# Hepatitis C Virus NS3 and Simian Virus 40 T Antigen Helicases Displace Streptavidin from 5′-Biotinylated Oligonucleotides but Not from 3′-Biotinylated Oligonucleotides: Evidence for Directional Bias in Translocation on Single-Stranded DNA†

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ABSTRACT: Helicases are enzymes that use energy from nucleoside triphosphate hydrolysis to unwind double-stranded (ds) DNA, a process vital to virtually every phase of DNA metabolism. Helicases have been classified as either 5′-to-3′ or 3′-to-5′ on the basis of their ability to unwind duplex DNA adjacent to either a 5′ or 3′ single-stranded (ss) DNA overhang. However, there has been debate as to whether this substrate preference is indicative of unidirectional translocation on ssDNA. We developed an assay that monitors the ability of a helicase to displace streptavidin from biotinylated oligonucleotides [Morris, P. D., and Raney, K. D. (1999) *Biochemistry 38*, 5164−5171]. Two helicases identified as having 5′-to-3′ polarity displaced streptavidin from the 3′-end of biotinylated oligonucleotides but not from the 5′-end. We performed similar experiments using the 3′-to-5′ helicases from the hepatitis C virus (NS3) and SV40 virus (SV40 T antigen). NS3 and SV40 T antigen were able to displace streptavidin from a 5′-biotinylated oligonucleotide but not from a 3′-biotinylated oligonucleotide. NS3 and SV40 T antigen enhanced the spontaneous rate of dissociation of streptavidin from biotin 340-fold and 1700-fold, respectively. The ssDNA binding protein, gp32, did not enhance dissociation of streptavidin from either end of an oligonucleotide. For NS3, the rate of displacement was faster from a 5′-biotinylated 60mer than from a 5′-biotinylated 30mer. The strong directional bias in streptavidin displacement activity exhibited by each helicase is consistent with a directional bias in translocation on ssDNA. The dependence of the reaction with NS3 on the oligonucleotide length suggests that multiple NS3 monomers are necessary for optimal activity.

Helicases are enzymes that unwind double-stranded (ds)[1] nucleic acids in processes such as replication and recombination where single-stranded (ss) nucleic acids are required intermediates (*1*, *2*). The energy needed to perform this function is provided by ATP hydrolysis. Much progress has been made toward understanding the biochemical mechanism(s) of ATP binding and hydrolysis for several helicases (*3*−*7*). Evidence has been presented for relating ATP binding and hydrolysis to conformational changes that are believed to occur during DNA unwinding for the PcrA helicase (*8*). However, the relationship of translocation on DNA to unwinding of DNA remains unclear.

Helicases are involved in many different processes including unwinding of secondary structure in ssRNA. For example, nonstructural protein 3 (NS3) is an RNA helicase from the hepatitis C virus that is thought to unwind dsRNA and remove secondary structure in ssRNA during replication of the HCV RNA genome (*9*). Translocation on ssRNA may be necessary for NS3 to traverse regions of ssRNA interspersed between regions of dsRNA. Enzymatic activity of helicases on ss nucleic acids has been inferred by measuring the ATPase activity of helicases as a function of varying ssDNA length or structure (*10*−*12*). These studies have provided evidence for a directional bias in translocation on ssDNA. We recently reported a new assay for studying the activity of a helicase on ssDNA. In this assay, the helicase-catalyzed enhancement in the rate of dissociation of streptavidin from biotinylated oligonucleotides is measured (*13*). Two helicases from bacteriophage T4, gp41 and Dda, were studied by this assay. gp41 forms hexamers in the presence of ATP or GTP (*14*), whereas Dda does not form stable oligomers (*15*). gp41 and Dda are 5′-to-3′ helicases, meaning that they unwind duplexes containing a single-stranded region that is 5′ to the duplex but do not readily unwind duplexes containing a single-stranded region that is 3′ to the duplex.

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[1] Abbreviations: ss, single-stranded; ds, double-stranded; NS3, nonstructural protein 3; SV40, simian virus 40; gp32, gene product of T4 gene 32; IPTG, isopropyl β-D-thiogalactoside; EDTA, ethylenediaminotetraacetic acid; BME, β-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenylpyruvate; BSA, bovine serum albumin; MOPS, 3-(morpholino)propanesulfonic acid; PK/LDH, pyruvate kinase/lactate dehydrogenase; gp41, gene product of T4 gene 41; Tag, T antigen; HCV, hepatitis C virus.

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Consistent with their presumed direction of translocation, gp41 and Dda are capable of displacing streptavidin from the 3′-ends of biotinylated oligonucleotides but not from the 5′-ends (*13*). These results provide evidence that helicases are capable of translocating on ssDNA with a directional bias, supporting previous evidence presented for gp41 (*11*) and Dda (*16*).

Helicases have been categorized into several superfamilies on the basis of sequence homology (*17*). Dda is a member of superfamily 1 and gp41 is a member of the F4 or DnaB-like superfamily. Thus, two helicases from different families exhibit similar polarity and are capable of displacing streptavidin from the 3′-end of biotinylated oligonucleotides. To determine whether helicases that exhibit 3′-to-5′ polarity are also capable of displacing streptavidin, two enzymes were investigated. NS3 of the hepatitis C virus (*9*) and the SV40 T antigen (*18*) are each reportedly 3′-to-5′ helicases. NS3 is a member of superfamily 2, and SV40 T antigen is a member of superfamily 3. Here we report that each helicase is capable of displacing streptavidin from the 5′-ends of oligonucleotides but not from the 3′-ends. The results indicate that the ability of a particular helicase to displace streptavidin from one end of biotin-labeled oligonucleotides, but not both ends, may be a general feature of these enzymes and that this activity may reflect the ability of the helicase to translocate with a directional bias on ssDNA.

## MATERIALS AND METHODS

*Materials.* Streptavidin, phosphoenolpyruvate (tricyclohexylammonium salt), ATP (disodium salt), ATP$\gamma$S, and pyruvate kinase/lactate dehydrogenase (in glycerol) were obtained from Sigma. T4 polynucleotide kinase was purchased from New England Biolabs. NZCYM and Bacto-agar were obtained from Difco Laboratories. [$\gamma$-$^{32}$P]ATP was purchased from Perkin-Elmer Life Sciences. DNA oligonucleotides (Operon Technologies) were purified by preparative denaturing polyacrylamide gel electrophoresis and stored in 10 mM Hepes (pH 7.5) and 1 mM EDTA. Purified oligonucleotides were quantified by UV absorbance at 260 nm in 0.2 M KOH by using calculated extinction coefficients. Recombinant gp32 in *Escherichia coli* N4830/pYS6 was expressed and purified as described (*19*). Full-length NS3 was expressed from the pET-ubiquitin vector system (*20*) and purified as described (*21*).

*Streptavidin Displacement Assay.* 5′- and 3′-biotinylated oligonucleotides contained a "bio-teg" {1-(dimethoxytrityloxy)-3-*O*-[(*N*-biotinyl-3-aminopropyl)triethyleneglycolyl]-glyceryl-2-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite} biotin label (*22*) incorporated into the DNA one nucleotide from the end of the strand. The sequence of the 3′-bio-62mer (61 nucleotides plus biotin) was 5′-TAACGTATTCAAGATACCTCGTACTCTGTACTGACTGCGATCCGACGTCCTGCATGATG**X**T-3′.

The sequence of the 5′-bio-60mer (59 nucleotides plus biotin) was 5′-**X**ACGTATTCAAGATACCTCGTACTCTGTACTGACTGCGATCCGACTGTCCTGCATGATG-3′.

The sequence of the 5′-bio-30mer (29 nucleotides plus biotin) was 5-G**X**ACGTATTCAAGATACCTCGTACTCTGTA-3′.

Placement of the biotin label is indicated by the **X** in the sequences.

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 [$\gamma$-$^{32}$P]ATP was removed by passing the labeled oligonucleotides through two Sephadex G-25 spin columns.

Streptavidin (100 $\mu$M) was prepared in buffer consisting of 25 mM Hepes, pH 7.4, 20% glycerol, and 10 mM NaCl. Solutions were stored at −80 °C. When ready for use, these stock solutions were diluted to 5 $\mu$M in buffer containing 25 mM Hepes, pH 7.5, 0.1 mg/mL BSA, 0.1 mM EDTA, and 1 mM BME. Oligonucleotide (10 nM) was incubated in reaction buffer [25 mM Hepes (pH 7.5), 12.5 mM $Mg(OAc)_2$, 150 mM KOAc, 4 mM PEP, 1 mM BME, and 0.1 mg/mL BSA for T antigen experiments; 25 mM MOPS (pH 7.0) was substituted for Hepes in experiments with NS3] along with 5 mM ATP, 300 nM streptavidin, and PK/LDH (10.8 and 16.6 units/mL, respectively) at 37 °C for 3 min. Following the 3 min incubation period, 6 $\mu$M biotin was added. The streptavidin displacement reaction was initiated by addition of helicase. In some cases, ATP was left out of the reaction mixture so that helicase could be incubated with the oligonucleotide and the reaction was initiated upon addition of ATP. At various times, 10 $\mu$L aliquots were removed from the reaction and mixed with 10 $\mu$L of quench solution [0.6% SDS, 200 mM EDTA, pH 8.0, 10 $\mu$M poly(dT), 0.08% xylene cyanol, 0.08% bromophenol blue, and 10% glycerol]. Samples were analyzed by electrophoresis on a 15% polyacrylamide gel in TBE. The quantity of radioactivity in the bands corresponding to free oligonucleotide and streptavidin-bound oligonucleotide in each sample was determined by use of a Molecular Dynamics 445-SI Phosphorimager with ImageQuant software. The fraction of free oligonucleotide, with a correction for the free oligonucleotide in the blank sample, was determined according to

$$FD_{c,t} = \frac{\left(\frac{FD_t}{FD_t + SD_t}\right) - \left(\frac{FD_b}{FD_b + SD_b}\right)}{1 - \left(\frac{FD_b}{FD_b + SD_b}\right)} \quad (1)$$

$FD_{c,t}$ is the fraction of free oligonucleotide corrected for the amount of free oligonucleotide in the blank sample. $FD_t$ is the radioactivity of free oligonucleotide DNA for each sample at time *t*. $SD_t$ is the radioactivity of streptavidin-bound oligonucleotide DNA at time *t*. $FD_b$ and $SD_b$ are the radioactivity of free oligonucleotide DNA and streptavidin-bound oligonucleotide DNA, respectively in the blank (b) at time zero.

*DNA Binding Assay.* 5′-Radiolabeled 3′-bio-62mer (10 nM) was incubated in the streptavidin displacement reaction buffer with 5 mM ATP$\gamma$S in the presence or absence of 1 $\mu$M streptavidin at 37 °C for 3 min. The samples were then incubated in the presence or absence of T antigen (3.5 $\mu$M) or NS3 (500 nM) for 10 min at 37 °C. Glycerol was added to a final concentration of 10% and the samples were analyzed by electrophoresis on a 4−15% polyacrylamide gel followed by phosphorimager analysis.

## RESULTS

*Requirements for the Streptavidin Displacement Reaction.* The 5′-to-3′ or 3′-to-5′ polarity exhibited by helicases during

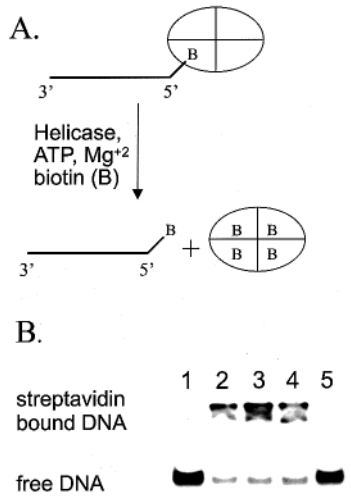


FIGURE 1: Requirements for streptavidin displacement from biotinylated oligonucleotides by NS3. (A) Diagram depicting helicase-catalyzed displacement of streptavidin from biotin-labeled oligonucleotides indicating the key components of the complete reaction mixture. (B) The complete reaction mixture was prepared by incubating 5′-bio-60mer (10 nM) with streptavidin (300 nM) for 3 min to prepare the substrate. NS3 (500 nM) was incubated with the substrate and the displacement reaction was initiated upon addition of 5 mM ATP, 10 mM $Mg(OAc)_2$, and 6 $\mu$M biotin. Various components of the reaction mixture were removed to illustrate the requirements for the reaction. Lane 1, complete reaction mixture excluding NS3 and streptavidin; lane 2, complete reaction mixture excluding NS3; lane 3, complete reaction mixture excluding biotin; lane 4, complete reaction mixture excluding ATP; lane 5, complete reaction mixture.

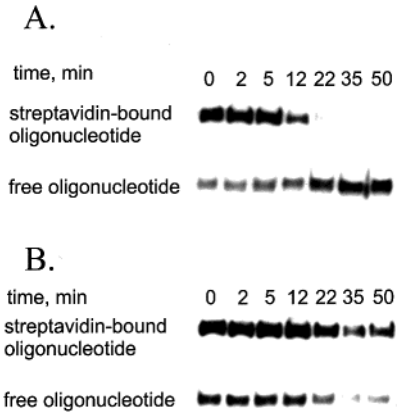


FIGURE 2: NS3-catalyzed displacement of streptavidin from 5′-bio-60mer and 3′-bio-62mer oligonucleotides. (A) NS3 (500 nM) was incubated with 10 nM 5′-bio-60mer in reaction buffer. Samples were removed at varying times and added to a quencher solution (0.6% SDS, 200 mM EDTA, 0.08% xylene cyanol, 0.08% bromophenol blue, and 10% glycerol) followed by separation of streptavidin-bound oligonucleotide from free oligonucleotide on a 15% polyacrylamide gel. (B) 3′-Bio-62mer was incubated with 500 nM NS3 under identical conditions as described for panel A.

unwinding of dsDNA suggests that these enzymes exhibit a directional bias for translocation on ssDNA. Previously, two 5′-to-3′ helicases, gp41 and Dda, were shown to displace streptavidin from a 3′-biotinylated oligonucleotide but not a 5′-biotinylated oligonucleotide (*13*). These results are consistent with a directional bias in translocation on ssDNA. To determine whether a trend is observed for streptavidin displacement with other helicases, two 3′-to-5′ helicases, NS3 and SV40 T antigen, were investigated. Necessary components for the streptavidin displacement experiment are indicated in Figure 1A. Several control experiments were performed to ensure that any observed displacement of streptavidin was due to helicase activity. Biotinylated oligonucleotide was incubated with streptavidin for 3 min to allow for association with streptavidin. Helicase was then incubated with the oligonucleotide and the reaction was initiated upon addition of ATP, $Mg^{2+}$, and biotin. Addition of biotin ensures that the biotinylated oligonucleotide will not reassociate with streptavidin after it has been displaced by the helicase. Samples were removed after 40 min and added to a quencher solution [0.6% SDS, 200 mM EDTA, pH 8.0, 10 $\mu$M poly(dT), 0.08% xylene cyanol, 0.08% bromophenol blue, and 10% glycerol] followed by separation of streptavidin-bound oligonucleotide from free oligonucleotide by 15% polyacrylamide gel electrophoresis. Control reactions are shown in Figure 1B. In the absence of streptavidin, the oligonucleotide runs free on the gel (Figure 1B, lane 1). In the absence of NS3, no displacement was observed (Figure 1B, lane 2). In the absence of biotin, no increase in the quantity of free oligonucleotide above background was observed, ruling out other explanations for the appearance of the free oligonucleotide such as a possible nuclease contamination (Figure 1B, lane 3). In the absence of ATP, no displacement was observed. NS3 is able to bind to ssDNA in the absence of ATP so this indicates that binding of NS3 to the oligonucleotide was not sufficient to displace the streptavidin (Figure 1B, lane 4). When all of the reaction components were included, displacement of streptavidin was observed (Figure 1B, lane 5).

*NS3 Displaces Streptavidin from a 5′-Biotinylated Oligonucleotide but Not from a 3′-Biotinylated Oligonucleotide.* Displacement of streptavidin from a 5′-bio-60mer and a 3′-bio-62mer by NS3 was examined. The reaction was initiated by addition of NS3. Aliquots were quenched at various times, followed by analysis by native polyacrylamide gel electrophoresis (Figure 2). The band due to streptavidin-bound oligonucleotide disappeared during the 50 min incubation of NS3 with the 5′-bio-60mer, indicating displacement of streptavidin from the biotin-labeled oligonucleotide (Figure 2A). The streptavidin displacement reaction was performed with the 3′-bio-62mer, but no displacement was observed over the 50 min reaction period (Figure 2B). These results support a strong, 3′-to-5′ directional bias in the activity of NS3 on ssDNA and the polarity is consistent with that observed for unwinding by NS3.

Streptavidin displacement experiments were performed at varying concentrations of NS3 (Figure 3A). In the presence of 10 nM 5′-bio-60mer, similar rates were obtained from 100 nM up to 2 $\mu$M NS3, suggesting that the substrate was saturated with respect to NS3 under these conditions (Figure 3B). Thus, binding to oligonucleotide does not limit the rate of streptavidin displacement by NS3 under these conditions.

*NS3 Displaces Streptavidin More Rapidly from Longer Oligonucleotides Than from Shorter Ones.* The dependence of the NS3-catalyzed streptavidin displacement reaction on the length of the oligonucleotide was investigated. NS3 was found to displace streptavidin from a 5′-bio-60mer more rapidly than from the 5′-bio-30mer (Figure 4). Under identical conditions, no displacement of streptavidin was observed from a 5′-bio-20mer or a 5′-bio-15mer (not shown). Thus, NS3-catalyzed displacement of streptavidin exhibits a clear dependence on the length of the oligonucleotide.

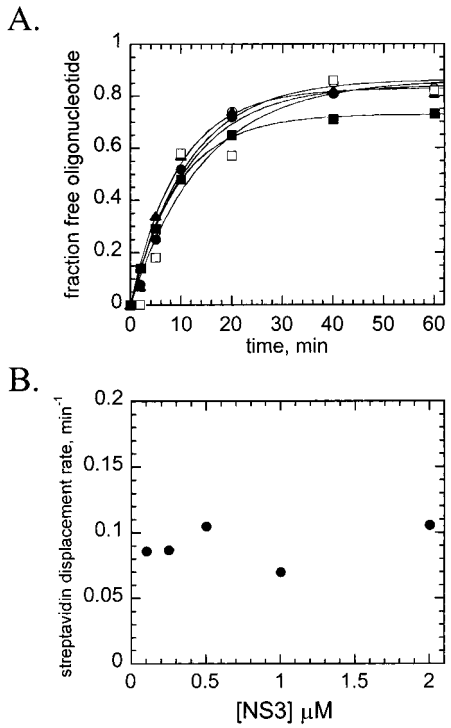


FIGURE 3: Streptavidin displacement from the 5′-bio-60mer oligonucleotide with varying concentrations of NS3. (A) 5′-Bio-60mer (10 nM) was incubated in complete reaction buffer with 100 nM NS3 (●), 250 nM NS3 (○), 500 nM NS3 (▲), 1 $\mu$M NS3 (□), or 2 $\mu$M NS3 (■). Data were fit to a single exponential by using KaleidaGraph software. (B) Rates of displacement of streptavidin from 5′-bio-60mer are plotted as a function of NS3 concentration. Observed rates at 100 nM, 250 nM, 500 nM, 1 $\mu$M, and 2 $\mu$M NS3 were 0.086, 0.087, 0.105, 0.070, and 0.106 min$^{-1}$, respectively.

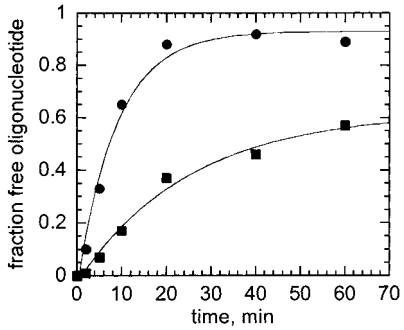


FIGURE 4: Displacement of streptavidin from biotinylated oligonucleotides of differing length by NS3. NS3 (250 nM) was incubated with 10 nM 5′-bio-60mer (●) or 10 nM 5′-bio-30mer (■) in reaction buffer. Aliquots were removed over time and analyzed as described for Figure 2. Data were fit to a single exponential, resulting in displacement rates of 0.039 and 0.087 min$^{-1}$ for the 5′-bio-30mer and the 5′-bio-60mer, respectively.

Previously, a similar dependence on oligonucleotide length was observed for Dda helicase, except that Dda displaces streptavidin from the 3′-end of the oligonucleotide rather than the 5′-end (*13*). The significance of these results is not known, but a possible explanation is that multiple NS3 monomers must bind simultaneously to the same strand of ssDNA in order to exhibit optimal activity. Indeed, oligomerization of the helicase domain of NS3 is likely required for optimal unwinding (*23*).

*SV40 T Antigen Displaces Streptavidin from the 5′ End of an Oligonucleotide but Not from the 3′-End.* To determine whether other 3′-to-5′ helicases are capable of displacing

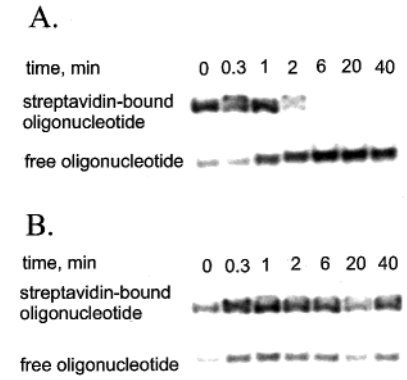


FIGURE 5: Analysis of SV40 T antigen-catalyzed displacement of streptavidin from biotinylated oligonucleotides. SV40 T antigen (4.6 $\mu$M) and biotin (6 $\mu$M) were added to reaction buffer containing 10 nM 5′-bio-60mer (A) or 10 nM 3′-bio-62mer (B). After quenching the reaction at various times, aliquots were analyzed by 15% polyacrylamide gel electrophoresis. The amount of streptavidin-bound oligonucleotide and free oligonucleotide were determined by use of a Molecular Dynamics phosphorimager with ImageQuant software.

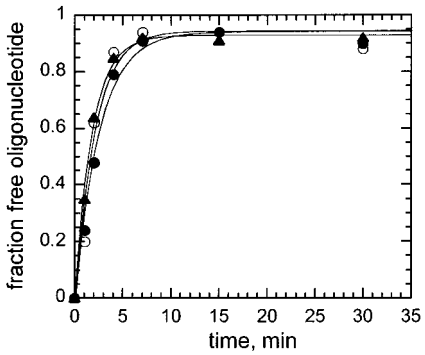


FIGURE 6: Streptavidin displacement from 5′-bio-60mer oligonucleotide at varying concentrations of SV40 T antigen. Displacement from 5′-bio-60mer by 4.6 $\mu$M T antigen (○), 5.9 $\mu$M T antigen (●), or 7.5 $\mu$M T antigen (▲). Displacement rates were determined by fitting the data to a single exponential. Rates of streptavidin displacement were 0.38 min$^{-1}$, 0.46 min$^{-1}$, 0.55 min$^{-1}$, at 4.6 $\mu$M, 5.9 $\mu$M, and 7.5 $\mu$M, respectively.

streptavidin, the SV40 T antigen was examined. SV40 T antigen is a 3′-to-5′ helicase that forms oligomeric structures, including hexamers and double hexamers (*24*, *25*), and interacts with ssDNA (*26*). Streptavidin displacement experiments were initiated by adding SV40 T antigen to assay buffer containing the 5′-bio-60mer or the 3′-bio-62mer. Aliquots taken at various times were analyzed for displacement by electrophoresis through a 15% polyacrylamide gel. Displacement of streptavidin was observed in the case of the 5′-bio-60mer (Figure 5A) but not in the case of the 3′-bio-62mer (Figure 5B). Thus, SV40 T antigen exhibits a 3′-to-5′ polarity in unwinding and a 3′-to-5′ polarity in streptavidin displacement. As with NS3, these results are consistent with 3′-to-5′ translocation on ssDNA.

Displacement from the 5′-bio-60mer was examined in the presence of increasing concentrations of SV40 T antigen. The rate of streptavidin displacement increased somewhat for each concentration tested (Figure 6A). The rates for streptavidin displacement at 4.6, 5.9, and 7.5 $\mu$M helicase were 0.38 ± 0.04, 0.46 ± 0.10, and 0.55 ± 0.04 min$^{-1}$, respectively.

*T Antigen and NS3 Bind to the Streptavidin-Bound 3′-Biotinylated Oligonucleotide from Which They Are Not*

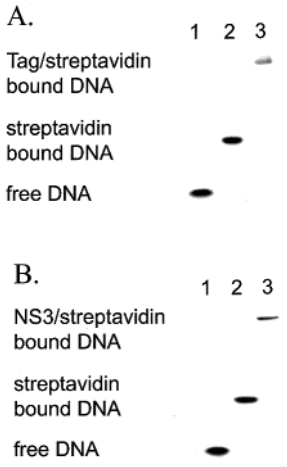


FIGURE 7: Binding of T antigen and NS3 to streptavidin-bound 3′-bio-62mer. Lane 1 is 3′-bio-62mer alone, and lane 2 is 3′-bio-62mer incubated with streptavidin. Lane 3 is 3′-bio-62mer preincubated with streptavidin, then incubated with T antigen (panel A) or NS3 (panel B).

*Able To Displace Streptavidin.* The inability of T antigen and NS3 to displace streptavidin from 3′-bio-62mer could be due to the inability of the enzymes to bind to this substrate or the inability to displace streptavidin from the substrate. To distinguish between these possible explanations, streptavidin-bound 3′-bio-62mer was incubated in the presence and absence of T antigen (Figure 7A) or NS3 (Figure 7B). A shift in the mobility of the streptavidin-bound 3′-bio-62mer was observed in the sample incubated with enzyme, indicating that the DNA is bound by the enzyme under these conditions. Therefore, the inability of T antigen and NS3 to displace streptavidin from 3′-bio-62mer is not due to an inability to bind to the substrate.

*ssDNA Binding Protein, gp32, Does Not Enhance Dissociation of Streptavidin from Biotin-Labeled Oligonucleotides.* An experiment was designed to determine whether streptavidin displacement could be enhanced as the result of cooperative protein binding in proximity to the streptavidin. The ssDNA binding protein from bacteriophage T4, gp32, is known to bind ssDNA cooperatively (reviewed in ref 27). gp32 was incubated with either 5′-bio-60mer or 3′-bio-62mer under conditions in which gp32 concentration was saturating with respect to DNA. No increase in free oligonucleotide was observed after 40 min in either case (Figure 8). Thus, binding alone does not increase the dissociation rate of streptavidin from biotin-labeled oligonucleotides by a measurable degree under the conditions described here.

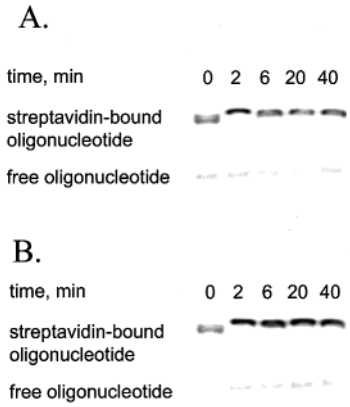


FIGURE 8: Incubation of streptavidin-bound oligonucleotides with single-stranded binding protein, gp32. Gp32 (2 $\mu$M) and biotin (6 $\mu$M) were added to reaction buffer and 10 nM 3′-bio-62mer (A) or 10 nM 5′-bio-60mer (B) in the presence of streptavidin (300 nM). Aliquots were removed at various times and analyzed by 15% polyacrylamide gel electrophoresis.

## DISCUSSION

The question of whether helicases translocate with a directional bias on ssDNA is of importance to the establishment of a mechanism for helicase-catalyzed unwinding of double-stranded nucleic acids. Attempts to answer this question have often been performed on substrates containing a duplex and a ssDNA tail that is 5′ or 3′ to the duplex (*28*). Most helicases unwind one or the other substrate with a strong preference. However, interpretation of these experiments is complicated due to the introduction of the second DNA strand. For example, the presence of the ss/dsDNA junction has been suggested to be the source of the strong polarity in unwinding, rather than a directional bias in translocation on ssDNA (*1*).

To study the activity of helicases on ssDNA, we have developed an assay using biotinylated DNA oligonucleotides bound to streptavidin. The activity of a helicase on ssDNA results in a dramatic increase in the dissociation rate of streptavidin from the biotin (*13*). In a previous study, two helicases thought to possess 5′-to-3′ directionality, the T4 gp41 and Dda helicases, were examined. gp41 forms hexameric structures in the presence of ATP or GTP (*14*) and is from the F4 family of helicases. Dda is from helicase superfamily 1. Evidence has been presented indicating that Dda can function as a monomer (*15*). gp41 and Dda were capable of displacing streptavidin from a 3′-biotinylated oligonucleotide but not a 5′-biotinylated oligonucleotide, consistent with their reported 5′-to-3′ polarity in unwinding. In this report, two helicases that possess 3′-to-5′ polarity in unwinding, NS3 and the SV40 T antigen, were found to displace streptavidin from a 5′-biotinylated oligonucleotide but not a 3′-biotinylated oligonucleotide. Thus, helicases from four different helicase families can enhance dissociation of streptavidin from biotinylated oligonucleotides with a strong directional bias that parallels the polarity observed during DNA unwinding.

The crystal structure of the NS3 helicase domain (NS3h) bound to ssDNA was recently reported (*29*). Based on the structure, a model for unwinding of dsDNA by NS3h was proposed in which the enzyme translocates with a 3′-to-5′ directional bias on ssDNA by using an "inchworm" mechanism. Our results are consistent with 3′-to-5′ translocation on ssDNA but do not address other aspects of this proposed mechanism.

The results obtained by varying NS3 concentrations are similar to those acquired previously with the Dda helicase (*13*) in that saturation of the rate of streptavidin displacement was achieved by increasing the enzyme concentration. The displacement of streptavidin appears to be the rate-limiting step of the reaction for NS3. DNA unwinding of oligonucleotides containing 15 base pairs occurs at a rate of $\sim$0.09 $s^{-1}$ at 25 °C (*21*), whereas streptavidin displacement occurs at a rate of 0.11 $min^{-1}$ at 37 °C (Figure 3). Under conditions in which NS3 is saturated with poly(U), the $k_{cat}$ for ATP

Table 1: Rate Enhancement of Streptavidin Dissociation Catalyzed by Helicases[a]

| helicase | rate of streptavidin displacement | rate enhancement | $\Delta\Delta G^{\ddagger a}$ (kcal/mol) |
|---|---|---|---|
| none | $3.24 \times 10^{-4}$ [b] | | |
| Dda[c] | 474 $min^{-1}$ | $1.5 \times 10^6$ | 8.4 |
| gp41[c] | 0.17 $min^{-1}$ | 525 | 3.7 |
| SV40 Tag | 0.56 $min^{-1}$ | 1700 | 4.4 |
| NS3 | 0.11 $min^{-1}$ | 340 | 3.4 |

[a] The rate of enhancement of streptavidin dissociation from biotin-labeled oligonucleotides was obtained by dividing the helicase-catalyzed rate by the spontaneous rate. The $\Delta\Delta G$ values were calculated according to eq 2. [b] Spontaneous rate of dissociation of biotin from streptavidin taken from ref 38. [c] Data from ref 13.

hydrolysis is ~30 $s^{-1}$ at 37 °C (Tackett and Raney, unpublished observations). Thus, multiple rounds of ATP hydrolysis are likely to occur during the time frame for unwinding and/or displacement of streptavidin. This suggests that the efficiency of the steptavidin displacement reaction is low with respect to ATP hydrolysis. SV40 T antigen reportedly unwinds dsDNA at a rate of a "few hundred" base pairs per minute (30) and hydrolyzes ATP at a rate of 18.5 $min^{-1}$ (*31*). The rate of streptavidin displacement by SV40 T antigen (0.55 $min^{-1}$, Figure 6) is somewhat slower, again suggesting that multiple rounds of ATP hydrolysis can occur during the time frame for streptavidin displacement.

NS3 displaces streptavidin from the 5′-end of a 60mer oligonucleotide more rapidly than from a 30mer, whereas no streptavidin displacement was observed from a 5′-biotin-labeled 20mer or 15mer over the 60 min reaction period. This result is qualitatively similar to that obtained with the Dda helicase (*13*). Dda was capable of displacing streptavidin much more rapidly than NS3 but displayed the similar characteristic of being able to catalyze the dissociation reaction more readily from longer oligonucleotides than from shorter ones. A model consistent with these results is that these enzymes may align along the ssDNA and function in a "cooperative" manner. Although evidence has been presented that suggests that the NS3 helicase domain can function as a monomer (*29*, *32*), others have reported that optimal activity is achieved with some form of oligomeric enzyme (*33*).

The role of helicase in enhancing the dissociation rate of streptavidin from biotin may be analyzed according to transition-state theory. The dissociation of streptavidin from biotin can be considered as the reaction being "catalyzed" by the helicase. The rate constant for dissociation ($k_1$) is enhanced by the activity of the enzyme. According to transition-state theory, the rate of enhancement is related to the energy of activation of the dissociation reaction by eq 2, assuming that the reaction pathway has only one transition state.

$$\frac{k_{1(\text{helicase})}}{k_{1(\text{spontaneous})}} = e\Delta\Delta G^{\ddagger}/\text{RT} \qquad (2)$$

By considering the enhanced rates of displacement of streptavidin as "rates of catalysis", the free energies required to achieve this rate acceleration are 8.4, 3.7, 4.4, and 3.4 kcal·mol-1 for Dda, gp41, SV40 Tag, and NS3, respectively (Table 1). All of these values are well within the range of free energy that is available through hydrolysis of ATP. Thus, it is feasible that a single catalytic event such as a conformational change associated with ATP hydrolysis is responsible for enhancing the rate of dissociation of streptavidin. The values described here may not reflect single catalytic events, especially if multiple helicase monomers are functioning together. If this is the case, then the displacement reaction is more complicated than the simple analysis described here. The main point illustrated by the calculations is that the free energy required to enhance the rate of streptavidin dissociation is not beyond that which can be achieved through a single ATP hydrolysis cycle, if that cycle were tightly coupled to displacement of streptavidin.

The mechanism in which helicase lowers the activation barrier for streptavidin dissociation has not been determined. We have described the enhancement of dissociation of streptavidin from biotinylated oligonucleotides as being due to force produced by the helicase as it translocates with a directional bias on ssDNA. The effect of the helicase on the streptavidin−biotin bond may be to distort the streptavidin as a result of a directionally biased conformational change of the helicase. The interaction between helicase and streptavidin may result in ground-state destabilization of the streptavidin−biotin bond, which would effectively lower the activation barrier by increasing the free energy of the ground state.

An alternative view is one in which the helicase serves to capture high-energy intermediates that form naturally between streptavidin and biotin. The bond distance between biotin and streptavidin varies due to thermal fluctuation, with the average bond distance representing the lowest potential energy conformation (*34*, *35*). The enhancement of dissociation of streptavidin from biotin due to the activity of a helicase may be due to stabilization of high-energy intermediates formed at the bonding interface of streptavidin and biotin. Stabilization could occur when the biotin is "partially" dissociated within the biotin-binding pocket of streptavidin, which would allow the enzyme to move along the DNA into a position that prevents the biotin−streptavidin complex from regaining the ground state. Molecular simulations predict that the rupture length for the biotin−streptavidin bond is 5 Å (*36*, *37*). The distance between base pairs is 3.4 Å in B-form DNA, although this distance might be different in ssDNA. Hence, the bond distance between streptavidin and biotin may increase transiently to accommodate a helicase with a step size that is equal to one base. Helicases with a step size that is larger than one base can also be accommodated because the starting point along the DNA lattice is variable due to random binding of the enzyme to ssDNA.

Whether the activity of a helicase on ssDNA pushes streptavidin from biotin (ground-state destabilization) or captures a high-energy intermediate (transition-state stabilization) remains to be determined. We suggest that the enzymes studied here undergo a directionally biased conformational change along the ssDNA that is driven by ATP hydrolysis. Depending on the particular helicase, this conformational change appears to occur in the 5′-to-3′ direction or the 3′-to-5′ direction. We further suggest that this conformational change occurs along the reaction pathway that ultimately leads to translocation of the helicase along ssDNA with a strong directional bias.

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