Increased DNA Unwinding Efficiency of Bacteriophage T7 DNA Helicase Mutant Protein 4A'/E348K*

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Bacteriophage T7 4A' protein is a DNA helicase that unwinds DNA in a reaction coupled to dTTP hydrolysis. To understand better its mechanism of DNA unwinding, we characterized a set of 4A' mutant proteins (Washington, M. T., Rosenberg, A. H., Griffin, K., Studier, F. W., and Patel, S. S. (1996) J. Biol. Chem. 271, 26825–26834). We showed here, using single turnover DNA unwinding assays, that the 4A'/E348K mutant protein had the unusual property of unwinding DNA (with a 5–6-fold slower rate) despite a significant defect in its dTTPase activity (a 25–30-fold slower rate). Comparing the DNA unwinding rates to the dTTPase rates, we estimated the DNA unwinding efficiencies of both wild-type (about 1 base pair unwound per dTTP hydrolysis) and mutant (4 to 6 base pairs unwound per dTTP hydrolysis). Thus the mutant had a 4–6-fold improvement in its DNA unwinding efficiency over that of the wild-type. We believe that this mutant undergoes less slippage (uncoupled dTTP hydrolysis) than the wild-type. We speculate that nature has selected for a high rate of DNA unwinding rather than a high efficiency of DNA unwinding. Thus even though the mutant is more efficient at DNA unwinding, the wild-type probably was selected because it unwinds DNA faster.

DNA helicases are motor proteins that catalyze processive DNA unwinding fueled by nucleoside triphosphate (NTP) hydrolysis (1). The bacteriophage T7 4A' helicase protein is a model enzyme for studying helicase mechanism. The 4A' protein assembles into a ring-shaped hexamer of identical subunits around ssDNA (2, 3). It is believed that when the 4A' protein binds to the replication fork, one strand of the double-stranded DNA is bound through the central hole of the 4A' protein ring and the complementary strand of DNA is excluded from the central hole (3–5). According to this model, DNA unwinding results from translocation along the DNA strand bound through the central hole (3–6). The translocation along DNA and the DNA unwinding are coupled to deoxothymidine triphosphate (dTTP) hydrolysis. Unfortunately this energy transduction process in DNA helicases is poorly understood.

Mutagenesis studies of the 4A' protein have been used to investigate the mechanism of DNA helicases (7–9). These studies have largely focused on highly conserved residues in the helicase-specific conserved motifs (10, 11). Amino acid residues in conserved motif 1, which is thought to be involved in dTTP binding and hydrolysis (12), have been changed in two site-directed mutant proteins (7, 8). These mutant proteins are defective in dTTP hydrolysis activity and DNA unwinding activity. Amino acid residues in conserved motif 4 have been changed in three other site-directed mutant proteins (9). These mutant proteins are defective, either directly or indirectly, in hexamer formation. In addition to the site-directed mutants of the 4A' protein, a series of random mutants of the 4A' protein were selected for in vivo defects in their DNA unwinding activity (13, 14). Biochemical characterization of nine of these mutant proteins revealed three general classes of mutant proteins that are defective in DNA unwinding (14). Mutant proteins in one class can hydrolyze dTTP with near wild-type activity, but these proteins are defective in coupling the energy from dTTP hydrolysis to DNA unwinding. Mutant proteins in another class are defective in DNA binding and DNA-stimulated dTTP hydrolysis. Mutant proteins in the final class are defective in DNA-independent dTTP hydrolysis and DNA-stimulated dTTP hydrolysis. In this report, we describe a more detailed characterization of one of these 4A' mutant proteins, the 4A'/E348K protein, that is a member of this last class.

The mutant 4A'/E348K protein forms stable hexamers and binds single-stranded DNA with a high affinity, but it is completely defective in DNA-independent dTTP hydrolysis activity and partially defective in DNA-stimulated dTTP hydrolysis activity (14). Here we report that despite a 25–30-fold defect in DNA-stimulated dTTP hydrolysis activity, the mutant 4A'/E348K protein surprisingly has a significant amount of DNA unwinding activity (only a 5–6-fold defect). Using steady-state and pre-steady state dTTP hydrolysis assays as well as single turnover DNA unwinding assays using oligodeoxynucleotide substrates, we have shown that the mutant 4A'/E348K protein has an improved DNA unwinding efficiency (the number of base pairs unwound per dTTP hydrolyzed). The mutant 4A'/E348K protein unwinds four to six times more DNA on an average per dTTP hydrolyzed than the wild-type 4A' protein. To our knowledge, this is the first mutant motor protein possessing a greater efficiency of work than the wild-type protein. We speculate that in the case of the bacteriophage T7 4A' helicase protein, nature has selected in favor of a high rate of DNA unwinding and not in favor of a high efficiency of DNA unwinding.

MATERIALS AND METHODS
Plasmids, Expression System, and Purification of 4A' and 4A'/E348K

Both the wild-type 4A' protein and the mutant 4A'/E348K protein were expressed in BL21(DE3) cells using the T7 expression system (15, 16). The pAR5018 plasmid, described by Rosenberg et al. (17), encodes the wild-type 4A' gene controlled by a T7 promoter. The pAR5056 plasmid encodes the mutant 4A'/E348K gene and was created by random mutagenesis of pAR5018 as described (13). Creation of the site-directed mutant 4A'/E348A gene was done using the QuikChange site-
directed mutagenesis kit (Stratagene) and the pAR5018 plasmid. The wild-type 4A' and the mutant 4A'/E348K and 4A'/E348A proteins were purified to greater than 95% homogeneity judged by SDS-polyacrylamide gel electrophoresis as described (14, 18, 19). Protein concentrations were determined by absorption measurements at 280 nm to 786 nm in TE buffer with 8 μM urea (76,100 μM^-1 cm^-1) (18) and by the Bio-Rad Protein Assay using bovine serum albumin as a reference.

**Nucleotides, Oligodeoxynucleotides, and Other Reagents**

Solid dTTP (Sigma) was dissolved in 50 mM Tris-Cl, and the pH was adjusted to 7.5 with NaOH. Concentration of dTTP was determined by measuring absorption at 260 nm. Radioactive [α-32P]dTTP and [γ-32P]ATP were purchased from Amersham. Four oligodeoxynucleotides were used to measure the DNA unwinding activity. The 5' (36'-66') oligodeoxynucleotide was a 102-mer with the sequence: 5'-TAGATCT-RGACGTCACCGTACGAGGCGAGCTACTACAGTACAGTATAGTACGACTATCCGACTATCCGAATCTCTAGAGTCTGAATTCTAATGTAGTATAGTAATCCGCTCATTGCTTGTATGGTC. These two oligodeoxynucleotides annealed to form a fork DNA with 66 base pairs of duplex DNA (the underlined portions). The 5' (36'-33') oligodeoxynucleotide was a 69-mer with the sequence: 5'-TAGATCTGACGTCACCGTACGAGGCGAGCTACTACAGTACAGTATAGTACGACTATCCGACTATCCGAATCTCTAGAGTCTGAATTCTAATGTAGTATAGTAATCCGCTCATTGCTTGTATGGTC. These oligodeoxynucleotides annealed to form a fork DNA with 33 bp of duplex DNA (the underlined portions). An additional oligodeoxynucleotide 60-mer was used in some DNA unwinding experiments to trap excess protein. Its sequence is: 5'-AATCGCTATGGCTAATCATGGTCATAGCTGTTTCCTCATGA-GAGTTGTCGATTAGGCTGTTACCTCTAATCACCAAGTATGCGT- TACGCTCAATACGGCTGCTTGTATGGTC. All oligodeoxynucleotides were purified with hydroxyapatite (12-16F) with urea (3-5 M) in TBE buffer. The oligodeoxynucleotides were electroeluted from the gel using an Elutrap apparatus (Schleicher and Schuell), and the concentration was determined spectrophotometrically at 260 nm. Single-stranded M13 DNA was prepared as described (20).

**Measurement of Helicase Activity**

Two fork DNA substrates were used to measure the DNA unwinding activity. Both DNA substrates contained a short duplex region with a 5' single-stranded tail (36 bases long) and a 3' single-stranded tail (15 bases long) on the same side of the duplex region. One substrate, which had a 66-base pair duplex region, was formed by annealing the 5'(36'-66') and the 3'(15'-68') oligodeoxynucleotides. The other substrate, which had a 33-bp duplex region, was formed by annealing the 5'(36'-33') and the 3'(15'-33') oligodeoxynucleotides. The annealing reaction was performed by incubating 1.5 μM 5'(3'-66') with DNA 1.0 μM 5'(3'-56') in 100 mM NaCl. The solution was heated to 95 °C and slowly cooled over 3 or 4 h to 4 °C. The DNA concentrations are expressed in terms of moles of DNA molecules, and the slight excess of 5' tail was not removed. The vast excess of protein over DNA substrates in these assays makes it highly unlikely that there is any effect from the presence of a small amount of the unlabeled single-stranded 5' oligodeoxynucleotide.

**Stopped-flow Helicase Assay**

The DNA unwinding or helicase activity of the wild-type 4A' protein and the mutant 4A'/E348K protein were measured at 18 °C by a stopped-flow assay (4) using the KinTek stopped-flow apparatus (State College, PA). The wild-type 4A' protein (1.33 μM hexamer concentration) or the mutant 4A'/E348K protein (1.3 μM hexamers, 2 μM dTTP, and 3 mM fork DNA for 90–120 min in 20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 10% glycerol. After the preincubation period, this solution was loaded into syringe A. Syringe B contained the mutant 4A'/E348K protein at 18 °C under the same conditions as above were also examined using the radiolabelled chemical quench flow apparatus. The wild-type 4A' protein was preincubated with annealing the fork, one of the two strands in each fork DNA sample with 32P-end labeled. The preincubated protein, fork DNA, and dTTP in buffer was loaded into syringe A and MgCl2 in buffer was added from syringe B to initiate the reaction. Samples were quenched with 100 μM EDTA, 1% SDS, and 10% glycerol. Some bromophenol blue was added to each quenched sample, and the samples were immediately run on a 12% polyacrylamide gel to separate the fork DNA substrate from the unwound ssDNA products. Some experiments were done in the presence of 60-mer trap DNA (4 μM) added either to syringe A (protein, fork DNA, and dTTP) before the protein was added or to syringe B. The fork DNA substrate and the unwound products were quantitated using the PhosphorImager. In each experiment, the fraction of ssDNA, normalized to a heat-denatured fork DNA sample, was plotted versus time of reaction, and the data were fit to an equation describing a single exponential function. The amplitude and first-order rate constant were obtained from this fit in each case.

**Measurement of DTTPase Activity**

**Steady-state Assay**—The DTTP hydrolysis activities of the wild-type 4A' protein and the mutant 4A'/E348K protein were measured at 18 °C. The enzyme (0.67 μM hexamers) was incubated in 20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 5 mM or 10 mM MgCl2, and 10% glycerol buffer in the presence of 50 nM M13 ssDNA and 50 μM dTTP for 20 min (30–50 μl reaction volumes). Reactions were initiated by adding various amounts of DTTP spiked with [γ-32P]dTTP (2 μCi/reaction). Aliquots (5 μl) were quenched with 10 μl of 1 M HCl and 20 μl of chloroform. Quenched reactions were neutralized by adding 1 μl of NaOH, 0.25 μl Tris base to reach pH 7 (about 8 μl), and 1 μl of the quenched reactions was spotted on a polyethyleneimine thin layer chromatography plate (Whatman). The radiolabeled dTTP and dTMP were separated using 0.3 μg potassium phosphate, pH 3.4, as the chromatography running buffer. The dTTP and dTMP spots were quantitated using the PhosphorImager (Molecular Dynamics).

**Pre-steady State Assay**—The DTTP hydrolysis activities of the wild-type 4A' protein and the mutant 4A'/E348K protein during DNA unwinding were examined at 18 °C under pre-steady state conditions using the KinTek rapid chemical quench flow apparatus. The wild-type 4A' protein or the mutant 4A'/E348K protein (1.3 μM hexamers), fork DNA with a 33-bp duplex region (4 μM), and dTTP (300 μM) were preincubated in 20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 10% glycerol for 1.5–2 h and loaded into syringe A of the quench flow. Syringe B contained 11 mM MgCl2 in the same buffer to initiate the reaction and a small amount of [γ-32P]dTTP (20 μCi). At various times, reactions were quenched with 1 mM HCl and chloroform (100 μl), neutralized with 1 μl NaOH, 0.25 mM Tris base, and analyzed by thin layer chromatography as described above.

**HPLC Gel Filtration**

HPLC size exclusion chromatography was used to examine the complex between the fork DNA substrate and the wild-type 4A' protein or the mutant 4A'/E348K protein. A Bio-Sil SEC 400–5 column (300 × 7.8 mm, 7 μM 4A' hexamers) was used to examine the elution of the 4A' hexamers. The elution was monitored with fluorescein fluorescence at 525 nm (excited at 494 nm). We compared the elution times of the wild-type 4A' hexamers and contained the wild-type 4A' hexamers and the mutant 4A'/E348K protein under standard conditions (1.3 μM 4A' protein hexamers, 8 μM 60-mer ssDNA, 1 mM dTMP-PCP in the same buffer with 10 mM MgCl2), in which we know hexamers are formed (22).

**RESULTS**

The mutant 4A'/E348K protein is a set in a set of randomly prepared mutant proteins of the bacteriophage T7 4A' helicase
protein that was genetically selected for the inability to support bacteriophage T7 DNA replication (13, 14). This amino acid substitution is adjacent to conserved motif 1a in the primary structure of the 4A' protein (Fig. 1). Here we report the DNA unwinding and dTTP hydrolysis activities of the mutant 4A'/E348K protein and show that this mutant protein has the unusual property of being able to unwind more base pairs of DNA per dTTP hydrolysis event than the wild-type protein. Thus the mutant 4A'/E348K protein is more efficient at unwinding DNA than the wild-type protein.

**dTTPase Activity of the Mutant 4A'/E348K Protein**—It has been shown previously (14) that the mutant 4A'/E348K protein is defective in dTTP hydrolysis, both in the absence and presence of ssDNA. To examine this activity in more detail, we measured both the intrinsic (DNA-independent) dTTPase activity and the DNA-stimulated dTTPase activities at 18 °C. In this assay, we incubated the wild-type 4A' or the mutant 4A'/E348K proteins (0.083 μM) with [α-32P]dTTP (0–500 μM) in the presence or absence of M13 circular ssDNA (50 nM), and monitored the conversion of dTTP to dTDP as a function of time. 50 nM M13 ssDNA was sufficient to achieve maximal DNA-stimulated dTTPase activity with both the wild-type and mutant proteins. Table I shows the kcat and Km steady-state parameters for the wild-type 4A' and the mutant 4A'/E348K proteins in the presence and absence of M13 ssDNA. The mutant 4A'/E348K protein had no detectable dTTPase activity in the absence of ssDNA. In the presence of ssDNA, the mutant 4A'/E348K protein had a 25–30-fold reduced kcat relative to the wild-type 4A' protein.

To compare directly the DNA unwinding rates with the DNA-stimulated dTTPase rates, we also measured the steady-state M13 ssDNA-stimulated dTTPase activity of the wild-type 4A' and the mutant 4A'/E348K proteins using similar protein concentration (0.67 μM), dTTP concentration (2 mM), and Mg2+ concentration (5 mM) used to measure DNA unwinding. These rate constants were used in the calculation of the DNA unwinding efficiency. For the wild-type 4A' protein, the rate constant of M13 ssDNA-stimulated dTTP hydrolysis was 26 ± 0.4 mol of dTTP hydrolyzed per mole of protein hexamer per second and for the mutant 4A'/E348K protein, the rate constant was 0.87 ± 0.014 mol of dTTP/mole of hexamer per second. This was a 30-fold reduction in the steady-state DNA-stimulated dTTPase activity of the mutant 4A'/E348K protein relative to the wild-type 4A' protein.

**DNA Unwinding Activity of the Mutant 4A'/E348K Protein**—Because dTTP hydrolysis is coupled to DNA unwinding, we expected the 25–30-fold decrease in the DNA-stimulated dTTPase activity of the mutant 4A'/E348K protein relative to the wild-type 4A' protein to result in a 25–30-fold decrease in the DNA unwinding activity. To test this, we used a single turnover stopped-flow assay to monitor the kinetics of DNA unwinding (4). Two DNA substrates (fork DNAs) were used: one with a 33-bp double-stranded DNA region and one with a 66-bp double-stranded DNA region. Both fork DNA substrates had two non-complementary single-stranded tails on the same end of the duplex region, a 36-base long 5' tail and a 15-base long 3' tail. These lengths were shown to provide optimal DNA unwinding activity (4). One strand of each fork DNA substrate was labeled at the end of the duplex region with a fluorescein, whose fluorescence increased upon DNA unwinding (4). Assays were done at a low DNA substrate concentration (1.5 nM) to reduce the amount of re-annealing of the two DNA strands following enzyme-catalyzed unwinding and a high protein hexamer concentration (0.67 μM) to achieve the maximum unwinding rate. Thus, there was a 450-fold excess of the enzyme (hexamer concentration) over the DNA substrate. The wild-type 4A' protein or the mutant 4A'/E348K protein was preincubated with the dTTP and the fork DNA in the absence of MgCl2. This preincubation period allowed a complex to form between the protein and the DNA fork, and the DNA unwinding reactions were initiated by the addition of MgCl2.

Fig. 2 shows the stopped-flow traces of the DNA unwinding reactions catalyzed by the wild-type 4A' and the mutant 4A'/E348K proteins. The stopped-flow traces show the exponential increase in the fluorescence of the DNA substrate due to DNA unwinding. We know that the change in fluorescence signal is due to DNA unwinding because the stopped-flow data overlay with the DNA unwinding data measured by the radiometric rapid chemical quench flow assay (4). The traces obtained from using the 33-bp fork substrate had a short lag period preceding the exponential increase, while the traces obtained from using the 66-bp fork substrate had a longer lag period. The presence of the lag is due to this assay measuring only the final ssDNA products and not any partially unwound DNA intermediates. The solid lines represent the fit of the data to Equation 1 (see “Materials and Methods”) which describes the stepwise unwinding of DNA. For the wild-type 4A' protein, the data obtained using the 33-bp substrate fit best with two steps (n = 2), and the data obtained using the 66-bp substrate fit best with three steps (n = 3). For the mutant 4A'/E348K protein, the data obtained using the 33-bp substrate fit best with one step (n = 1), and the data obtained using the 66-bp substrate fit best with two steps (n = 2). We have used this equation in our analysis of the stopped-flow DNA unwinding data only because it is the proper way to obtain the overall rate of DNA unwinding in the presence of a lag (21). Although this analysis provides an estimate of the size of each step, a more detailed study of the unwinding activity using many lengths of duplex DNA is required to obtain accurate values for the size of each step. For each stopped-flow trace, the overall rate of DNA unwinding was calculated from the number of steps and the first-order rate constant for each step, both of which were obtained from the fit to Equation 1. Table II lists the overall rate constants of the DNA unwinding activities (in forks unwound per second).
The DNA unwinding efficiency is the ratio of the overall rate constant of DNA unwinding to the rate constant of dTTP hydrolysis. The overall rate constant of DNA unwinding is expressed both as the number of fork DNAs unwound/s and the number of base pairs unwound/s. Therefore, the overall DNA unwinding rate in units of base pairs unwound per second is:

\[ \text{overall DNA unwinding rate} = \frac{\text{rate constant of DNA unwinding}}{\text{rate constant of dTTP hydrolysis}} \]

By multiplying the overall DNA unwinding rate (forks/s) by the length of DNA unwound in each fork substrate, we calculated the overall DNA unwinding rates in units of base pairs unwound per second. We found that the wild-type 4A' protein unwound the 33-bp substrate with a rate of 27 bp/s and the 66-bp substrate with a rate of 19 bp/s. Because these two rates were similar, it is highly unlikely that there is an additional kinetic step, such as a conformational change, occurring prior to DNA unwinding and contributing to the lag. Thus the rate measured under the given conditions is actually the unwinding rate of double-stranded DNA. The mutant 4A'/E348K protein unwound the 33-bp substrate with a rate of 6.6 bp/s and the 66-bp substrate with a rate of 3.3 bp/s. Again the similar rates mean that we are actually measuring the DNA unwinding rate.

The above data show that despite a 20–30-fold decrease in the dTTPase activity, there is only a 5–6-fold reduction in the DNA unwinding activity of the mutant 4A'/E348K protein relative to the wild-type 4A' protein.

Since the DNA unwinding activity of the mutant 4A'/E348K protein required less dTTP hydrolysis than the wild-type 4A' protein, we checked whether the unwinding activity of the mutant protein was dependent upon dTTP hydrolysis. DNA unwinding was measured in the presence of the nonhydrolyzable analog of dTTP, dTMP-PCP, and in the absence of dTTP. DNA unwinding was not detected in either case (data not shown), indicating that the DNA unwinding activity of the mutant 4A'/E348K protein was dependent upon dTTP hydrolysis.

### Table II

| Protein     | Fork DNA size (bp) | \(k_{\text{overall}}^a\) | \(k_{\text{overall}}^b\) | \(k_{\text{dTTPase}}^b\) | Efficiency$^c$ (bp/dTTP) |
|-------------|-------------------|----------------|----------------|----------------|------------------|
| 4A'         | 33                | 0.82           | 27             | 26             | 1.0              |
| E348K       | 33                | 0.17           | 5.6            | 0.87           | 6.4              |
| 4A'         | 66                | 0.29           | 26             | 0.73           | 1.0              |
| E348K       | 66                | 0.050          | 3.3            | 0.87           | 3.8              |
| E348A       | 33                | 0.15           | 5.0            | 4.8            | 1.0              |

*The overall rate constant of DNA unwinding was calculated from the rate constants obtained from fitting the DNA unwinding stopped-flow traces (Fig. 1) to Equation 1 (see "Materials and Methods"). The overall rate constant of DNA unwinding is expressed both as the number of fork DNAs unwound/s and the number of base pairs unwound/s.

*The M13 ssDNA stimulated dTTPase activities were measured under identical conditions as the DNA unwinding assays: 20 mM TrisCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 10% glycerol, 4 mM protein, 2 mM dTTP, and 50 nM M13 ssDNA. The units for the rate constant of dTTP hydrolysis are mol of dTTP hydrolyzed/mol of protein hexamer per second.

*The DNA unwinding efficiency is the ratio of the overall rate constant of DNA unwinding to the rate constant of dTTP hydrolysis.
Fig. 2. HPLC gel filtration analysis of the complex between the fork DNA and 4A' or mutant 4A'/E348K proteins. Gel filtration elution profiles of fluorescein-labeled fork DNA and a mixture of fork DNA and 4A' or 4A'/E348K proteins are shown. Samples contained 4A' or mutant 4A'/E348K proteins (0.67 μM hexamers), 2 mM dTTP, and 15 mM fork DNA with a 33-bp duplex region in 20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 10% glycerol. The fluorescence (λex = 494 nm and λem = 525 nm) of the fork DNA was monitored as described (see "Materials and Methods"). The free fork DNA eluted at 10.5 min, fork DNA complexed with one hexamer eluted at 9.5 min, and fork DNA complexed with two hexamers eluted at 8.7 min. The arrows at 9.3 and 8.5 min indicate the elution times of the standards: one 4A' hexamer bound to 60-mer ssDNA and one 4A' dodecamer bound to 60-mer ssDNA, respectively.

DNA, eluted at 8.7 min, and the other peak, corresponding to one hexamer bound to the fork DNA, eluted at 9.5 min. The proportion of fork DNAs bound to one versus two hexamers was approximately the same for the wild-type 4A' protein and the mutant 4A'/E348K protein. The significance of two hexamers binding to the same fork DNA is unclear. Thus, both the wild-type and mutant proteins formed the same complex with forked DNA during the unwinding preincubation period.

Next we did additional DNA unwinding experiments in the presence of a 60-mer ssDNA trap, which was used to eliminate any DNA unwinding that resulted from protein not complexed with the fork DNA during the preincubation period. These experiments would determine if the same protein hexamer that was bound initially to the fork DNA was catalyzing the DNA unwinding reaction once MgCl₂ was added. These experiments were designed to investigate possible DNA unwinding, in the case of the 4A'/E348K protein, by a completely different mechanism, such as cooperative binding of several hexamers to the fork DNA. DNA unwinding was measured by the radiometric assay (4) using a rapid chemical quench flow instrument in the absence or presence of the trap DNA. In a control experiment the 60-mer DNA was added to the fork DNA substrate prior to preincubating with the protein. When the trap DNA was included in the preincubation mixture, no unwinding was detected (Fig. 4, A and C).

Fig. 4 shows the DNA unwinding kinetics of 4A' and 4A'/E348K proteins in the absence and presence of trap DNA using the 33- or 66-bp fork DNA. In each case, the data were fit to single-exponential functions which provided the amplitudes and first-order rate constants for DNA unwinding. The DNA unwinding rates in the absence and presence of the trap were generally similar. The largest difference was seen with the wild-type 4A' using the 33-bp fork DNA substrate, and this difference was less than 2-fold. The DNA unwinding rates for the mutant 4A'/E348K protein in the presence and absence of the trap DNA were almost identical. The amplitudes of DNA unwinding, which reflects the fraction of fork DNA substrate unwound, did differ in the absence or presence of trap DNA. When trap DNA was included, a smaller fraction of the fork DNA was unwound, most likely due to the dissociation of the inactive or nonproductive complex. A 2-fold difference in amplitude of unwinding the 66-bp fork DNA was seen with the mutant 4A'/E348K protein, and a 1.4-fold difference was seen for the 4A' protein. These experiments show that DNA unwinding was catalyzed by a single 4A' or 4A'/E348K protein hexamer that was bound to the fork DNA substrate during the preincubation period and that DNA unwinding was not catalyzed by a completely different mechanism in the case of the mutant 4A'/E348K protein.

dTTPase Activity of the Mutant 4A'/E348K during DNA Unwinding—We have measured the dTTPase activity of the proteins with the fork DNA to examine any differences between the M13 ssDNA-stimulated dTTPase rates and the dTTPase rates in the time scale of DNA unwinding. The dTTPase activity was measured using a rapid chemical quench flow instrument since unwinding is completed within a few seconds. The wild-type 4A' protein or the mutant 4A'/E348K protein was preincubated with an excess of the 33-bp fork DNA. The dTTP concentration of 150 μM was used to observe a better signal of dTTP hydrolysis. We examined time points below the t₁/₂ of DNA unwinding of the 33-bp fork DNA (0.85 s for the wild-type 4A' protein and 4.2 s for the mutant 4A'/E348K protein).
measure the dTTPase activity during unwinding. Fig. 5 shows examples of the dTTPase kinetics of both the wild-type and mutant proteins. For the wild-type 4A' protein, the rate constant of dTTP hydrolysis during DNA unwinding ranged from 5.5 to 9 mol of dTTP hydrolyzed per mole of protein hexamer per second, and for the mutant 4A'/E348K protein, it ranged from 0.3 to 0.35 mol of dTTP hydrolyzed per mole of protein hexamer per second. The dTTPase activity during DNA unwinding of the mutant 4A'/E348K protein was about 20–25-fold lower than that of the wild-type 4A' protein. The dTTPase rates with the fork DNA are slightly lower (3–4-fold) than the rates obtained from the steady-state dTTPase assay using M13 ssDNA. This was most likely due to subsaturating concentration of dTTP.

Properties of the Site-directed Mutant 4A'/E348A Protein—To understand better at the structural or molecular level how the Glu-348 to lysine substitution leads to an increase in the efficiency of DNA unwinding, we created and characterized a site-directed mutant of Glu-348. Because glutamate to lysine is a radical change, we examined the properties of a site-directed mutant protein with a more conservative change, glutamate to alanine. Table II lists the rate constants of the DNA unwinding and the M13 ssDNA-stimulated dTTPase activities of the site-directed mutant 4A'/E348A protein. Like the mutant 4A'/E348K protein, the site-directed mutant 4A'/E348A protein had a 5-fold lower DNA unwinding rate than the wild-type protein. Unlike the mutant 4A'/E348K, the site-directed mutant 4A'/E348A had only a 5-fold lower M13 ssDNA-stimulated dTTPase rate than the wild-type protein. Therefore, the DNA unwinding efficiency of the site-directed mutant 4A'/E348A protein is the same as that of the wild-type protein, about 1 bp of DNA unwound per dTTP hydrolyzed.

DISCUSSION

We have examined the kinetics of dTTP hydrolysis and DNA unwinding by the wild-type 4A' protein and a mutant 4A'/E348K protein to estimate the DNA unwinding efficiencies of both proteins. The DNA unwinding efficiency of the wild-type 4A' protein is close to 1 bp/dTTP hydrolysis and the DNA unwinding efficiency of the mutant 4A'/E348K protein is in the range of 4–6 bp per dTTP hydrolysis. The HPLC size exclusion chromatography (Fig. 3) and the unwinding experiments done in the presence of trap DNA (Fig. 4) show that both proteins unwind the DNA substrates using the same general mechanism. They both bind to the DNA substrates in the presence of dTTP and absence of MgCl2 but do not unwind it. In the case of both proteins, once MgCl2 is added, the proteins hexamers that are bound to the DNA substrate translocate along the DNA and unwind the DNA.

In estimating the DNA unwinding efficiencies of the wild-type 4A' protein and the mutant 4A'/E348K protein, we have compared DNA unwinding rates to the steady-state rate of M13 ssDNA-stimulated dTTP hydrolysis. We used the steady-state M13 ssDNA-stimulated rates for three reasons. First, these rates are readily measurable under the same buffer conditions and same dTTP concentrations as used in the unwinding assay. Second, the steady-state rates measured with M13 ssDNA are nearly 10-fold higher than the steady-state rates measured with short oligodeoxynucleotides. This is due to the fact that falling off the end of the DNA is rate-determining under steady-state conditions, and because M13 ssDNA has no end, the M13-DNA-stimulated rates better reflect the rates of dTTP hydrolysis during translocation along the DNA and DNA unwinding. Third, studies with the hexameric Escherichia coli Rho helicase, which unwinds RNA/DNA heteroduplexes, have shown that the rate of ATP hydrolysis of the Rho protein while it is translocating along ssRNA is identical to the rate of ATP hydrolysis while it is unwinding RNA/DNA heteroduplexes (23).

In previous studies, the efficiency of DNA (or DNA/RNA heteroduplex) unwinding by the E. coli Rho protein, the E. coli RecBCD protein, and the E. coli Rep protein have been reported. Rho protein was reported to unwind 0.5–1 bp per ATP hydrolyzed (23), Rep helicase unwinds 0.5 bp per ATP hydrolyzed (24), RecBCD helicase unwinds between 0.25 and 0.33 bp per ATP hydrolyzed (25, 26), and RecBC enzyme (missing the D subunit) unwinds close to 1 bp per ATP hydrolyzed (27). The apparent increase in DNA unwinding efficiency when the D subunit of the RecBCD helicase is omitted is not surprising because the D subunit is itself an ATPase not required for DNA unwinding.

Three of these previous studies used very long double-stranded DNA substrates and contained other protein components, such as single-stranded DNA-binding protein (24–26) or recJ/exol (27) to indirectly monitor single-stranded DNA generated by the helicase-catalyzed DNA unwinding. The Rho study (24) and our study used short oligodeoxynucleotide substrates and monitored DNA unwinding directly in single-turnover assays. Despite these different approaches, the value for the DNA unwinding efficiency of the wild-type 4A' protein and these other helicase proteins are in close agreement. However, the 4–6-fold increase in DNA unwinding efficiency observed with the mutant 4A'/E348K protein is significantly greater than the values reported for these other helicases.

Without a high resolution structure of the 4A' protein, it is difficult to understand at the structural or molecular level how the Glu-348 to lysine change leads to an increase in the effi-

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2 M. T. Washington and S. S. Patel, unpublished data.
ciency of DNA unwinding. Glutamate to lysine is a radical change, so we examined the properties of a site-directed mutant protein 4A’/E348A, which has a more conservative change. It seems that a conservative change of Glu-348 to alanine reduces the DNA unwinding activity of the 4A’ protein the same as the radical change of Glu-348 to lysine does (about 5-fold). However, the radical change of Glu-348 to lysine reduces the ssDNA-stimulated dTTPase activity of the 4A’ protein more than a conservative change of Glu-348 to alanine does (only about 5-fold). Therefore the mutant 4A’/E348K protein has the same DNA unwinding efficiency as the wild-type 4A’ protein.

From the structure of the E. coli Rep helicase (28), it is clear that motif 1a, the conserved motif containing Glu-348, is involved in ATP hydrolysis. Motif 1a contains a highly conserved glutamate, Glu-62 in the Rep helicase, Glu-71 in the PcrA helicase (29), and Glu-348 in the 4A’ helicase, that according to the Rep helicase structure is part of the nucleotide-binding site (28). Also, residues at the beginning of motif 1a, in the position of residues 435 to 438 in the primary structure of the 4A’ protein, bind ssDNA in the Rep protein structure (28). It was speculated that motif 1a may transmit conformational changes between the nucleotide-binding site and the DNA-binding site that are required for coupling (28). In fact, mutagenesis studies of the 4A’ protein show that point mutation S345F results in a protein that is defective in coupling dTTPase activity and DNA unwinding activity (14). It is likely that this motif is playing an important role in the conformational changes necessary for energy transduction, and that point mutations in the region can affect the efficiency of DNA unwinding positively (as is the case of E348K) or negatively (as is the case of S345F).

In mechanistic terms, how can the DNA unwinding efficiency of the mutant 4A’/E348K protein be increased relative to the wild-type 4A’ protein? DNA helicases couple the energy of NTP hydrolysis to DNA unwinding, and they do this by strictly following a specific biochemical cycle, called an NTPase translocation cycle (30). In every NTPase translocation cycle, the helicase protein must hydrolyze one NTP and it must move one microstep size along the DNA unwinding it. The microstep size is the average size in base pairs of each step unwound by the helicase as a result of one NTPase translocation cycle. It is important to note that the DNA unwinding efficiency (number of base pairs unwind per NTP hydrolyzed) is less than the microstep size number of base pairs unwound per NTPase translocation cycle) if there is slippage. Slippage is the additional hydrolysis of NTP that is not linked to translocation and DNA unwinding. Since the DNA unwinding efficiency and the microstep size are identical if there is no slippage, the DNA unwinding efficiency is a lower limit of the microstep size.

Based on this mechanism, there are two ways in which the DNA unwinding efficiency of the mutant 4A’/E348K protein can be increased 4–6-fold over that of the wild-type 4A’ protein: either the microstep size of the mutant protein is increased 4–6-fold over that of the wild-type protein or the frequency of slippage by the mutant protein is decreased 4–6-fold with respect to that of the wild-type protein. A 4–6-fold increase in the microstep size of the mutant protein is unlikely because we believe that the microstep size is determined largely by the geometry of the hexameric ring and the magnitude of certain conformational changes needed to move along the DNA and unwind it. A point mutation that dramatically changes to such a degree either the geometry of the hexameric ring or the magnitude of conformational changes will almost certainly result in a total loss of DNA unwinding activity. Instead, we believe that the mutant 4A’/E348K protein slips 4–6 times less frequently than the wild-type 4A’ protein. Slippage can occur to a very large extent in DNA helicases. An extreme case is the E. coli Rep helicase, which hydrolyzes about 150 molecules of ATP per single step along ssDNA (31).

If the wild-type 4A’ protein and the mutant 4A’/E348K protein have the same microstep size steps for unwinding DNA as we have argued, then the DNA unwinding efficiency of the mutant 4A’/E348K protein provides a lower limit on the microstep size of the 4A’ protein, 4–6 bp/step. The thermodynamics of DNA unwinding provide an upper limit on the microstep size of the 4A’ protein. The hydrolysis of one NTP should provide enough energy to unwind 9–12 bp of duplex DNA (1) assuming it takes 1–1.5 kcal/mol on average to unwind 1 bp of DNA. Clearly the Rho helicase, the Rep helicase, the RecBCD helicase, and the wild-type 4A’ protein are operating at a level far below this theoretical optimum efficiency. The mutant 4A’/E348K protein is more efficient than these other proteins, but it is still not optimal. Since the 4A’ protein hydrolyzes one dTTP at a time (6), then the theoretical optimum of DNA unwinding efficiency is an upper limit on the microstep size of the 4A’ protein. In the case of the 4A’ protein, no step can be larger than 9–12 bp of DNA, because every step is linked via a dTTPase translocation cycle to only one dTTP hydrolysis. Thus the microstep size of the 4A’ protein must be between 4–6 and 9–12 bp per step, probably much closer to the lower limit.

This study shows that a point mutation can make the wild-type 4A’ protein more efficient as a helicase. One may ask why is the wild-type not as efficient as it clearly can be. We showed that the mutant 4A’/E348K protein, while it unwinds DNA more efficiently than the wild-type 4A’ protein, does it more slowly than the wild-type 4A’ protein. The mutant 4A’/E348K protein goes through the dTTPase translocation cycles slower than the wild-type, and so it hydrolyzes less dTTP and unwinds less DNA than the wild-type in the same length of time. We speculate that there may be a trade-off between the rate of the dTTPase translocation cycles and the degree of slippage in the 4A’ protein. If one wants to make the helicase protein unwind DNA more efficiently, one probably has to slow down the rate of the dTTPase translocation cycles which will make the helicase unwind DNA more slowly. It appears that in the case of bacteriophage T7 DNA replication, nature has selected for the appropriate balance between speed and efficiency. The wild-type 4A’ protein was selected because it is fast, even though it is less efficient. The mutant 4A’/E348K protein, while it is more efficient than the wild-type protein does not support the growth of the bacteriophage (13), possibly because it is too slow in unwinding DNA. Such a trade off between the rate of work and the efficiency of work may be a general feature of motor proteins.

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