleotides was determined by UV absorbance at 260 nm in 0.2 M NaOH using extinction coefficients that were calculated from the DNA sequence. The oligonucleotides were stored in 10 mM Hepes, pH 7.5, and 1 mM EDTA at 10 °C. A crystalline suspension of phosphoenolpyruvate kinase/lactate dehydrogenase (Sigma) was diazylated against Hepes, 25 mM, pH 7.5, 2 mM 1-mercaptoethanol, 20% glycerol and stored at −70 °C. Phosphoenolpyruvate (tricyclohexylammonium salt), ATP (disodium salt), Hepes, and Trizma base were from Sigma.

Preparation of DNA Fork Substrates—Purified 60-mer oligonucleotides were mixed in a one to one ratio in 20 mM Tris, pH 7.5, and annealed by heating at 95 °C for 5 min followed by slow cooling to room temperature. The resulting 60-mer fork was purified by preparative gel electrophoresis using a native 15% polyacrylamide gel. The DNA fork was quantitated by UV absorbance using a calculated extinction coefficient based on the DNA sequence (ε260 = 1.42 × 10^5 M⁻¹ cm⁻¹). Absorbance was measured in 0.2 M NaOH at 40 °C to disrupt secondary structure. Alternatively, a small quantity of DNase I (2 μl of a 2 mg/ml stock) was added to a 1-ml aliquot of the DNA fork in 20 mM Tris, pH 7.5, to degrade the DNA fork to smaller oligonucleotides, and the A260 was obtained. Both methods gave identical results. DNA forks containing sequence-directed DNA bends or biotin labels were prepared in a
DNA Fork Substrate

\[ \text{C}_{\text{t}}(t) + \text{C}_{\text{heat}}(t) + \text{C}_{\text{heat}}(\text{heat}) \]

DNA Unwinding Assays—Rapid-quench experiments were performed at 37 °C using a three-syringe quench-flow instrument (Johnson, 1986). The DNA fork substrates were 5'-end-labeled using γ-32P-ATP and polynucleotide kinase. Excess γ-32P-ATP was removed by passing the labeled DNA through two G-25 spin columns. The experiments were carried out by pricking reagents in helicase assay buffer within the two load syringes of the instrument and then rapidly mixing equal volumes (40 μl) to initiate the reaction. Helicase assay buffer contained 25 mM Tris·HCl, pH 7.5, 150 mM KOAc, 5 mM Mg(OAc)2, 1 mM dithiothreitol, and 20% glycerol) and concentrated using an Amicon concentrator to -0.6 mg/ml. Further concentration of 41 led to precipitation owing to the protein’s low solubility (Marracli et al., 1994; Dong et al., 1995). 41 was filtered through a 0.2-μm filter, and 100-μl aliquots were frozen in liquid N2 prior to storing at -80 °C. The concentration of 41 was determined by UV absorbance at 280 nm using the calculated extinction coefficient of 7.1 × 10^4 M^-1 cm^-1 (Young et al., 1994).

The 59 protein was purified as described (Spacciapoli and Nossal, 1994), dialyzed against 25 mM Hepes, pH 7.5, 50 mM KOAc, 1 mM dithiothreitol, and 20% glycerol and frozen in liquid N2 in small aliquots for storage at -80 °C. The concentration of 59 was determined by UV absorbance at 280 nm using the extinction coefficient calculated from the amino acid sequence, ε280 = 3.78 × 10^4 M^-1 cm^-1.

DNA Unwinding Assays—Rapid-quench experiments were performed at 37 °C using a three-syringe quench-flow instrument (Johnson, 1986). The DNA fork substrates were 5'-end-labeled using γ-32P-ATP and polynucleotide kinase. Excess γ-32P-ATP was removed by passing the labeled DNA through two G-25 spin columns. The experiments were carried out by pricking reagents in helicase assay buffer within the two load syringes of the instrument and then rapidly mixing equal volumes (40 μl) to initiate the reaction. Helicase assay buffer contained 25 mM Tris·HCl, pH 7.5, 150 mM KOAc, 5 mM ATP, 0.1 mg/ml bovine serum albumin, and 1 μM β-mercaptoethanol. 41 was preincubated with helicase assay buffer, 20 mM Mg(OAc)2, an ATP regenerating system, and, in some instances, the 59 protein. The ATP regenerating system contained 4 μM phosphoenolpyruvate and 10 units/ml phosphoeholpyruvate kinase (Sigma). The DNA fork was preincubated in the second syringe in helicase assay buffer alone, or, in some experiments, with the 59 protein and the DNA trapping strand. The trapping strand was a 30-mer that was complementary to the base-pairing region of one of the DNA strands and included of the 30-mer prevented reannealing of products (see “Results”). After rapid mixing, reagents were added to various times ranging from 300 μl to 40 s and then mixed with the quench solution (68 mM EDTA, pH 8.0, and 0.33% SDS, final concentrations). Samples were diluted 4:1 with non-denaturing gel loading buffer (30% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol), electrophoresed on a nontoxic acrylamide gel, and quantitated using a Molecular Dynamics PhosphorImager.

The fraction of DNA that had been unwound by the helicase was corrected for efficiency of the trapping strand in preventing reannealing of products. This correction factor was determined for each experiment by heating a sample of the DNA fork substrate at 95 °C for 5 min in the presence of the trapping strand (Equation 1). The quantity of DNA fork that was prevented from reannealing was typically 95%.

\[ \text{fraction ssDNA}_{\text{heat}} = \left( \frac{C_{\text{t}}(t) + C_{\text{heat}}(t)}{C_{\text{heat}}(\text{heat})} \right) \] (Eq. 1)

C_{\text{t}}(t) and C_{\text{heat}}(t) are the counts in the gel bands corresponding to single-stranded and duplex DNA, respectively, at time t. C_{\text{heat}}(\text{heat}) and C_{\text{heat}}(\text{heat}) are the counts in the gel bands corresponding to single-stranded and duplex DNA, respectively, in the heated sample.

(fraction ssDNA) = \left( \frac{C_{\text{t}}(0) + C_{\text{t}}(0)}{C_{\text{t}}(0) + C_{\text{t}}(0)} \right) \] (Eq. 2)

Equation 2 was used to determine the fraction of DNA fork that was unwound by the helicase with correction for the amount of ssDNA.

RESULTS

Development of a Rapid Kinetic Assay for Measuring DNA Unwinding—A DNA unwinding assay using rapid kinetics was developed for measuring helicase activity on oligonucleotide substrates (Fig. 1). The 41 helicase has been shown to require a minimum of 29 nucleotides of ssDNA on the 5' and 3' regions adjacent to the DNA fork for maximum activity (Richardson and Nossal, 1989). Two partially complementary oligonucleotides were annealed to form a DNA fork species containing two 30-nucleotide regions of ssDNA and 30 base pairs. The unwinding reaction produced two 60-mer DNA strands that can spontaneously hybridize to reform substrate. Thus, a trapping strand that is complementary to the base-pairing region of one of the 60-mers was added to the assay in excess to prevent reannealing of products (Fig. 2A).

Rapid kinetic experiments using chemical quench-flow have been reported in which the helicase was preincubated with an oligonucleotide substrate and then mixed with ATP to initiate the reaction (Amaratunga and Lohman, 1993; Bjornson et al., 1994). However, 41 has been shown to oligomerize into hexamers in the presence of ATP and ATPγS (Dong et al., 1995); thus, the appropriate preincubation conditions that are neces-
FIG. 2. A, the DNA unwinding assay that was used for measuring helicase activity in which two partially complementary oligonucleotides, 60-mers, are used to make the substrate. Helicase-induced unwinding of this DNA fork gives rise to ssDNA products, 60-mer lagging strand and 60-mer leading strand, which can immediately reanneal to reform the substrate. A 30-mer oligonucleotide that is complementary to the base-pairing region of the substrate was included to prevent reannealing. The resulting single-strand DNA and trapped DNA products are separated from the DNA fork substrate by native acrylamide gel electrophoresis. B, the experimental setup for rapid quench studies of DNA unwinding. 41 was preincubated with ATP in helicase assay buffer in one syringe of the instrument (see "Experimental Procedures" buffer

[Diagram showing DNA fork substrate with degradation products and experimental setup]

C

| time, s | 0 | 0.3 | 0.6 | 1.0 | 1.5 | 2.5 | 3.5 | 5.0 | 7.5 | 10 | 15 | 20 | 40 | heated |
|---------|---|----|----|----|----|----|----|----|----|----|----|----|----|-------|
| DNA fork substrate | ![DNA fork substrate gel image] |
| trapped 60-mer | ![Trapped 60-mer gel image] |
| 60-mer | ![60-mer gel image] |
sary to rapidly assemble the active species of the helicase were investigated. Rapid-quench experiments were performed in which 41 was preincubated with various components of the reaction mixture and then rapidly combined with the remaining components to initiate the unwinding reaction. Preincubation of 41 in the absence of ATP led to distinct lag phases of ~1 s in the unwinding reaction (data not shown). Addition of ATP to 41 prior to mixing with additional reaction components led to even longer lag phases (data not shown). Preincubation of 41 with 5 mM ATP and the ATP regenerating system (see “Experimental Procedures”) followed by rapid mixing with 59 and the DNA fork led to rapid unwinding of the substrate. The presence of 59 in the preincubation mixture with 41 had only a small effect on the resulting unwinding kinetics as long as ATP was present during the preincubation (see below). When 41 and 59 were preincubated in the absence of ATP, precipitation of the proteins occurred. Fig. 2B illustrates the most favorable preincubation conditions determined for performing DNA unwinding assays using these proteins. The products of the reaction were readily separated on a native 15% polyacrylamide gel, and radiolabeled DNA was detected and quantitated using a Molecular Dynamics PhosphorImager (Fig. 2C).

The Effect of the Trapping Strand on the Kinetics of DNA Unwinding—Previous experiments using the T4 Dda helicase indicated that the presence of a trapping strand during unwinding led to slower observed unwinding rates, presumably due to binding of the trapping strand by the helicase (Raney et al., 1994). Thus, in initial experiments, the trapping strand was placed in the receiving vial so that substrate renaturation was prevented after the reaction was quenched but allowed to occur during the time frame of the reaction. The spontaneous hybridization rate of the ssDNA 60-mers was measured directly using stopped-flow absorbance spectroscopy providing a bimolecular rate constant of 1.3 × 10^5 M^{-1}s^{-1} at 37°C (data not shown). Unwinding experiments were performed at 50 nM and 1 nM DNA fork; concentrations at which little renaturation of fork was expected to occur. The half-life for duplex formation at 50 nM and 1 nM DNA fork is ~15 s and 770 s, respectively. However, the data for unwinding indicate that a plateau was reached with less than 100% substrate unwound (Fig. 3). The plateau at 1 nM DNA fork occurred with ~90% of the substrate unwound while only 60% of the substrate was unwound at 50 nM DNA fork. The partition toward more product at lower DNA fork concentration suggests that the plateau represents a steady-state between unwinding by the helicase and renaturation of the products. Kinetic simulation of the unwinding curves indicates that the spontaneous hybridization rate was too slow to account for the observed plateau, and, therefore, renaturation of the duplex was enhanced by the 41 and/or 59 proteins. Preliminary experiments support a 10-fold faster hybridization rate for the 60-mers in the presence of 41, 59, and ATP. 2

To prevent renaturation of the single-stranded products during the reaction period, the trapping strand was included within the reaction mixture by preincubating it with the DNA fork and, in some instances, 59. The possibility that the addition of ssDNA might sequester the helicase and slow the observed unwinding rate was investigated by performing the experiment under conditions in which the 41 concentration was in excess of the sum of DNA fork and DNA trap concentrations. The concentration of 41 was sufficiently high so that even if the trap sequestered the helicase, the DNA fork would still be unwound by the remaining excess 41. Under these conditions, all of the DNA fork was unwound, and the data were fit to a single exponential providing a rate constant of 0.75 ± 0.08 s^{-1} (Fig. 4A). The experiment was then performed under conditions in which the trap concentration was in excess of 41 in hexamer concentration. The resulting data provided a first order rate constant (0.79 ± 0.08) that was within error of that obtained at low trap concentration suggesting that 41 binds and releases the trapping strand very rapidly, and the rate-limiting step for unwinding of the substrate is not affected by variation in the trap concentration under these conditions (Fig. 4B). Thus, subsequent experiments were performed in the presence of trapping strand to prevent renaturation during the time frame of the reaction.

Steady-state Unwinding: Varying 59 Concentration—DNA unwinding was carried out using a concentration of DNA fork (285 nM) that was in excess of 41 concentration (100 nM hexamer). 41 was preincubated with 59 in these experiments, and, at the high 41 concentration, the DNA fork was unwound in 20 s and no distinct burst phase was observed (Fig. 5A). The steady-state unwinding rates were obtained by fitting the early portion of the unwinding curves to a linear function, providing steady-state rates of 46.3 and 19.7 nM-s^{-1} at 100 nM and 50 nM 41 hexamer, respectively (Fig. 5B). The unwinding rate at 100 nM 41 hexamer in the absence of 59 was 0.29 nM-s^{-1} (Fig. 5C). When 59 was preincubated with the DNA fork rather than with

2 T. E. Carver and S. J. Benkovic, unpublished observations.
41, the steady-state rate was $59 \text{nM} \cdot \text{s}^{-1}$ (data not shown) providing an overall rate enhancement for DNA unwinding of 200-fold by the 59 protein. Increasing the DNA fork concentration to 700 nM led to only a small increase ($\leq 10\%$) in the steady-state unwinding rate (data not shown) indicating that the DNA fork concentration of 285 nM was saturating under these conditions allowing an estimate of the $K_m$ for this substrate of less than $60 \text{nM}$.

Reaction conditions similar to those in Fig. 5 were utilized to investigate the effect of varying the 59 concentration in the presence of a fixed concentration of 41 (100 nM hexamer). The steady-state rate increased linearly with increasing concentrations of 59 until 59 was equimolar with 41 monomers (Fig. 6). Further increase in concentration of 59 led to no further enhancement in the unwinding rate indicating that 59 forms a 1:1 complex with 41. The linear increase in the steady-state rate suggests that the 41 concentration was well above the $K_p$ for binding to 59. Thus, since 41 was 600 nM monomer, we estimate that the $K_p$ for 41 binding to 59 is less than 120 nM.

Steady-state Unwinding: Varying 41 Concentration—Steady-state unwinding was carried out under conditions in which the concentration of 41 was varied in the presence of excess 59. The 59 protein (750 nM) was preincubated with the DNA fork substrate in excess of the 41 helicase concentration (in hexamers). Experiments in which 59 was included were performed using the rapid quench instrument. 41 helicase (100 nM hexamer (●), 50 nM hexamer (○)) preincubated in assay buffer was rapidly mixed with 59 protein (750 nM (●), 375 nM (○)), DNA fork (335 nM), and trapping strand (3.5 mM) in assay buffer. All of the DNA fork substrate was unwound at the highest 41 concentration while no distinct burst phase was observed (A). The early time points of the reactions were used to obtain steady-state unwinding rates (B) providing velocities of $46.3 \pm 0.6 \text{nM} \cdot \text{s}^{-1}$ and $19.7 \pm 0.7 \text{nM} \cdot \text{s}^{-1}$ at 100 nM and 50 nM 41 hexamer. Steady-state unwinding in the absence of 59 lead to an unwinding rate of $0.29 \text{nM} \cdot \text{s}^{-1}$ (C).

59 protein (750 nM) was preincubated with the DNA fork (315 nM) in these experiments owing to the slightly faster rates obtained using this procedure. The steady-state unwinding rate constant, $k_{cat}$, was plotted as a function of 41 concentration resulting in a sigmoidal curve that indicates a highly cooperative transition occurring at $\sim 65 \text{nM}$ 41 (Fig. 7). The 59 and DNA fork concentrations are relatively high in these experiments, well above the respective $K_p$ and $K_m$ estimated for these components, suggesting that the transition involves formation of the 41 hexamer from 41 monomers rather than interaction of 41 with 59 or with DNA.

Unwinding under Single-turnover Conditions—The first or-
der rate constant for unwinding of the DNA fork was determined under two different preincubation conditions. The DNA fork (50 nM) was preincubated with the DNA trapping strand (1 mM) while 41 (1.2 mM) was preincubated with 59 (1.0 mM) in helicase assay buffer. The first order rate constant obtained under these conditions was 0.85 ± 0.08 s⁻¹ (Fig. 8A). Increasing one or both proteins did not lead to enhancement of the rate constant, indicating that these protein concentrations were saturating with respect to the DNA fork concentration. A similar first order rate constant of 1.0 ± 0.08 s⁻¹ was obtained under conditions in which 59 was preincubated with the DNA rather than with 41 (Fig. 8B). Thus, preincubation of 59 with 41 or with the DNA fork gave rise to similar rate constants for unwinding under single turnover conditions.

Unwinding of DNA Fork Substrates Containing Sequence-directed DNA Bending—The mechanism of DNA unwinding by the 41:59 helicase complex was investigated under single-turnover conditions. We sought to determine if 41:59 utilizes the duplex region of the DNA fork during unwinding. The overall topology of this region was altered using DNA fork substrates that contained sequence-directed DNA bends within the duplex region of the fork (Fig. 1). Duplex DNA containing A-tracts at appropriate intervals has been shown to bend ~18° per A-tract (Crothers et al., 1990). Two sequences were designed with the A-tract offset by 5 base pairs. This arrangement alters the resulting DNA bend in bent DNA fork 1 (BDF1) by 180° with respect to the bend in BDF2 (Crothers et al., 1990).

Single-turnover conditions were used for unwinding in which 59 (1.5 mM) was preincubated with the bent DNA fork (50 nM) and trapping strand (1.0 mM) in assay buffer. The first order rate constant obtained was 0.85 ± 0.08 s⁻¹. Increasing the concentration of 41 or 59 did not increase the observed rate constant indicating that the substrate was saturated. 41 (200 nM hexamer) was preincubated with only ATP and assay buffer, while 59 (2.5 mM) was preincubated with the DNA fork (50 nM) and trapping strand (1.0 mM). The first order rate constant obtained was 1.00 ± 0.08 s⁻¹.
Unwinding of DNA fork substrates containing sequence-directed bends or biotin/streptavidin blocks

| Substrate                        | Unwinding rate constant, s⁻¹ |
|----------------------------------|------------------------------|
| Normal DNA fork                  | 1.00 ± 0.08                  |
| Bent DNA fork 1                  | 0.85 ± 0.08                  |
| Bent DNA fork 2                  | 0.81 ± 0.08                  |
| Lag biotin DNA fork              | 0.69 ± 0.05                  |
| Lead biotin DNA fork             | 0.87 ± 0.08                  |
| SA                               |                              |
| +SA                              |                              |

The unwinding experiment with the bent DNA substrates was performed using the protocol described for the normal DNA substrate. The first order rate constant for unwinding obtained with each bent DNA fork was similar to that determined for the normal DNA fork substrate (Table I). Thus, 41:59 is insensitive to this type of structural alteration within the duplex region of the DNA fork.

Unwinding of DNA Fork Substrates Containing Biotin/Streptavidin Blocks—The DNA strand preference of 41:59 during dsDNA unwinding was investigated under single-turnover conditions using substrates containing a biotin label within the duplex region of either the leading or lagging strand of the DNA fork (Fig. 1). 41 (1.2 μM) was preincubated in assay buffer while 59 (1.5 μM) was preincubated with DNA fork (50 nM) and trapping strand (1 μM). In the absence of SA, the rate constant for unwinding of the biotinylated DNA fork substrates were similar to that obtained with the unlabelled substrate (Table I). In the presence of SA, very little difference was observed for unwinding of the substrate containing the biotin label on the leading strand (lead-bio-DF) while an 8-fold reduction in the rate constant for unwinding was observed for the substrate containing the biotin label within the lagging strand (lag-bio-DF). This result strongly implies that the lagging strand is primarily involved in 41:59 interactions with DNA during unwinding.

DISCUSSION

Kinetic studies of DNA helicase-mediated DNA unwinding have been limited due to the unique nature of the assay. Upon unwinding of dsDNA, the ssDNA products can spontaneously reanneal to reform substrate. A previous kinetic study on the bacteriophage T4 Dda helicase utilized excess DNA fork substrate (250 nM) over enzyme concentration (50 nM) (Raney et al., 1994). These conditions were optimized to study a fluorescence change derived from unpairing of 2-aminopurine residues that were incorporated into the DNA fork substrate. However, the relatively high DNA concentration led to reannealing of product strands during the time frame of the unwinding reaction, and kinetic simulations were required to extract the rate constant for DNA unwinding. Other work has been performed at very low DNA concentration, 1 nM substrate, precluding strand renaturation during the unwinding reaction of E. coli Rep helicase (Amaratunga and Lohman, 1993; Bjornson et al., 1994). In this report, attempts to use very low DNA fork concentrations to circumvent the reannealing phenomenon indicated that even at 1 nM substrate, a steady-state between unwinding and reannealing was observed (Fig. 3), owing to the enhancement of the spontaneous annealing rate of ssDNA 60-mer by the 41:59 helicase. A trapping strand was therefore included in the reaction mixture to prevent reannealing, and the presence of this DNA did not affect the observed unwinding rate (Fig. 4).

The unwinding assay was used to probe the interaction of 41 with the T4 59 protein. 59 has been shown to enhance the ability of 41 to hydrolyze ATP (Morrical et al., 1994) and to unwind DNA (Yonesaki, 1994). Unwinding under conditions of excess DNA fork did not show burst kinetics when observed within a pre-steady-state time frame (Fig 5A). The rate-limiting step for the unwinding reaction must therefore be similar to the overall rate-limiting step for the steady-state reaction with this substrate. The DNA fork concentration (285 nM) was saturating with respect to helicase concentration (100 nM in hexamers), suggesting that the Kₚₕ for the substrate is <60 nM (assuming that the concentration needed to saturate is ~5-fold greater than the Kₚₕ). The unwinding rate was measured as a function of 59 concentration revealing a 1:1 stoichiometry on a molar basis between 59 and 41 monomers (Fig. 6). The steady-state unwinding rate of 41 in the absence of 59 was 0.29 nM⁻¹s⁻¹ whereas unwinding in the presence of a slight excess of 59 (with respect to 41) was 59.3 nM⁻¹s⁻¹, providing a 200-fold enhancement. The linear increase in unwinding activity observed at increasing concentrations of 59 suggests that the concentration of 41 (600 nM) was well above the Kₚₕ for these two proteins. Thus, an upper estimate of the Kₚₕ for 41:59 is ~120 nM (assuming that saturating concentration of 41 is >5-fold above the Kₚₕ).

41 forms a hexameric ring structure in the presence of ATP (Dong et al., 1995). The mechanism of oligomerization has been proposed to involve formation of a dimer followed by dimerization of dimers to form a tetramer, followed by binding of an additional dimer to form the hexamer. The Kₐₕ for dimer formation in the absence of ATP was reported to be 1.4 × 10⁻⁶ M⁻¹.

The steady-state unwinding assay was used to probe 41:41 interactions by varying the 41 concentration in the presence of excess 59 and DNA fork concentrations. The Kₐₕ for unwinding is reflective of the active helicase species, presumably the hexamer, and when plotted as a function of 41 concentration resulted in a sigmoidal curve indicative of a cooperative interaction (Fig. 7). The inflection point in the curve occurs at ~65 nM 41 and is likely the limiting concentration for hexamer formation. Thus, in the presence of ATP, 41 hexamer forms at low nanomolar concentration which is on the same order as the estimated Kₐₕ for 41:59 interaction (~120 nM) and Kₚₕ for 41:59 DNA fork interaction (~60 nM).

Conditions for unwinding all of the DNA fork in a single turnover were determined using two preincubation conditions. The rate constant for unwinding was similar regardless of whether 59 was preincubated with 41 (in the presence of ATP) or with the DNA fork (Fig. 8). The single-turnover reaction conditions and modified DNA fork substrates were used to investigate the mechanism of unwinding. DNA bending has been proposed to be energetically coupled to DNA melting by molecular modeling studies; i.e. DNA that contains bending is more apt to melt (Ramstein and Lavery, 1988). Thus, bent DNA might be an intermediate along the reaction pathway for DNA unwinding and a DNA sequence that contains a bend might be hyper-reactive toward DNA helicase. To test this hypothesis, DNA fork substrates were designed that contained A-tracts within the duplex region (Fig. 1). Appropriately spaced A-tracts lead to DNA bending with a angle of ~18° per A-tract (Crothers et al., 1990). The A-tracts were placed such that the bend in BDF1 would be oriented 180° with respect to the bend in BDF2. Unwinding of these substrates under single turnover conditions led to first order rate constants that were very similar to the rate determined with the normal DNA fork (Table I). Thus, 41:59 is insensitive to sequence-directed DNA bends in the
duplex region of the DNA fork substrate.

DNA fork substrates containing biotin labels in the duplex region of the leading or lagging strand were designed to further address the strand preference of 41:59 during DNA unwinding (Fig. 1). In the presence of SA, these substrates should provide evidence as to whether the strand to which the SA is bound is utilized during the unwinding reaction. The biotin-labeled DNA fork substrates were unwind with similar rates as the normal substrate in the absence of SA (Table I). In the presence of SA, the rate constant for unwinding of the lag-bio-DF substrate was reduced by 8-fold, while the rate constant for unwinding of the lead-bio-DF substrate was reduced only by 10%. These results strongly imply that 41:59 utilizes the lagging strand primarily during the unwinding process. Most helicases have been characterized as 5′ to 3′ or 3′ to 5′ based on the apparent direction in which they translocate during DNA unwinding. However, with a few exceptions, unidirectional travel on ssDNA has not been demonstrated (Raney and Benkovic, 1995; Young et al., 1994). 41 is a 5′ to 3′ helicase (Venkatesan et al., 1982) and evidence has been presented which supports unidirectional translocation (Young et al., 1994). This evidence, coupled with the results reported here using the substrates containing biotin/SA blocks, suggests that unwinding can occur by a simple displacement mechanism, whereby 41:59 translocates in a 5′ to 3′ direction on the lagging strand of the DNA fork and displaces the leading strand possibly owing to steric interactions at the ss/dsDNA junction. The observation by Nossal (1994) that the helicase requires a ssDNA tail on the 3′ side of the fork may simply reflect the need for the DNA to be somewhat frayed at the ss/dsDNA junction. Recent work suggests that the need for the 3′-ssDNA tail is reduced in the presence of 59 (Yonesaki, 1994).

The RuvB helicase, T7 gene 4 helicase, and SV40 T antigen have been shown to exist as hexamers, and biochemical and electron microscopy studies have revealed that DNA can bind to these oligomeric proteins by passing through the hole of the hexameric helicase (Stasiak et al., 1994; Egelmann et al., 1995; Dean et al., 1992). RuvB was shown to encircle dsDNA, and SV40 T antigen was proposed to encircle dsDNA at the replication origin, while T7 gene 4 helicase was found to encircle only ssDNA. The question of whether one or both strands of DNA pass through the hole during the unwinding reaction remains unanswered, although a mechanism for unwinding by RuvB in which both DNA strands pass through the hole has been proposed (Adams and West, 1995). 41 exists as a hexamer and is very similar in size and shape to the RuvB and T7 gene 4 helicases and is likely to bind DNA in a similar fashion (Dong et al., 1995).

The manner for unwinding proposed here requires only the lagging strand of the DNA fork to pass through the center of the helicase. In the presence of SA, which has a diameter of 45 Å, the DNA strand containing the biotin label should not be able to pass through the hole of the hexameric 41 helicase, which has a diameter of −25 Å (Dong et al., 1995), unless the helicase can partially open while translocating past the biotin/SA block. The 8-fold reduction in the unwinding rate in the presence of SA on the lagging strand may be due to just such a process. Alternatively, 41:59 might stall at the biotin/SA block, and a second 41:59 helicase complex might bind on the 5′ side of the block, completing the unwinding process. The mechanism of the translocation process remains to be determined, but the evidence suggests that 41:59 might utilize primarily the lagging strand during the unwinding reaction.

CONCLUSION

The stoichiometry of the T4 primosome has been partially defined and contains one 59 monomer per 41 monomer. 59 enhances the rate of DNA unwinding by 200-fold over the rate with 41 alone in the steady-state assay described here. Oligomerization of 41 occurs with strong cooperativity at a limiting concentration of −65 nM, presumably giving rise to the 41 hexamer. The 41:59 helicase complex unwinds the DNA fork substrate described here with a rate of 30 base pairs s−1 and is insensitive to sequence-directed DNA bends in the duplex region of this substrate. 41:59 strongly prefer the lagging strand of the DNA fork and is unaffected by steric blocks placed within the duplex region of the leading strand of the substrate. The possibility that one strand is primarily involved during the unwinding reaction is consistent with evidence for unidirectional translocation of 41 on ssDNA (Young et al., 1994) and with the recently proposed mechanism of homologous recombination in which 41:59 plays a role in driving branch migration (Salinas and Kodadz, 1995).

REFERENCES

Adams, D. E., and West, S. C. (1995) J. Mol. Biol. 247, 404–417

Amaratunga, M., and Lohman, T. M. (1993) Biochemistry 32, 6815–6820

Barry, J., and Alberts, B. (1994) J. Biol. Chem. 269, 33049–33062

Bjornson, K. P., Amaralunga, M., Moore, K. J. M., and Lohman, T. M. (1994) Biochemistry 33, 13406–13416

Bujalski, W., Klonowska, M. M., and ezezewska, M. J. (1994) J. Biol. Chem. 269, 31350–31358

Burke, R. L., Munn, M., Barry, J., and Alberts, B. M. (1985) J. Biol. Chem. 260, 2171–2172

Crotthers, D. M., Haran, T. E., and Nadeau, J. G. (1990) J. Biol. Chem. 265, 7093–7096

Dean, F. B., Borowiec, J. A., Eki, T., and Hurwitz, J. (1992) J. Biol. Chem. 267, 14229–14237

Dong, F., Gogol, E. P., and von Hippel, P. H. (1995) J. Biol. Chem. 270, 7462–7473

Egelmann, E. H., Yu, X., Wild, R., Hingorani, M. M., and Patel, S. S. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3869–3873

Hinton, D. M., and Nossal, N. G. (1987) J. Biol. Chem. 262, 10873–10878

Hinton, D. M., Silver, L. L., and Nossal, N. G. (1985) J. Biol. Chem. 260, 12851–12857

Johnson, K. A. (1986) Methods Enzymol. 134, 677–705

Kreuzer, K. N., and Morrical, S. W. (1994) in Molecular Biology of Bacteriophage T4 (Karem, J. D., ed) pp. 28–42, American Society for Microbiology, Washington, D.C.

Liu, C.-C., and Alberts, B. M. (1981) J. Biol. Chem. 256, 2821–2829

Morrical, S. W., Hempstead, K., and Morrical, M. (1994) J. Biol. Chem. 269, 33069–33081

Nossal, N. G. (1994) in Molecular Biology of Bacteriophage T4 (Karem, J. D., ed) pp. 43–53, American Society for Microbiology, Washington, D.C.

Ramestein, J., and Lavery, R. (1988) Proc. Natl. Sci. U. S. A. 85, 7231–7235

Raney, K. D., and Benkovic, S. J. (1995) J. Biol. Chem. 270, 22236–22242

Raney, K. D., Sowers, L. C., Millar, D. P., and Benkovic, S. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 6644–6648

Reha-Krantz, L. J., and Hurwitz, J. (1978) J. Biol. Chem. 253, 4043–4050

Richardson, R. W., and Nossal, N. G. (1989) J. Biol. Chem. 264, 4725–4731

Salinas, F., and Kodeyke, T. (1995) Cell 82, 111–119

Spacapoli, P., and Nossal, N. G. (1994) J. Biol. Chem. 269, 447–455

Stasiak, A., Taneva, I., 59, S. C., Bason, C. J., Yu, S., and Egelmann, E. H. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7618–7622

Tarumi, K., and Yonesaki, T. (1995) J. Biol. Chem. 270, 2614–2619

Taneva, I., Müller, B., and West, S. C. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1315–1319

Venkatesan, M., Silver, L. L., and Nossal, N. G. (1982) J. Biol. Chem. 257, 12426–12434

Wahle, E., Lasken, R. S., and Kornberg, A. (1989) J. Biol. Chem. 264, 2463–2468

Yonesaki, T. (1994) J. Biol. Chem. 269, 1284–1289

Young, M. C., Schultz, D. E., Ring, D., and von Hippel, P. H. (1994) J. Mol. Biol. 235, 1447–1458
