DNA Helicases Displace Streptavidin from Biotin-Labeled Oligonucleotides†

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ABSTRACT: Helicases are enzymes that use energy derived from nucleoside triphosphate hydrolysis to unwind double-stranded (ds) DNA, a process vital to virtually every phase of DNA metabolism. The helicases used in this study, gp41 and Dda, are from the bacteriophage T4, an excellent system for studying enzymes that process DNA. gp41 is the replicative helicase and has been shown to form a hexamer in the presence of ATP. In this study, protein cross-linking was performed in the presence of either linear or circular single-stranded (ss) DNA substrates to determine the topology of gp41 binding to ssDNA. Results indicate that the hexamer binds ssDNA by encircling it, in a manner similar to that of other hexameric helicases. A new assay was developed for studying enzymatic activity of gp41 and Dda on single-stranded DNA. The rate of dissociation of streptavidin from various biotinylated oligonucleotides was determined in the presence of helicase by an electrophoretic mobility shift assay. gp41 and Dda were found to significantly enhance the dissociation rate of streptavidin from biotin-labeled oligonucleotides in an ATP-dependent reaction. Helicase-catalyzed dissociation of streptavidin from the 3′-end of a biotin-labeled 62-mer oligonucleotide occurred with a first-order rate of 0.17 min^{-1}, which is over 500-fold faster than the spontaneous dissociation rate of biotin from streptavidin. Dda activity leads to even faster displacement of streptavidin from the 3′ end of the 62-mer, with a first-order rate of 7.9 s^{-1}. This is more than a million-fold greater than the spontaneous dissociation rate. There was no enhancement of streptavidin dissociation from the 5′-biotin-labeled oligonucleotide by either helicase. The fact that each helicase was capable of dislodging streptavidin from the 3′-biotin label suggests that these enzymes are capable of imparting a force on a molecule blocking their path. The difference in displacement between the 5′ and 3′ ends of the oligonucleotide is also consistent with the possibility of a 5′-to-3′ directional bias in translocation on ssDNA for each helicase.

DNA helicases are molecular motors that transduce the energy obtained from hydrolysis of nucleoside triphosphates (NTP)[1] to perform the mechanical work of unwinding double-stranded (ds) DNA (*1*−*5*). These enzymes are ubiquitous and necessary for most aspects of nucleic acid metabolism, including replication, repair, and recombination. Several disease states have recently been associated with defective helicases, such as Bloom's syndrome (*6*) and xeroderma pigmentosum (*7*). However, the biochemical mechanism(s) of helicases are largely unknown. The *Escherichia coli* Rep helicase has been closely studied and a mechanism has been proposed for this dimeric enzyme (*2*, *5*). One subunit of Rep is proposed to bind ssDNA while the other binds and unwinds dsDNA in a cyclic process coordinated by binding and hydrolysis of ATP. The preferential affinity of one subunit of the dimer for dsDNA over ssDNA at a ds/ss DNA junction is proposed to drive translocation of the enzyme via a "rolling" or "subunit switching" mechanism. This mechanism predicts that little or no directional bias occurs during translocation on ssDNA substrates. In contrast, others have proposed that some helicases translocate unidirectionally on ssDNA and that such activity is important to the overall mechanism of dsDNA unwinding (*8*). A model for translocation by the *E. coli* transcription termination protein Rho has been provided that includes a directionally biased random walk along RNA (*9*). Evidence has been provided suggesting that the bacteriophage helicases gp41 and Dda translocate unidirectionally on ssDNA (*10*, *11*).

gp41 can oligomerize into a hexamer in the presence of ATP or GTP (*12*) and serves as the replicative helicase of bacteriophage T4. Investigators using electron microscopy have determined that some hexameric helicases bind to DNA by encircling it, such that the DNA passes through the central channel of the hexamer (reviewed in ref *3*). Results from biochemical experiments (*13*) may be interpreted as being consistent with similar DNA binding by gp41. In this report, we provide additional evidence based on biochemical studies that suggests that gp41 encircles ssDNA.

One outstanding question regarding helicase function deals with the direction and mechanism of translocation on ssDNA.

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[1] Abbreviations: NTP, nucleoside triphosphate; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; gp41, gene product of the T4 41 gene; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PK/LDH, phosphoenolpyruvate kinase/lactate dehydrogenase; DTT, dithiothreitol; DSP, dithiosuccinimido propionate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminotetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BME, β-mercaptoethanol.

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Studies of another molecular motor protein, the F_1-ATPase, may provide clues toward answering this question. The hexameric form of helicases with DNA passing through the central channel is somewhat analogous to the F_1-ATPase, which consists of a hexamer of $\alpha_3\beta_3$ subunits encircling a γ subunit (*14*). Rotation of the γ subunit within the cylinder of the hexamer has been directly observed in the presence of ATP (*15*). Recently, the dTTPase activity of the hexameric gene 4 helicase from bacteriophage T7 was found to resemble the mechanism of the F_1-ATPase, in which three of the six potential nucleotide binding sites on the hexamer are catalytic sites and three are noncatalytic sites (*16*). A rotational movement of the gene 4 hexamer around the ssDNA was proposed to result in unidirectional translocation and unwinding of duplex DNA.

Others have recently proposed an inchworm mechanism involving unidirectional translocation on RNA or ssDNA based on the crystal structure of the nonstructural protein 3 (NS3) helicase from the hepatitis C virus (*17*). If helicases translocate unidirectionally on ssDNA, they may produce a force in the direction of translocation. The effect of force on biochemical kinetics has recently received much attention (*18*). Technical innovations with atomic force microscopy (AFM), optical tweezers, and other methods have allowed direct measurement of the forces involved in binding of a ligand to its receptor as well as the forces generated by a translocating enzyme (*19*). In this report, we provide evidence for force production by DNA helicases that appears to have a strong directional bias on ssDNA. When challenged with biotinylated oligonucleotide substrates to which streptavidin has been bound, the gp41 and Dda helicases can rapidly displace the streptavidin, suggesting that these enzymes impart a strong force on the streptavidin.

EXPERIMENTAL PROCEDURES

Materials. Chemicals and where they were purchased are as follows: streptavidin and PK/LDH (Sigma); ATP, biotin, and acrylamide (Fisher); radioactive ATP (New England Nuclear); DSP (Pierce); T4 polynucleotide kinase and M13 ssDNA (New England Biolabs); SYBR Green II nucleic acid stain (Molecular Probes). Oligonucleotides were synthesized by Operon with the Bio-TEG biotin label. Dda helicase was overexpressed and purified as described (*11*, *20*).

gp41 Purification. gp41 was expressed in *E. coli* strain OR1265/pDH518 by temperature induction at 42 °C for 3 h (*21*). Cells were suspended in lysis buffer (0.2 mg/mL lysozyme, 0.5 mM PMSF, 50 mM Tris acetate, pH 7.4, and 1 mM EDTA), sonicated, and centrifuged at 16 000 rpm for 30 min. The pellet was resuspended in extraction buffer as described previously (*22*) (20 mM Tris acetate, pH 7.4, 10 mM $MgCl_2$, 1.0 mM DTT, 2.0 M urea, and 10% sucrose). Barry and Alberts (*22*) reported that utilization of urea in the buffer facilitates solubilization of gp41 and results in protein with the same or higher specific activity as gp41 prepared by alternative protocols (*21*). After 1 h at 4 °C, this solution was centrifuged at 40 000 rpm for 2 h in a Beckman Ti70 rotor, leaving the protein in the supernatant. gp41 was then applied to a macroprep High-Q strong anion-exchange resin (Bio-Rad) equilibrated with extraction buffer and eluted with a linear NaCl gradient from 0 to 500 mM. Fractions containing gp41, which eluted at 200−250 mM NaCl, were identified by SDS−PAGE, pooled, and dialyzed into extraction buffer. gp41 was then applied to a ssDNA−cellulose column (Amersham Pharmacia Biotech) and eluted with a linear NaCl gradient from 0 to 2.0 M, and fractions were analyzed by SDS−PAGE. gp41 eluted at 80−380 mM NaCl. Protein was dialyzed into MOPS extraction buffer (25 mM MOPS, pH 6.7, 1.0 M urea, 10 mM $MgCl_2$, 1 mM DTT, and 5% glycerol) and applied to a macro-prep High-S strong cation-exchange resin (Bio-Rad). Protein was eluted with a linear gradient from 0 to 500 mM NaCl, with gp41 eluting at 315−450 mM NaCl, and fractions were analyzed by SDS−PAGE. At this point, the protein appeared to be >95% pure. Half of the purified protein was dialyzed into a buffer of 25 mM Hepes, pH 8.0, 1.0 mM EDTA, 20% glycerol, 50 mM KOAc, and 5 mM BME. The other half was dialyzed into the same buffer without BME; this stock was used for experiments involving DSP cross-linking. Aliquots of protein were stored at −80 °C.

Oligonucleotide Purification and Labeling. Oligonucleotides were purified by denaturing 20% polyacrylamide gel electrophoresis and electroeluted from the gel with an Elutrap apparatus (Schleicher & Schuell). DNA was desalted with a Waters Sep-Pak column and dried via Speed-Vac (Savant). Oligos were resuspended in 10 mM Hepes, pH 7.5, and 1 mM EDTA and quantitated by their A_{260} after dilution in 0.2 M KOH using calculated extinction coefficients. Oligonucleotides were 5′-radiolabeled with T4 polynucleotide kinase at 37 °C for 1 h. The kinase was inactivated by heating to 85 °C for 10 min. Unincorporated [γ-^{32}P]ATP was removed by twice passing the labeled oligos through a Sephadex G-25 spin column. Oligonucleotide sequences for the streptavidin displacement experiments were as follows (with **X** signifying a biotin label): 5′-bio-60-mer, 5′-G**X**ACGTATTCAAGATACCTCGTACTCTGTACTGAC-TGCGATCCGACTGTCCTGCATGATG-3′; 3′-bio-62-mer, 5′-TAACGTATTCAAGATACCTCGTACTCTGTACTGA-CTGCGATCCGACGTCCTGCATGATG**X**T-3′; 3′-bio-30-mer, 5′-CTGACTGCGATCCGACTGTCCTGCATGA**X**G-3′; 3′-bio-21-mer, 5′-ATCCGACTGTCCTGCATGA**X**G-3′; 3′-bio-16-mer, 5′-TCCTGCATGATGAG**X**T-3′; 3′-bio-11-mer, 5′-TGCATGATG**X**T-3′.

Binding Topology Experiment. A 5′-radiolabeled 50-mer oligonucleotide was used for the linear DNA portion of this experiment. In four samples, 25 nM 50-mer was incubated with 25 mM Hepes, pH 8.2, 100 μM EDTA, and 5.8 μM gp41 at 37 °C for 5 min. ATP (10 mM) was then added, and the binding reaction was allowed to proceed for 10 min. This was followed by addition of 1 mM DSP cross-linker to samples 3 and 4. Cross-linking was allowed to proceed for 4 min at 37 °C, after which the reaction was quenched by addition of 0.1 M glycine. Samples 2 and 4 were subjected to denaturing conditions (0.1% SDS, 50 °C for 5 min). All four samples were then analyzed by electrophoresis through a native 15% polyacrylamide gel in running buffer containing 500 μM ATP. Gel bands were visualized with a Molecular Dynamics 445-SI Phosphorimager.

M13 ssDNA (New England Biolabs) was used for the circular DNA portion of this experiment. Four samples of 19 μM M13 ssDNA (concentration in nucleotides), 25 mM Hepes, pH 8.2, 100 μM EDTA, and 6.5 μM gp41 were incubated at 37 °C for 5 min. ATP (10 mM) was added, and after 10 min DSP (1 mM) was added to samples 3 and

4. Cross-linking was quenched after 4 min by addition of 0.1 M glycine, and samples 2 and 4 were subjected to denaturing conditions by treatment with 0.1% SDS at 50 °C for 5 min. The four samples were then analyzed by electrophoresis on a 1.0% agarose gel containing 500 μM ATP. Gel bands were visualized by staining the ssDNA with SYBR Green II (Molecular Probes).

Streptavidin Displacement Experiment. 5′-Radiolabeled oligos with biotin on either their 5′ or 3′ end were utilized in this experiment. Oligonucleotide (10 nM) was incubated in helicase reaction buffer [25 mM Hepes, pH 7.5, 12.5 mM $Mg(OAc)_2$, 150 mM KOAc, 4 mM phosphoenolpyruvate (PEP), 1 mM BME, and 0.1 mg/mL bovine serum albumin (BSA)], along with 5 mM ATP and 300 nM streptavidin at 37 °C for 2−3 min. PK/LDH, 10.8 units/mL and 16.7 units/mL, respectively, and 6 μM free biotin trap were added. The reaction was initiated upon addition of helicase at the concentrations described in the figure legends. At various times, 10 μL aliquots were removed and mixed with 10 μL of helicase quench buffer [0.6% SDS, 200 mM EDTA, pH 8.0, and 10 μM poly (dT)]. Gel loading buffer (0.1% xylene cyanol, 0.1% bromophenol blue, and 10% glycerol) was added to each sample, followed by electrophoretic analysis on a native 15% polyacrylamide gel. The fraction of free oligonucleotide and streptavidin-bound oligonucleotide was determined for each sample with the Molecular Dynamics Phosphorimager. Oligo length for each experiment ranged from 11 to 62 nucleotides, and gp41 concentration varied from 250 nM to 4 μM as described in the figure legends.

For the Dda helicase, a Kintek rapid chemical quench-flow instrument (Kintek, Inc., State College, PA) was used to measure very fast dissociation of streptavidin from biotin-labeled oligonucleotides. The reaction was performed with two different protocols. In the first method, Dda in helicase reaction buffer was rapidly mixed with the biotin-labeled oligonucleotide (10 nM after mixing) and ATP (5 mM after mixing). The reaction mixture was incubated for varying times and then stopped by rapidly mixing with helicase quench buffer. In the second method, Dda in helicase assay buffer was preincubated with the biotin-labeled oligonucleotide, followed by initiation of the reaction by mixing with ATP. The concentration of Dda for each experiment is listed in the figure legends. The receiving vial for each sample contained poly(dT) (5 μM after addition of the reaction mixture) in order to prevent a gel shift of the biotin-labeled oligonucleotide due to helicase binding. An aliquot (25 μL) of each sample was mixed with nondenaturing gel loading buffer (4 μL), followed by analysis of samples by gel electrophoresis, visualization with a phosphorimager, and quantitation by Imagequant software (Molecular Dynamics).

RESULTS

gp41 Hexamer Binds ssDNA by Encircling the DNA Strand. Studies using electron microscopy, as with gene 4 protein and *E. coli* RuvB, and biochemical techniques, as with SV40 T antigen, have revealed that hexameric helicases can bind to their DNA substrates by encircling them (*23*−*25*). gp41 is a hexamer with dimensions similar to those of the gene 4 helicase (*12*). Previous work using biotinylated oligonucleotides bound with streptavidin suggested that one strand of DNA passes through the channel of gp41 during

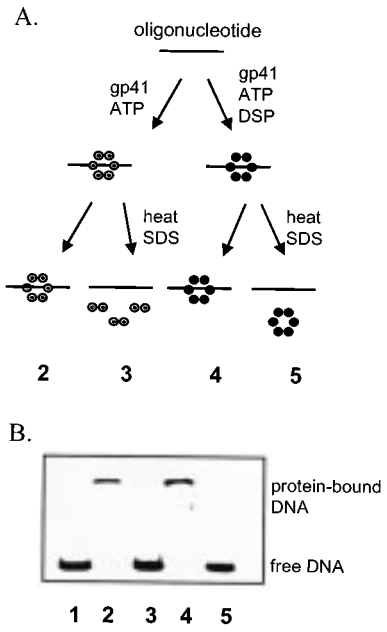

FIGURE 1: Cross-linking, gel-shift assay with single-stranded linear DNA. (A) Illustration of experimental protocol. Radiolabeled 50-mer oligonucleotide was incubated with gp41 and EDTA in Hepes (pH 8.2), followed by addition of ATP. This mixture was divided into two parts, and one was treated with DSP cross-linker. The two mixtures were split again, with half of each subjected to denaturing conditions for 5 min. The samples were then analyzed by native polyacrylamide gel electrophoresis. (B) Results from electrophoretic mobility shift assay. Lane 1 depicts radiolabeled 50-mer. The remaining four lanes correspond to the numbered samples in panel A.

DNA unwinding (*13*). To further investigate this possibility, we have utilized protein cross-linking experiments to analyze the binding topology of the hexamer in a manner similar to that described for the gene 4 helicase (*23*). gp41 can be readily cross-linked as a hexamer by the chemical cross-linker dithiosuccinimido propionate (DSP) in the presence of nucleoside triphosphates ATP or GTP, as well as the nonhydrolyzable analogues ATPγS and GTPγS (*12*). Chemical cross-linking of gp41 has been used in conjunction with electrophoretic gel mobility shift analyses of ssDNA to investigate whether gp41 encircles the DNA.

Figure 1 shows the results of gp41 hexamer cross-linking in the presence of linear ssDNA. In the absence of DSP, gp41 binding retards DNA migration through the gel. When the non-cross-linked gp41 is subjected to denaturing conditions, the DNA shift disappears. In the presence of DSP, gp41 again shifts DNA, unless it is subjected to denaturing conditions, in which case the shift disappears. This result indicates that DSP does not cross-link gp41 to the DNA.

Figure 2 shows the results of gp41 hexamer cross-linking in the presence of M13 circular ssDNA. In the absence of DSP, gp41 hexamer binding causes DNA to be shifted, and as with the oligonucleotide, this shift disappears with the introduction of denaturing conditions (lane 3, Figure 2B). In the presence of DSP, the DNA band is shifted regardless of the presence or absence of denaturing conditions. These results can be interpreted as hexameric gp41 binding ssDNA by encircling the DNA strand. In the absence of DSP, heat and SDS treatment causes the hexamer to dissociate from both linear and circular ssDNA, due to disruption of both DNA−protein binding and protein−protein interactions

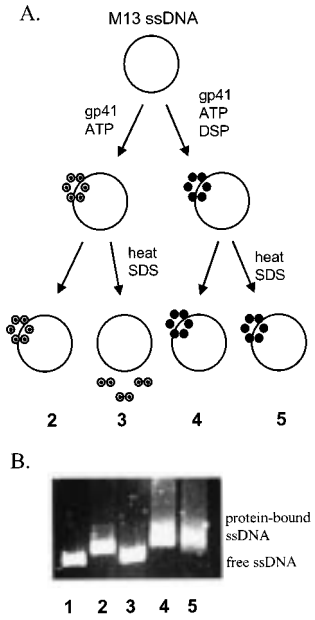

FIGURE 2: Cross-linking, gel-shift assay with circular ssDNA (M13). (A) Illustration of experimental protocol. M13 ssDNA was incubated with gp41 and EDTA in Hepes (pH 8.2), followed by addition of ATP. This mixture was divided into two parts, and one part was treated with DSP. The two mixtures were split again, with half of each being subjected to denaturing conditions for 5 min. The samples were then analyzed by agarose gel electrophoresis. (B) Results from electrophoretic mobility shift assay. Lane 1 depicts M13 ssDNA. The remaining four lanes correspond to the numbered samples in panel A.

between hexameric subunits. When gp41 hexamer is treated with DSP, the protein subunits are linked by covalent bonds that are not broken by denaturation, although DNA−protein binding is lost as shown with the oligonucleotide (Figure 1). Denaturing treatment causes the cross-linked hexamer to dissociate from linear ssDNA, because gp41 binding to DNA is disrupted and the hexamer can slide off the end of the DNA strand. The cross-linked hexamer will not, however, dissociate from circular ssDNA under denaturing conditions. Binding between gp41 and DNA is disrupted, but there is no free DNA end for the cross-linked hexamer to slide off, and the DNA band remains shifted on the gel.

gp41 and Dda Are Capable of Displacing Streptavidin from the 3′ End, but Not the 5′ End, of a Biotinylated Oligonucleotide. Previous work from the von Hippel laboratory indicated that gp41 translocates with a 5′-to-3′ directional bias on ssDNA (*10*). Kinetic analysis of the ATPase activity of gp41 in the presence of varying length ssDNA strongly suggested that this enzyme moves primarily in one direction. We have provided evidence that Dda also translocates with a 5′-to-3′ directional bias (*11*). The ATPase activity of Dda was measured on ssDNA substrates containing biotin−streptavidin blocks, and the resulting alteration in the ATPase kinetics on 5′-biotinylated oligonucleotides versus 3′-biotinylated oligonucleotides suggested that Dda travels with a 5′-to-3′ directional bias. However, each of these approaches relied on kinetic analysis of ATPase activity, and we sought a more direct approach for studying translocation on ssDNA.

Translocation directionality was investigated by analyzing the ability of each helicase to displace streptavidin from biotinylated oligonucleotides. A gel mobility shift assay was utilized to separate biotinylated oligonucleotides bound by

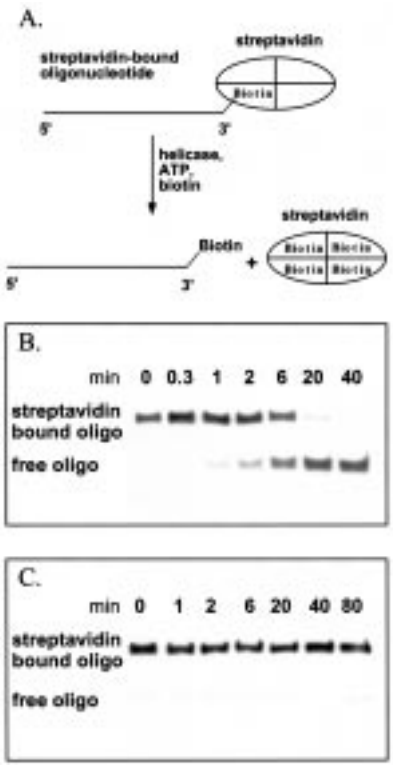

FIGURE 3: Helicase-mediated displacement of streptavidin from a biotinylated oligonucleotide. (A) Illustration of experimental protocol. Biotinylated oligonucleotide was preincubated with ATP in Hepes (pH 7.5). After 2−3 min, free biotin trap was added, along with varying concentrations of helicase. Aliquots were removed at varying times and the helicase reaction was quenched by addition of SDS and EDTA. Any streptavidin displaced from the oligonucleotide is prevented from rebinding by free biotin. The samples were analyzed by native 15% polyacrylamide gel electrophoresis, and the quantity of streptavidin-bound oligonucleotide and free oligonucleotide was determined with a phosphorimager. (B) Phosphorimage of streptavidin displacement from a 3′-biotinylated oligonucleotide. (C) Phosporimage of streptavidin displacement from a 5′-biotinylated oligonucleotide.

streptavidin from free biotinylated oligonucleotides (Figure 3A). Prior to addition of helicase, the majority of 3′-biotinylated 62-mer is shifted due to binding of streptavidin (Figure 3B). After addition of 2 μM gp41 and ATP, this shift disappears over the time course of the experiment. After 40 min, virtually all of the 62-mer runs free of streptavidin on the gel. This suggests that gp41 activity on ssDNA is capable of producing a force great enough to disrupt the interaction between streptavidin and biotin. SDS (0.3% final concentration) and poly(dT) were included in the quencher to prevent a complicating gel shift arising from binding of gp41 to the oligonucleotide (data not shown).

Several control experiments were performed to ensure that streptavidin dissociation was due to helicase activity rather than a contaminating nuclease. The presence of contaminating nuclease activity could remove the biotinylated region of the oligonucleotide, leading to the observed loss of band shifting. In one control, the displacement experiment was performed in the absence of ATP, resulting in formation of no free 62-mer throughout the 80 min time course of the experiment, suggesting that streptavidin was not removed in the absence of actively translocating gp41 (data not shown). In the second control, the displacement experiment was performed in the absence of excess biotin trap. If gp41

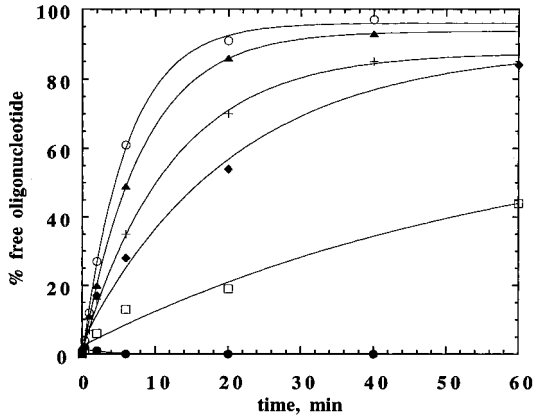

FIGURE 4: Streptavidin displacement from a 3′-biotinylated, 62-mer oligonucleotide at varying concentrations of gp41. Displacement rates were determined by fitting the data to a single exponential with the program KaleidaGraph. Displacement from 3′-biotinylated oligonucleotide: (○) 4 μM gp41, (▲) 2 μM gp41, (+) 1.0 μM gp41, (◆) 0.5 μM gp41, (□) 250 nM gp41. Displacement from the 5′ biotinylated oligonucleotide: (●) 4 μM gp41. Rate constants are listed in Table 1.

displaces streptavidin in the absence of free biotin, the streptavidin should rebind to the oligonucleotide, and no loss of band shifting will be observed. This was indeed the case, suggesting that any loss of band shifting in the displacement experiments is due to gp41 activity and not degradation of the oligonucleotide (data not shown).

Figure 3C shows results of the displacement experiment in the presence of the 5′-biotinylated 60-mer. Prior to gp41 addition, the majority of the oligonucleotide is shifted due to streptavidin binding. Addition of 2 μM gp41 and ATP causes no loss of band shifting, even over a period of 80 min. Thus, while gp41 activity is capable of dislodging streptavidin from the 3′ end of an oligonucleotide, it cannot dislodge it from the 5′ end. Although this does not prove unidirectional translocation, it is consistent with the idea of gp41 moving primarily in a 5′ to 3′ direction.

The results for streptavidin displacement at increasing helicase concentration are plotted in Figure 4. The rate of displacement of streptavidin increased with increasing gp41 even at the highest helicase concentration. The solubility limit for gp41 is ~8 μM under the conditions used here. The highest concentration of gp41 tested was 4 μM final, and the rate of displacement of streptavidin still did not appear to be saturating with respect to gp41 concentration (Table 1). The fastest rate of displacement measured for gp41 was 0.17 min^{-1}, which is ~500-fold faster than the spontaneous dissociation rate of streptavidin from biotin, 3.3×10^{-4} min^{-1} (*26*).

Data for streptavidin displacement by the Dda helicase are shown in Figure 5. Dda was much more effective than gp41 at displacing the 3′-streptavidin, although the 5′-streptavidin was not displaced, just as with gp41. Measurement of the rate of streptavidin displacement by Dda required use of a rapid mixing instrument (Kintek RQ3, Kintek, Inc.). Two conditions for initiating the reaction were examined. Dda was rapidly mixed with the oligonucleotide and ATP in one case, while in the other case Dda was preincubated with the oligonucleotide, followed by rapid mixing with ATP. Results shown in Figure 5 show that the fastest rates were obtained when the enzyme and DNA were preincubated.

Table 1: Rates of Dissociation of Streptavidin from 3′-Biotinylated 62-mer Oligonucleotide[a]

streptavidin concn (μM)	Dda, k (s^{-1})	gp41, k (min^{-1})
0.25	3.9 (2.5)[b]	0.03
0.5	nd[c]	0.05
1.0	7.9	0.08
2.0	6.5	0.12
4.0	nd	0.17

[a] Rates were determined as described in Figures 4 and 5. The error in fitting of the data was typically ± 0.5 s^{-1} for rates pertaining to Dda and ± 0.01 min^{-1} for rates pertaining to gp41. [b] Result obtained by initiating reaction by rapid mixing of Dda with the biotin-labeled oligonucleotide. All other rates pertaining to Dda were obtained by preincubating Dda with the biotin-labeled oligonucleotide followed by rapid mixing with ATP. Rates constants determined for gp41 were independent of the order of mixing of reagents. [c] Not determined.

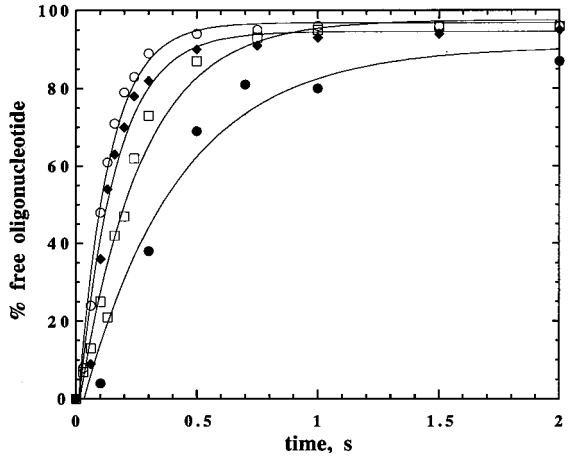

FIGURE 5: Dda-catalyzed displacement of streptavidin from a 3′-biotinylated, 62-mer oligonucleotide at varying helicase concentrations and mixing conditions. The lines through the data represent the best fit to a single exponential with the program KaleidaGraph. The reaction was initiated by mixing 0.25 μM Dda with the oligonucleotide and ATP (●). Alternatively, 0.25 μM Dda was preincubated with the oligonucleotide, followed by rapid mixing with ATP, which provided faster displacement rates (□). Similar rates were obtained at 1 μM Dda (○) and 2 μM Dda (◆). Rate constants are listed in Table 1.

Rates of displacement of streptavidin were measured at varying concentrations of Dda in the presence of 10 nM oligonucleotide. The rate of displacement at 1 μM enzyme was similar to that measured at 2 μM, suggesting that saturating conditions were obtained with respect to Dda (Table 1). The rate of displacement of streptavidin from the 3′ end of the 62-mer was 7.9 s^{-1}, which is over a million-fold faster than the spontaneous dissociation rate (*26*).

Displacement of Streptavidin from Biotin-Labeled Oligonucleotides of Varying Length. Streptavidin displacement assays were performed with a series of 3′-biotinylated oligonucleotides of varying lengths (Figure 6A). The rate of streptavidin displacement by gp41 (2 μM) was similar for the 62-mer, 30-mer, and 21-mer oligonucleotides. There was a decrease in the rate of displacement for the 16-mer and a very sharp decrease in the rate of displacement for the 11-mer, suggesting that approximately 20−30 nucleotides is the optimal length for gp41 binding and translocation. This number of nucleotides corresponds to the number estimated to be bound by gp41 from previous gel shift experiments

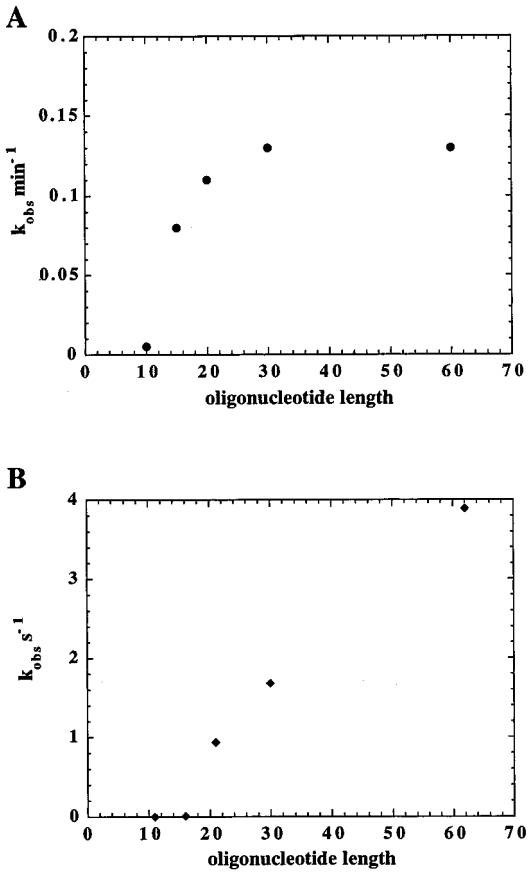

FIGURE 6: Rate of streptavidin displacement from 3′-biotinylated oligonucleotides of varying lengths. (A) Displacement rates in the presence of 2 μM gp41 are plotted versus oligonucleotide lengths of 11, 21, 30, and 62 nucleotides. (B) Displacement rates in the presence of 0.25 μM Dda versus oligonucleotide length.

(*10*) and is similar to the 29 nucleotides that are sequestered upon binding of gene 4 helicase to ssDNA (*23*).

Displacement of streptavidin from varying length oligonucleotides by Dda follows a different patten than gp41. The rate of displacement is fastest with the 62-mer and decreases somewhat linearly with the 30-mer and 21-mer oligos. The rate decreases sharply when the 21-mer is compared to the 16-mer, and very little displacement is observed for the 11-mer (Figure 6B). The faster rates for Dda with longer oligonucleotides may represent the need for Dda to oligomerize along the DNA in order to exhibit maximal activity, although the oligomeric form of this helicase has not been defined. Dda is known to dissociate rapidly from DNA (*27*, *28*) and therefore may function in a different manner than gp41, which is known to exhibit high processivity (*29*). Further studies of Dda's possible oligomeric nature and its binding site size will be necessary to fully explain the results in Figure 6B.

DISCUSSION

ssDNA Passes through the Central Channel of the gp41 Hexameric Helicase. Work from several laboratories has shown that many helicases adopt a hexameric form in the presence of a nucleoside triphosphate (*23*−*25*), or Mg^{2+}, as in the case of the *E. coli* DnaB helicase (*30*). The oligomeric form of the gene 4 helicase from bacteriophage T7 has been studied closely by electron microscopy, and the results from these studies suggest that ssDNA passes through the central channel of the hexamer, interacting with only one or two of the six subunits (*31*). Electron microscopy images of gp41 taken in the presence of ssDNA indicate increased electron density within the central channel of the hexamer, suggesting that the DNA may pass through the center hole of this enzyme as seen with gene 4 helicase (*12*). We have conducted a gel mobility shift assay in which a covalently closed hexamer was found to be linked to a covalently closed segment of ssDNA in a concatenated structure (Figure 2). These results support the conclusion that ssDNA passes through the central channel of gp41, similar to other DNA helicases.

This work also helps to interpret previous experiments. By use of biotin−streptavidin blocks that were placed on one strand or the other of an unwinding substrate for gp41, helicase activity was observed to be inhibited only when the block was attached to the strand on which gp41 is believed to bind, the 5′-to-3′ strand (*13*). In light of the current study, the results with the streptavidin block strongly suggest that only one strand of the DNA substrate passes through the hexamer during the unwinding reaction. Similar conclusions were drawn from results of DNA unwinding experiments performed with the gene 4 helicase and biotinylated oligonucleotide substrates bound to streptavidin (*32*). Thus, the biochemical experiments are consistent with the structural data and suggest a mechanism for unwinding whereby the helicase translocates on one strand of DNA with a directional bias, leading to unwinding of the duplex by occlusion of the complementary strand from the central channel of the hexamer (*13*, *16*, *23*, *32*).

Helicases Can Impart a Force on Proteins in Their Path. Results from previous DNA unwinding experiments in which a streptavidin block was placed on either strand of a fork substrate are slightly different for the gene 4 helicase than for the gp41 helicase. While the block on the 5′-to-3′ strand was able to completely inhibit unwinding by gene 4, the block only impeded gp41 unwinding by 8-fold (*13*, *32*). Either gp41 was able to somehow bypass the streptavidin block, or the streptavidin was being displaced from the biotin. We initially considered the latter possibility unlikely, due to the strong interaction between biotin and streptavidin, which has a dissociation constant of $\sim 10^{-14}$ M^{-1} (*26*). More importantly, the disruption force required to remove streptavidin from biotin was measured by atomic force microscopy and found to be ∼250 pN (*33*, *34*). For comparison, the force that can be generated by a molecular motor enzyme such as kinesin has been determined to be 5−10 pN (*35*, *36*), while the largest force that has thus far been measured for any enzyme that translocates on DNA is 14 pN for RNA polymerase (*37*). The disruption force measured for removal of steptavidin from biotin is much larger than that previously measured for any translocating enzyme. However, in light of the result from the previous unwinding experiments in which gp41 unwound duplex DNA despite the streptavidin block, we investigated whether the activity of a helicase on ssDNA could cause disruption of the streptavidin−biotin bond.

Using a gel shift assay that separates biotin-labeled oligonucleotides bound to streptavidin from those that are not, we determined that the two helicases studied here can rapidly displace streptavidin. At the highest protein concen-

tration used, gp41 was capable of displacing streptavidin from biotin with a dissociation rate ~500-fold faster than the spontaneous dissociation rate of biotin from streptavidin (Table 1; *26*). The reaction was dependent on the presence of ATP and the presence of excess free biotin, indicating that the displacement of streptavidin was indeed due to helicase activity. Dda was capable of displacing streptavidin at even faster rates, resulting in greater than a million-fold enhancement in streptavidin displacement from biotin when compared to the spontaneous dissociation rate (Table 1).

These results indicate that gp41 and Dda can impart a force upon proteins blocking their path on ssDNA. The directional bias of the force was shown in experiments in which the biotin label was placed on the 5′-end of oligonucleotides. Neither Dda nor gp41 was able to displace any of the 5′-streptavidin under conditions in which all of the 3′-streptavidin label was rapidly removed (Figure 3). Hence, the results are consistent with the idea that these helicases translocate unidirectionally in a 5′ to 3′ manner, as has been reported (*10*, *11*). The oligomeric nature of Dda has not been defined; therefore the mode of unwinding for this enzyme remains to be determined. gp41 functions as a hexamer, with only one strand of the DNA duplex passing through the central channel. The role of force production may serve to separate the two strands, with the protein acting as a wedge and the displaced strand passing on the outside of the hexamer. In regard to models for helicase function, the production of force with a directional bias suggests that the enzyme may actively participate in the melting of duplex DNA due to translocation on ssDNA, perhaps by enhancing formation of ssDNA that already exists naturally due to thermal fluctuations at the ss/ds DNA junction (*38*).

Whether the displaced DNA strand interacts with gp41 in a formal manner remains to be determined, although in previous experiments, placement of a streptavidin block on the displaced strand did not impede progress of the enzyme (*13*). Inconsistent results have been reported regarding the interaction of the displaced strand with the gene 4 helicase. Benzo[*a*]pyrene−DNA adducts inhibited gene 4 helicase in a strand-specific manner, suggesting a significant difference between interactions of the helicase and the two DNA strands (*39*). Hacker and Johnson (*32*) reported that little interaction with the displaced strand is necessary for the unwinding reaction to occur. These authors suggested that the displaced strand need simply be excluded from the central channel of the hexamer in order for unwinding to proceed. This is supported by an experiment in which unwinding was observed for a substrate that did not contain a 3′-tail. A streptavidin block was placed on the 3′ end of the displaced strand, which excluded the strand from the central channel of the gene 4 helicase. Ahnert and Patel (*40*) have reported results in which the 3′-tail of the displaced strand was required, based on the observation of reduced unwinding when the 3′-ssDNA tail was converted to a dsDNA tail. The discrepancy in these results may lie in the conditions used to perform the experiments. Ahnert and Patel performed unwinding experiments at 4 °C while Hacker and Johnson performed their experiments at 10 °C or higher, which may have led to more fraying of the ss/ds DNA. Thus, the role of the 3′ tail in unwinding by gp41, gene 4 helicase, and other hexameric 5′-to-3′ helicases remains to be resolved.

Dda displaces streptavidin much faster than gp41 (Table 1). These results correlate with results from DNA unwinding studies that indicate that Dda unwinds oligonucleotide substrates much faster than gp41 (*13*, *27*). The rate-limiting step in the displacement reaction may be different for the two helicases, and experiments are underway to determine the biochemical events that give rise to the difference in streptavidin displacement rates.

Models for Helicase Function. An important aspect of the work described here is that the force generated by these helicases is produced solely on ssDNA substrates. No unique structures are required, such as a ss/dsDNA junction or concomitant binding of ssDNA and dsDNA. It is tempting to speculate that the functional requirements of gp41 and Dda are satisfied with only ssDNA and may not require dsDNA during the reaction cycle. In light of the fact that only one strand of DNA passes through the central channel of gp41, DNA unwinding may be a *consequence* of the ability of the enzyme to translocate unidirectionally on ssDNA. A similar suggestion has been made for the helicase activity of the rho transcription termination factor (*41*). Rho transcription termination factor may produce a strong force in the direction of translocation based on its ability to displace RNA polymerase from a growing transcript (*41*). The fact that gp41 and Dda can cause displacement of streptavidin from biotin argues favorably for these enzymes being capable of displacing a complementary strand of DNA due to translocation. Such a mechanism could be accommodated by an inchworm model of translocation for a monomeric or dimeric helicase, as has been proposed for the NS3 helicase of the hepatitis C virus (*17*). For the hexameric helicases, unidirectional translocation may be driven by rotational catalysis, based on similarities between the ATPase activity of the gene 4 helicase with the F_1-ATPase. The F_1-ATPase is known to rotate about a polypeptide subunit, which may be analogous to the possible rotation of a hexameric helicase about ssDNA (*16*). Some form of "subunit switching", in which one subunit of the enzyme moves relative to a second subunit that remains bound to DNA, may be responsible for translocation, as has been suggested for the dimeric Rep helicase (*1*). Mechanisms involving unidirectional translocation on ssDNA have been characterized as "passive", although the results described here suggest that helicases can in fact impart a strong force on objects in their path, which would likely include complementary strands of DNA.

How can gp41 and Dda produce enough force to dislodge streptavidin from biotin? If the streptavidin−biotin bond were treated as a static system, then the force required to displace streptavidin would need to be greater than the measured disruption force. However, noncovalent bonds are reversible, meaning that ultimately no force is required to separate the molecules (*42*). Fluctuations in the free energy minimum of the streptavidin−biotin bond, produced by thermal vibrations, will eventually cause the bond to break. Application of a force, even a weak one, will reduce the lifetime of the bond by diminishing the free energy minimum to an extent that may be proportional to the exerted force (*42*, *43*, *44*). Thus, gp41 and Dda need not apply a force that is equal to the measured disruption force of the streptavidin−biotin bond in order to significantly enhance the dissociation rate of streptavidin from biotin.

The thermodynamic relationship between the force required to break the biotin−streptavidin bond has been found to correlate with the activation enthalpy rather than the overall free energy of the bond (*34*). The theoretical relationship between the kinetics of dissociation for a protein−protein or protein−ligand interface as a function of applied force has been reported to be logarithimic (*42*, *44*, *45*). The possibility exists that the interaction between the helicases and streptavidin causes some distortion in the streptavidin that lowers its affinity for biotin, which would complicate any attempt to relate the dissociation rates described here with the apparent force imparted by the helicase. If such a relationship can be established, then the method described here may be a simple way in which to estimate the force produced by enzymes that translocate on DNA. Others have confirmed our results regarding the ability of gp41 to dislodge streptavidin from biotin-labeled oligonucleotides (F. Dong, E. Gogol, and P. von Hippel, personal communication). The results described here do not prove unidirectional translocation on ssDNA by a helicase, but they are consistent with the notion. It will be interesting to further examine the relationship between streptavidin displacement and translocation on ssDNA by helicases.

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