Displacement of a DNA binding protein by Dda helicase

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ABSTRACT

Bacteriophage T4 Dda helicase has recently been shown to be active as a monomer for unwinding of short duplex oligonucleotides and for displacing streptavidin from 3′-biotinylated oligonucleotides. However, its activity for streptavidin displacement and DNA unwinding has been shown to increase as the number of Dda molecules bound to the substrate molecule increases. A substrate was designed to address the ability of Dda to displace DNA binding proteins. A DNA binding site for the *Escherichia coli trp* repressor was introduced into an oligonucleotide substrate for Dda helicase containing single-stranded overhang. Here we show that a Dda monomer is insufficient to displace the *E.coli trp* repressor from dsDNA under single turnover conditions, although the substrate is unwound and the repressor displaced when the single-stranded overhang is long enough to accommodate two Dda molecules. The quantity of product formed increases when the substrate is able to accommodate more than two Dda molecules. These results indicate that multiple Dda molecules act to displace DNA binding proteins in a manner that correlates with the DNA unwinding activity and streptavidin displacement activity. We suggest a cooperative inchworm model to describe the activities of Dda helicase.

INTRODUCTION

Helicases are ubiquitous molecular motors which utilize the energy of nucleotide triphosphate (NTP) hydrolysis to separate the strands of duplex nucleic acids or move along DNA (1–4). This strand separation ability makes them essential for virtually all processes in DNA and RNA metabolism, including replication, recombination, transcription and DNA repair (5). Helicases have been classified into five families based on sequence homology (6) and a number of helicase motifs have been identified (7). It has become clear that the roles of enzymes containing helicase motifs extend beyond simply separating dsDNA into ssDNA. For example, removing proteins from nucleic acids is now believed to be an important role for both DNA helicases (8,9) and RNA helicases (10,11).

Helicases and other enzymes that move along DNA or RNA are likely to encounter proteins that are bound to the nucleic acid. The collision between these proteins can lead to stalling of the motor, displacement of the motor or displacement of the DNA binding protein. The probable outcome of such collisions may depend on the relative affinity of the motor protein compared with the DNA binding protein, as well as the force imparted by the molecular motor. The biological relevance of such collisions is becoming increasingly apparent. For example, RNA polymerase can encounter protein roadblocks that prevent transcription of essential genes. In *Escherichia coli*, stalled RNA polymerase can be 'pushed' by the translocase activity of a helicase-like protein called Mfd (12). Mfd can push RNAP to correctly position the polymerase active site after stalling. Alternatively, Mfd can completely displace RNAP from the DNA. In other cases, the translocase activity of a polymerase can accelerate the rate of a helicase. Another clear example of a collision between a DNA-dependent ATPase and a DNA binding protein occurs with chromatin remodeling proteins such as those in the SWI2/SNF2 class (13). Chromatin remodelers disrupt the interaction between DNA and histones but do not necessarily unwind dsDNA. A number of models have been proposed for how remodeling can occur including nucleosome sliding, nucleosome dissociation or histone replacement with a variant histone. Chromatin remodelers share regions of homology to DNA helicases (14).

The mechanism for helicases has been under intense study by a number of laboratories over the past decade. One aspect of the mechanism that has provided much discussion is in regards to the optimal functional form of helicases. For those enzymes of superfamily 1 (SF1) and SF2, different

models have been proposed. Evidence for functional, dimeric forms of helicases has been reported for the *E.coli* Rep helicase (15,16) and *E.coli* UvrD helicase (17). The proposed inchworm mechanism for helicase activity (18,19) places no requirements on the oligomeric structure of the active helicase species. A model for the inchworm mechanism for PcrA helicase has been provided based on biochemical and X-ray crystallographic evidence (19,20). The SF2 helicase, NS3 helicase domain, from the hepatitis C virus has been proposed to function as a monomer (21), although functional cooperativity between monomers has been suggested for optimal activity (22). The full-length NS3 helicase has been proposed to function as a dimer (23), although optimal activity may require a larger species (24).

Dda helicase has been shown to be capable of functioning as a monomer for unwinding of short duplex oligonucleotides (25,26) and for displacing streptavidin from 3′-biotinylated oligonucleotides (27). Dda also does not appear to form stable oligomers even in the presence of ATP, Mg^{2+} and ssDNA (25). Dda's activity for displacement of streptavidin from 3′-biotinylated oligonucleotides (27) and for unwinding of short duplex oligonucleotides has been shown to increase as the number of Dda molecules bound to the DNA substrate increases (28). This led to the proposal of a model for Dda whereby monomeric forms of the enzyme can function in an inchworm-like fashion, yet multiple molecules can function together to enhance the overall activity of the enzyme (27).

Although unwinding of duplex DNA is a physiologically relevant reaction, displacement of streptavidin from biotinylated oligonucleotides is a process which Dda does not perform *in vivo*. However, the displacement of streptavidin may mimic Dda's ability to displace DNA binding proteins in the path of the replication complex (29–31). We sought to determine whether the cooperativity observed for streptavidin displacement (27) was also exhibited for displacement of a protein bound to duplex DNA. The *E.coli trp* repressor was chosen because it binds to the *trpEDCBA* operator sequence with an equilibrium dissociation constant of 3.2 × 10^{-10} (32) in the presence of L-Trp. This value is intermediate in the range of dissociation constants for DNA binding proteins present in *E.coli* (Table 1).

MATERIALS AND METHODS

Reagents

Poly(dT) was purchased from Amersham Pharmacia Biotech. BSA, L-Trp and Sephadex G-25 were from Sigma. ATP, Hepes, KOAc, BME and EDTA were obtained from Fisher. [γ^{32}-P]ATP was purchased from PerkinElmer Life Sciences and T4 polynucleotide kinase was obtained from New England Biolabs. Fluorescein-labeled oligonucleotides were purchased from Operon Technologies. Unlabeled oligonucleotides were purchased from Integrated DNA Technologies. Dda (25) and *E.coli trp* repressor (33) were overexpressed and purified as described.

Oligonucleotides

All the substrates used contain a 30 bp duplex (Figure 1B). The first 18 nt are the sequence of the *E.coli trpEDCBA*

Table 1. Equilibrium dissociation constants of selected cellular and phage DNA binding proteins in *E.coli*

DNA binding protein	Binding site	Dissociation constant (M)
lac repressor[a]	*lac* operator	2E−13[b]
P22 *mnt* repressor	*mnt* operator	1E−11[c]
λ cI repressor	O_R1	2.0E−10[d]
	O_R2	2.3E−9[d]
	O_R3	7.5E−9[d]
trp repressor	*trpEDCBA* operator	3.2E−10[e]
	aroH operator	3.4E−10[e]
	trpR operator	2.2E −10[e]
RNA polymerase	*E.coli* and phage promoters	10^{-6}–10^{-9f}
λ Cro	λ O_R operator/λ O_L operator	1E−9[g]
Factor H₁	λ or φ80 DNA	1E−8[h]
P22 Arc repressor[i]	*mnt* operator	1E−7[f]

[a]One of the tightest binding *E.coli* proteins (51).
[b]Riggs *et al*. (52).
[c]Youderian *et al*. (53).
[d]Hawley *et al*. (54).
[e]Liu and Matthews(32).
[f]McClure (51).
[g]Takeda *et al*. (55).
[h]Spassky and Buc (56).
[i]One of the weakest repressors (51).

operator (a palindromic sequence) and the remaining 12 nt are a random, nonpalindromic sequence. The 5′-single-stranded overhang consists of a varying number of thymidines. DNA duplexes and oligonucleoitdes were purified by PAGE as described previously (25). Concentrations were determined using the absorbance at 260 nM in 0.2 M KOH and calculated extinction coefficients (34). The loading strand (top strand in Figure 1B) was radiolabeled (25).

Displacement of and unwinding in the presence of *trp* repressor

Reactions in which the *E.coli trp* repressor was displaced from its binding site were performed at 25°C using a Kintek rapid chemical quench-flow instrument maintained. An illustration of the reaction appears in Figure 1A. All concentrations listed are after mixing unless otherwise noted. Dda (at the concentrations indicated in the figure legends) was incubated with the indicated concentration of ^{32}P-labeled DNA substrate (Figure 1B) in reaction buffer (25 mM Hepes pH 7.5, 10 mM KOAc, 0.1 mM EDTA, 2 mM BME, 0.1 mg/ml BSA and 0.5 mM L-Trp). The reaction was initiated by the addition of ATP (5 mM), Mg(OAc)₂ (10 mM), 75 μM (in nucleotides) poly(dT) and a 25-fold excess of a re-annealing trap. The reaction was quenched at various times with 200 mM EDTA (concentration before mixing). A control sample to test for the efficiency of the annealing trap was heated to 95°C for 10 min and cooled on ice. Samples were mixed with 0.1% xylene cyanol, 0.1% bromophenol blue and 10% glycerol and loaded immediately on a 20% native polyacrylamide gel containing 10 mM TrisCl pH 7.5 and 0.1 mM L-Trp while running at 250 V in a buffer containing 10 mM TrisCl pH 7.5 and 0.1 mM L-Trp with a circulating water bath maintaining the temperature at 4°C. The fraction of *trp* repressor bound dsDNA, free dsDNA and ssDNA in each sample were determined using a Molecular Dynamics 445-SI PhosphorImager and ImageQuant software.
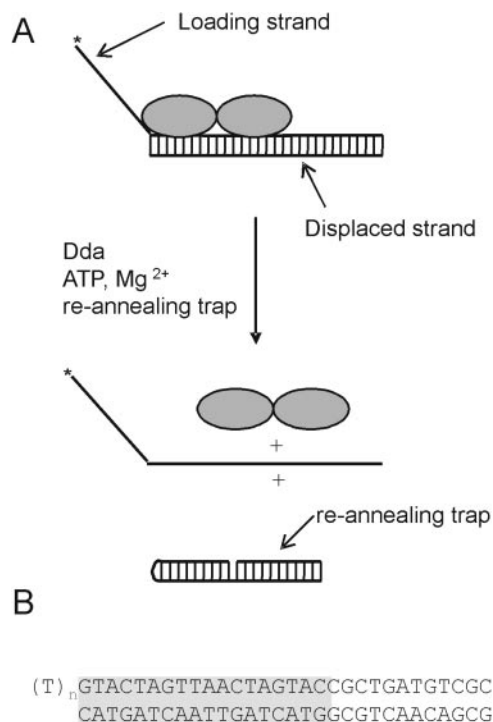
Figure 1. Illustration of the helicase-catalyzed, protein-displacement reaction. (**A**) In the presence of ATP and Mg^{2+}, Dda is capable of displacing the *E.coli trp* repressor from the *trpEDCBA* operator and unwinding the duplex. A re-annealing trap complimentary to the non-palindromic portion of the displaced strand (the 12 nt closest to the 5′ end of the displaced strand) was included in the reaction to prevent the displaced strand of DNA from re-annealing to the loading strand. The *trp* repressor only binds to duplex DNA so it is unable to bind to the ssDNA product after displacement from the substrate. This assay allows both unwinding and protein displacement to be monitored simultaneously. A protein trap was also included to prevent Dda that dissociates from the substrate from rebinding to the substrate. (**B**) The substrates used contain the *E.coli trpEDCBA* operator sequence (highlighted) at the ssDNA–dsDNA junction, followed by 12 bp of non-palindromic sequence. The single-stranded portion of the substrate consists of a variable number of thymidine residues.

For unwinding in the presence of *trp* repressor, *trp* repressor bound and free dsDNA were both counted as substrate. For displacement of the *trp* repressor, free dsDNA and ssDNA were both counted as product. Data were fit using Kaleidagraph software to a stepping equation (35). A minimum of four steps were required to fit the lag phase with a 30 bp duplex (Equation 1).

$$y = A\left\{1 - \left(1 + k_{\text{obs}}t + \frac{(k_{\text{obs}}t)^2}{2} + \frac{(k_{\text{obs}}t)^3}{6}\right)e^{-k_{\text{obs}}t}\right\}, \qquad 1$$

where A is the amplitude of product formation, t is the time and the rate constant k_{obs} reflects the contribution of the unwinding and dissociation rates to the observed rate. Dissociation of Dda from the substrate is not considered explicitly in this approach in which the amplitude (fraction substrate converted to product) is the primary parameter being sought.

DNA unwinding in the absence of bound *trp* repressor

Unwinding reactions were performed at 25°C using a Kintek rapid chemical quench-flow instrument as described

previously (26) in reaction buffer. Dda (750 nM) and 100 nM DNA (final concentrations) were pre-incubated and the reactions were initiated by addition of ATP and Mg(OAc)$_2$, poly(dT) (75 μM, in nucleotides) and DNA trap (5 μM). Data were fit to a stepping equation and a minimum of three steps were required to fit the data (Equation 2).

$$y = A\left\{1 - \left(1 + k_{\text{obs}}t + \frac{(k_{\text{obs}}t)^2}{2}\right)e^{-k_{\text{obs}}t}\right\} \qquad 2$$

Protein–DNA binding. The equilibrium dissociation constant for Dda binding to the substrate with an 8 nt ssDNA tail was calculated after measuring the polarization of 0.5 nM fluorescein-labeled DNA pre-incubated with Dda in reaction buffer using a Beacon fluorescence polarization spectrophotometer (PanVera) as described previously (25) in the presence and absence of 25 nM *trp* repressor. The substrate used was identical to the displacement substrate with the 8 nt single-stranded region except that the 5′ end of the single-stranded region was labeled with fluorescein.

RESULTS

### Displacement of the *trp* repressor is dependent on ATP and Dda

A substrate was designed to allow examination of helicase-catalyzed displacement of a DNA-binding protein along with DNA unwinding in the same assay. The standard helicase assay (Figure 1) must allow the ssDNA products to be separated from dsDNA by native polyacrylamide gel electrophoresis. This is done by adding a strand of DNA that can hybridize to one of the ssDNA products produced by the helicase activity. This additional strand is referred to as the re-annealing trap. Another feature of helicase experiments described here is that single turnover conditions with respect to the DNA substrate were maintained. Helicases require ATP hydrolysis to unwind dsDNA, therefore the enzyme can be incubated with the DNA substrate prior to initiating the reaction. Single turnover conditions can be obtained by introducing a large amount of ssDNA such as poly dT at the same time as the ATP. The poly dT serves as a protein trap that binds to any enzyme that is free in solution or dissociates from the substrate. Thus, in addition to the DNA substrate, the reaction contains a re-annealing trap and a protein trap.

The *trp* repressor was chosen based on the ease of the protein purification and the well-characterized affinity for duplex dsDNA (Table 1) (36). The addition of a DNA-binding protein contributes additional complications to the assay. The *trpEDCBA* operator is a palindromic sequence which could be problematic for trapping of the ssDNA reaction products in the helicase assay. For this reason, the substrate was designed with 12 nt of non-palindromic sequence adjacent to the *trpEDCBA* operator sequence to aid in substrate preparation and increase trapping efficiency (Figure 1B). To test the effectiveness of the DNA trap, an unwinding reaction was performed with 750 nM Dda and 100 nM substrate containing a 12 nt single-strand overhang in the absence of a protein trap. Under these conditions, all of the substrate would be

expected to be converted to product, allowing the trapping efficiency to be determined. No substrate was present after 1 s (Figure 2A), indicating that the displaced strand can be effectively trapped using a re-annealing trap complimentary to the 12 nt of non-palindromic sequence in the displaced strand (Figure 1A).

Next we sought to determine whether displacement of *trp* repressor from the substrate was dependent on ATP and Dda. In the absence of ATP, no displacement was observed after 20 s, but the substrate was converted to product in the presence of 5 mM ATP (Figure 2B). Product was also not formed in the absence of Dda, but substrate was completely
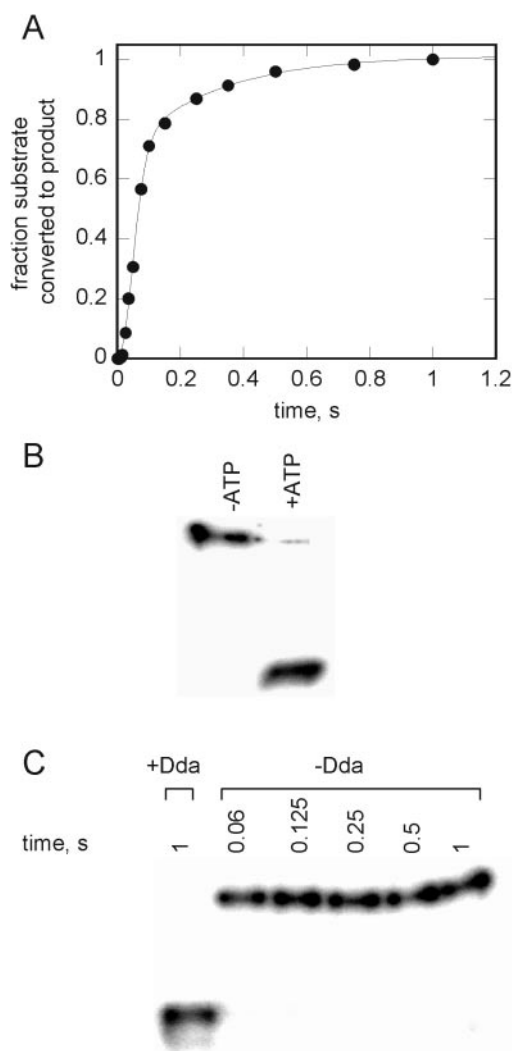
Figure 2. Assessment of the DNA unwinding and protein displacement assay. (**A**) Product formation is plotted for unwinding in the absence of a protein trap to illustrate that the re-annealing trap functions well even though part of the duplex sequence is a palindrome. Substrate (100 nM) containing a 12 nt single-strand was pre-incubated with Dda (750 nM) and the reaction was initiated by the addition of ATP, Mg^{2+} and a re-annealing trap. (**B**) Dda (750 nM) does not displace the *trp* repressor from 100 nM substrate containing a 12 nt single-strand in the absence of ATP in 20 s. (**C**) The repressor is not displaced in the absence of Dda from 100 nM substrate containing a 12 nt single-strand. Complete displacement is seen in the same time frame in the presence of 750 nM Dda.

converted to product in the presence of 750 nM Dda (Figure 2C). This indicates that the *trp* repressor is displaced by Dda as it unwinds the DNA substrate.

Helicase-catalyzed displacement of the *E.coli trp* repressor and DNA unwinding

Dda sequesters 6 nt when bound to ssDNA (27). Based on this, a substrate with an 8 nt single-strand overhang should easily be able to accommodate one Dda molecule. Since the *trp* repressor binding site is at the ssDNA–dsDNA junction, it is unlikely that much fraying occurs, so binding of more than one Dda molecule would be unlikely. Dda helicase exhibits higher processivity when more than one molecule of the enzyme binds to the substrate (28). We wished to compare the protein displacement activity of monomeric Dda with that of multiple molecules of Dda, so substrates with 12 and 24 ssDNA overhangs were designed that should be able to bind two and four Dda molecules when saturated, respectively.

Displacement of *trp* repressor from and unwinding in the presence of *trp* repressor of substrates with 8, 12 and 24 nt single-strands were compared. Dda was incubated with substrate, and the reactions were initiated by addition of ATP, Mg^{2+}, and protein and re-annealing traps. The products were separated by PAGE in the presence of L-Trp (Figure 3A). The *trp* repressor bound, dsDNA substrate could be separated from the unbound dsDNA substrate, allowing for protein displacement to be measured in the absence of DNA unwinding. However, the unbound dsDNA appeared only transiently in the reaction, and almost all of dsDNA substrate from which *trp* repressor was displaced and was quickly melted by the helicase. Reaction progress curves (Figure 3B) show that the unwinding reaction is completed rapidly after the protein is displaced. The same quantities of product are formed in both reactions, which illustrates that Dda is capable of displacing a protein and continuing with the unwinding reaction after displacement since the *trp* repressor must be displaced before the final 12 bp can be unwound. No unwinding or displacement is seen when the substrate contains only 8 nt of single-strand, suggesting that a single Dda molecule is not capable of displacing the *trp* repressor while unwinding DNA under single cycle conditions. A monomer is capable of unwinding naked DNA (25,26) but for unwinding of this substrate to occur, the bound protein must first be displaced. The displacement of *trp* repressor and unwinding of the DNA require multiple steps, as indicated by the initial lag in product formation (Figure 3C). The lag phases are identical, again suggesting that protein displacement limits the overall reaction rate.

In order to ensure that the substrate was saturated with respect to Dda, the reaction with the three substrates was conducted at two concentrations of Dda with each substrate (Figure 3D). No increase in the rate or amplitude was observed when Dda was raised from 750 nM to 1 μM (Figure 3D). Therefore, 750 nM Dda is sufficient to saturate the rate on 100 nM of all the three substrates.

Unwinding of unbound dsDNA

To determine whether protein displacement limits the reaction rate and quantity of product formed, unwinding reactions were performed in the absence of *trp* repressor on
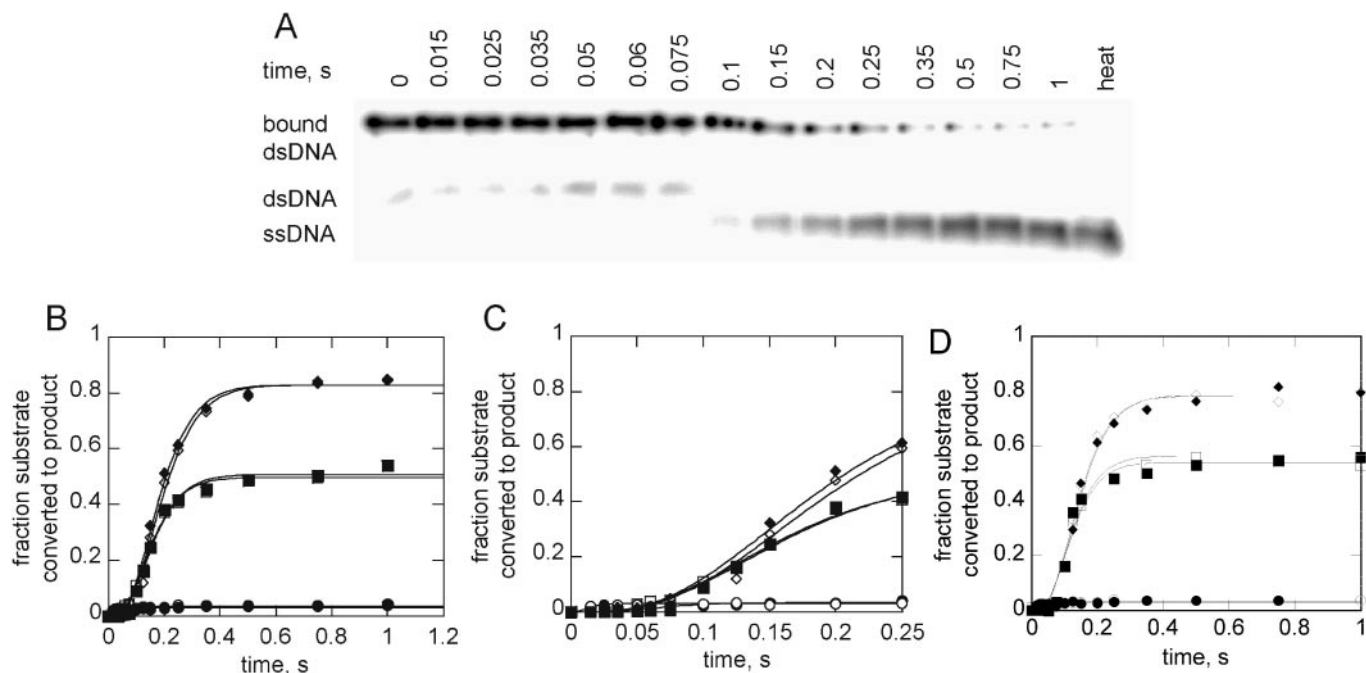
Figure 3. DNA unwinding and *trp* repressor displacement is dependent on the length of the ssDNA overhang. (**A**) Separation of products by native polyacrylamide gel electrophoresis shows product formation for 100 nM substrate containing a 24 nt single-strand by 750 nM Dda. (**B**) Reaction progress curves for displacement of and unwinding in the presence of *E.coli trp* repressor. No product formation was observed from the substrate with the 8 nt ssDNA overhang for displacement (filled circles) or unwinding (open circles). Rates obtained from fits to a four-step sequential mechanism (Equation 1) were 29.1 ± 3.6 s^{-1} and 24.7 ± 3.1 s^{-1} for *trp* repressor displacement from substrates containing 12 (filled squares) and 24 (filled diamonds) nt overhangs, respectively, and unwinding rates were 31.7 ± 4.6 s^{-1} and 24.8 ± 4.4 s^{-1} for substrates with 12 (open squares) and 24 (open diamonds) nt ssDNA overhangs, respectively. (**C**) Displacement and unwinding data from panel B are shown to 0.25 s to illustrate the lag phase in product formation. (**D**) No displacement was observed from a substrate (100 nM) containing 8 nt of single-strand by 750 nM (filled circles) or 1 μM (open circles) Dda. Displacement by 750 nM (filled squares) and 1 μM (open squares) Dda occurred at rates of 31.7 ± 4.6 and 29.9 ± 2.0 s^{-1}, respectively, from the substrate containing a 12 nt ssDNA overhang. The displacement rates were 24.8 ± 4.4 and 25.5 ± 1.8 s^{-1} from the substrate with 24 nt ssDNA overhang in the presence of 750 nM (filled diamonds) and 1 μM (open diamonds) Dda.

each substrate. Reactions were performed as described for the *trp* repressor displacement experiments except the *trp* repressor was omitted. Samples were analyzed by PAGE (Figure 4A). Reaction progress curves (Figure 4B) show that the quantity of product formed increases as the length of the ssDNA overhang increases. Interestingly, product is observed with the substrate containing 8 nt ssDNA overhang (filled circles in Figure 4B), whereas in the presence of *trp* repressor, no product was observed (filled circles in Figure 3B). Unwinding of the free 30 bp duplex also exhibits a lag phase (Figure 4C), although the lag phase is shorter than that for unwinding in the presence of the *trp* repressor (Figure 3C), indicating that fewer steps are required for unwinding of free DNA.

Comparison of quantities of product formed and rates in the presence or absence of *trp* repressor

The quantity of product formed (Figure 5A) and the rate at which it is formed (Figure 5B) are compared in Figure 5. The major difference between DNA unwinding and *trp* repressor displacement occurs on the substrate containing the 8 nt ssDNA overhang. Little product for DNA unwinding or *trp* repressor displacement was observed with this substrate in the presence of the repressor. However, the substrate was rapidly unwound in the absence of *trp* repressor. The quantity

of product formed for protein displacement and DNA unwinding are similar in the presence of *trp* repressor with the substrates containing the 12 nt ssDNA overhang, but the rate for DNA unwinding is somewhat faster in the absence of *trp* repressor. The magnitude of these differences decreases as the length of the single-stranded portion of the substrate increases from 12 to 24 nt, suggesting that the more Dda molecules bound, the more efficient the displacement of the protein bound in its path. The rate and quantity of product formation in the presence and absence of *trp* repressor are nearly identical when the substrate can accommodate up to four Dda molecules. The fact that unwinding occurs at nearly identical rates to *trp* repressor displacement in the presence of *trp* repressor, compared with somewhat faster rates for unwinding in the absence of *trp* repressor, indicates that the unwinding reaction is more rapid than protein displacement.

Dda does bind to the substrate with an 8 nt single-strand when *trp* repressor is bound

No displacement or unwinding in the presence of *trp* repressor was observed from the substrate with an 8 nt single-strand. Since the *trp* repressor binding site is at the ssDNA–dsDNA junction, it is possible that the *trp* repressor protein overhangs the single-stranded region of the substrate somewhat and prevents Dda from binding. The equilibrium
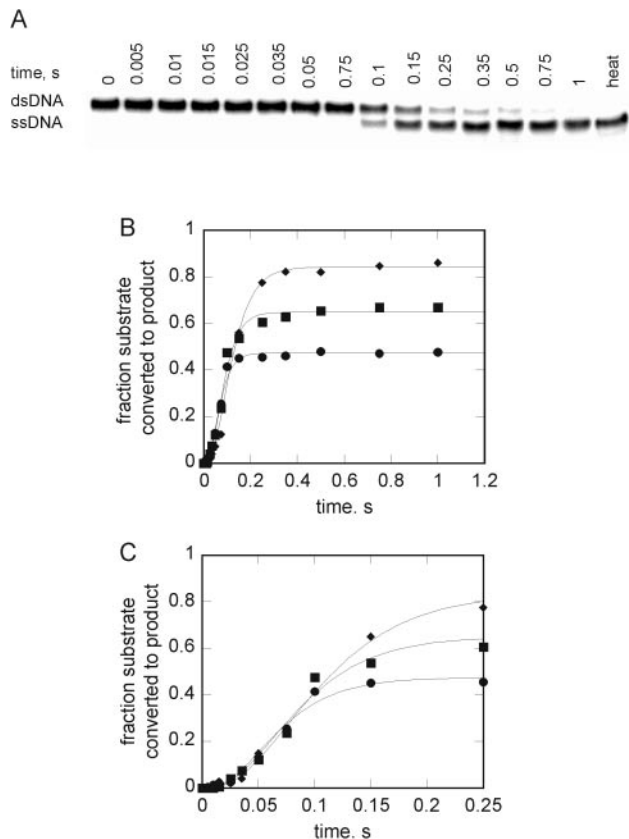
Figure 4. DNA unwinding kinetics in the absence of bound *trp* repressor. (**A**) Products of unwinding reactions were separated by electrophoresis on 20% polyacrylamide gels (shown is unwinding of 100 nM substrate containing a 24 nt ssDNA overhang by 750 nM Dda). (**B**) Product formation was plotted using Kaleidagraph software for unwinding of 100 nM substrate by 750 nM Dda. Unwinding rates obtained from fits to a three-step mechanism (Equation 2) were 55.0 ± 0.4, 44.1 ± 1.0 and 30.0 ± 1.3 s^{-1} for unwinding of substrates containing 8 (filled circles), 12 (filled squares) and 24 (filled diamonds) nt of single-strand, respectively. (**C**) Data in panel B are shown to 0.25 s to illustrate the initial lag phase in product formation.
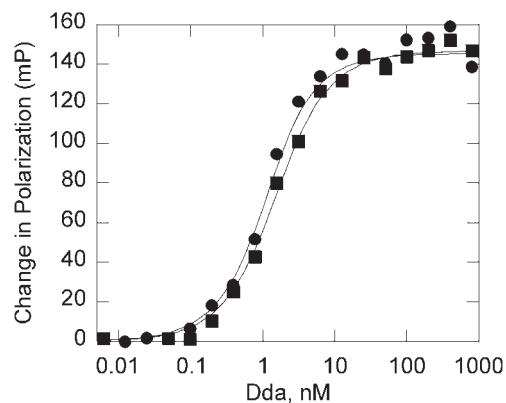
dissociation constants for Dda from a substrate containing an 8 nt single-strand in the presence and absence of bound *trp* repressor were found to be nearly identical (Figure 6), suggesting that Dda is bound to the substrate with the 8 nt single-strand even when the *trp* repressor is bound. However, a single Dda molecule is not able to displace the bound protein.

DISCUSSION

DNA and RNA helicases are motor proteins that manipulate nucleic acids in virtually all aspects of nucleic acid metabolism. The primary role for DNA helicases is believed to be in melting of dsDNA, which is likely to be the role in most cases. The role for helicases in RNA metabolism is not as clear, although for both RNA and DNA, collisions between helicases and proteins bound to nucleic acids are highly probable to occur. These collisions may lead to dissociation of the translocating helicase, or the bound protein might be displaced by the helicase. Numerous examples of collisions between DNA or RNA translocases have been reported, but the mechanism for protein displacement has not
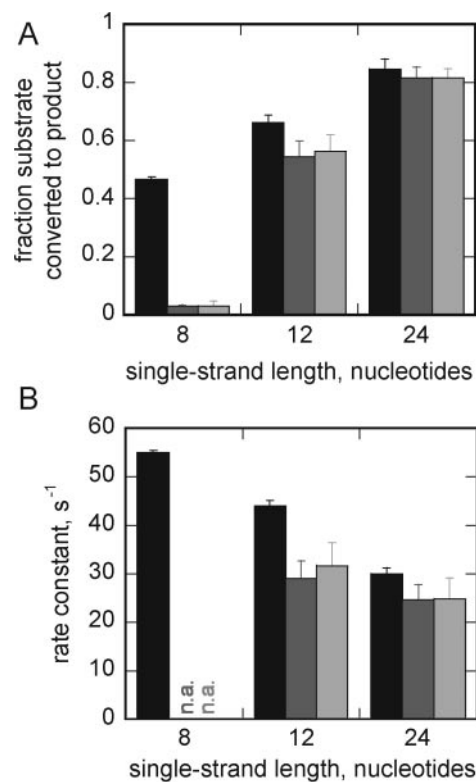
Figure 5. Comparison of the quantities of product formed and the rates at which it was formed. The fraction of substrate converted to product (**A**) and the rate at which this occurs (**B**) for DNA unwinding in the absence of *trp* repressor (black), in the presence of *trp* repressor (dark gray). For comparison, the quantity and rate of displacement of *trp* repressor are shown (light gray). The error bars represent the SD of three experiments.

Figure 6. Determination of equilibrium dissociation constants in the presence and absence of bound *trp* repressor. The K_D values for Dda binding to the substrate containing an 8 nt single-strand were 0.7 ± 0.2 and 0.9 ± 0.3 nM in the presence (filled circles) and absence (filled squares) of *E.coli trp* repressor bound to the duplex.

been described. For example, DNA replication termination in *E.coli* has been shown to occur at defined sequences due to specific interaction between the DnaB helicase and the replication terminator protein Tus (37). Other protein–nucleic acid complexes do not impede DnaB helicase. Kaplan and O'Donnell showed that DnaB can displace dsDNA-binding

proteins during translocation on dsDNA (38). This activity of DnaB was proposed to facilitate branch migration in DNA recombination or to strip proteins away from sites of DNA repair. In *Saccharomyces cerevisiae*, termination of replication forks is controlled in a sequence-specific fashion by the interaction of the Fob1p replication terminator protein (39). Stalled replication forks can be released by the Rrm3p helicase (termed a 'sweepase'). The action of Rrm3p is modulated by other proteins such as Tof1p-Csm3p complex (40). Hence, a complex series of protein collisions controls the number of replication forks that can proceed through normal termination sites in this organism.

Removal of proteins from RNA was shown *in vitro* in the case of NPH-II helicase illustrating that RNA helicases can participate in structural reorganization of ribonucleoprotein complexes (11). The ability of RNA helicases to remove proteins from dsRNA in the absence of RNA unwinding has also been demonstrated (10). Evidence has been presented for a functionally significant role for two putative yeast helicases, Sub2 and Prp28, in disruption of protein–RNA complexes during spliceosomal assembly (41,42).

We have designed a substrate for evaluation of the biochemical mechanism of displacement of protein–DNA complexes by Dda helicase. The substrate was designed so that the monomeric form of Dda could be compared with multimeric forms of the enzyme by increasing the length of the ssDNA loading strand. Dda has been found to function as a monomer in unwinding short oligonucleotide substrates, but the processivity of the enzyme is enhanced by increasing the number of molecules bound to a DNA substrate (28). Dda also displaces streptavidin from biotin labeled oligonucleotides, and the activity exhibits positive cooperativity, again illustrating the idea that more molecules of helicase appear to function together. The data for streptavidin displacement activity support a model whereby two or more Dda molecules interact to enhance the activity of the enzyme. This might occur in a manner where a trailing molecule of helicase pushes a leading molecule along the nucleic acid strand, or prevents the lead molecule from slipping during translocation. The data for DNA unwinding by Dda can be interpreted in terms of 'functional' cooperativity, which does not rely on protein–protein interactions *per se*. The latter term has been applied to the NS3 helicase domain to explain how multiple molecules can work on the same substrate to enhance the rate and quantity of ssDNA formed from dsDNA substrates (22). Functional cooperativity can occur when the trailing molecule of helicase continues unwinding dsDNA after the lead molecule dissociates, thereby leading to increased product formation. Also, the trailing molecule can prevent re-annealing of the ssDNA products that might occur after the lead molecule unwinds a given number of base pairs. It is likely that some of the biochemical activities of Dda require interactions between helicase monomers and other activities do not require protein–protein interactions.

A single Dda molecule was not capable of displacing the *E.coli trp* repressor from the *trpEDCBA* operator sequence or unwinding DNA with a *trp* repressor molecule bound under single turnover conditions (Figure 3), although it was able to unwind the DNA in the absence of *trp* repressor (Figure 4). Two Dda molecules were sufficient to convert about half of the substrate to product in the presence of *trp*

repressor (Figure 3), although, more unwinding was observed in the absence of bound protein (Figure 4). The longest ssDNA overhang used here (24 nt) can bind up to four Dda molecules. When this substrate was examined, almost all of the *trp* repressor was displaced and nearly all of the substrate unwound (Figures 3 and 4). In fact, the quantity of product formed and rate of product formation for protein displacement, unwinding in the presence of bound *trp* repressor, and unwinding of naked DNA were nearly identical when four Dda molecules were bound to the substrate. This suggests that although a Dda monomer is sufficient to unwind the 30 bp of DNA, it cannot produce the force necessary to unwind DNA and displace a protein bound to the duplex at the same time under single turnover conditions. Two Dda molecules can accomplish this task, although the presence of the protein slows the unwinding process. When more than two molecules of Dda are bound to the substrate, progression of the unwinding reaction is unaffected by the presence of the bound protein, indicating that multiple Dda molecules have increased activity in protein displacement.

The unwinding reaction was limited by protein displacement, as illustrated by the faster DNA unwinding in the absence of *trp* repressor. The longer lag phase in the presence of *trp* repressor indicates that Dda needed additional catalytic cycles to displace the *trp* repressor (compare Figure 3C with Figure 4C). This observation is consistent with the idea that Dda may slip when encountering a block or challenge in its path, therefore requiring additional steps to complete the process. This also illustrates the need for multiple Dda molecules to remove *trp* repressor, because monomeric Dda is not highly processive, therefore it is not able to undergo as many catalytic cycles after encountering the protein block. The fact that protein displacement and dsDNA unwinding were correlated so tightly for the substrates with longer ssDNA overhangs indicates that the displacement reaction was probably a direct consequence of the DNA unwinding. This suggests that the DNA melting activity of Dda is responsible for protein displacement.

Others have shown that Dda can displace proteins from DNA including the *E.coli* lac repressor (30) and the *E.coli* Ter protein (43). However, not all protein–DNA complexes are displaced by Dda. Dda was unable to dislodge a GAL4–DNA complex, even under conditions which should favor binding of more than one molecule of Dda to the substrate (44). Therefore, some specific protein–DNA complexes are able to sequester Dda in a manner that appears to trap Dda and reduce the ATP hydrolysis activity of the enzyme. It is possible that the GAL4–DNA complex adopts a unique structure that perturbs Dda's interaction with DNA.

A model for Dda helicase activity is emerging that illustrates a role for multiple helicase molecules per substrate, whether DNA unwinding is measured or protein displacement. The work here illustrates that the idea of cooperative function of Dda put forth for streptavidin displacement applies for displacement of DNA-binding proteins as well; i.e. increasing the number of Dda molecules leads to enhanced activity. This model suggests that multiple helicase molecules can cooperate to unwind DNA and displace proteins more effectively than a single molecule. The activity continues to increase as more helicase molecules bind to the same substrate. We suggest the term 'cooperative
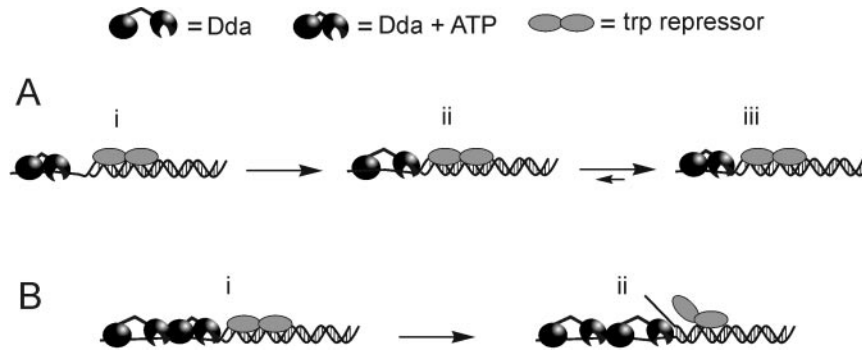
Figure 7. Model depicting displacement of the *trp* repressor by Dda helicase. (**A**) Monomeric Dda is shown bound to a substrate to which *trp* repressor is bound (i). Upon hydrolyzing ATP, the helicase moves forward while adopting an open conformation (ii). Upon binding ATP, the domains change conformations to adopt the closed conformation (iii). The presence of the *trp* repressor prevents the helicase from moving forward after ATP hydrolysis, therefore the enzyme slips backward or dissociates from the substrate. (**B**) When two or more helicase molecules are bound to the substrate, the trailing molecule can act as a brake to prevent slipping by the lead molecule (i). The lead molecule is able to translocate forward, thereby unwinding the DNA and simultaneously displacing the *trp* repressor (ii).

inchworm' to describe Dda, which illustrates the ability of individual monomers to function, but the increased activity as more than one molecule binds to a substrate. Some challenges presented to the helicase, such as protein displacement or unwinding of long stretches of dsDNA, require the action of multiple helicase molecules, whereas short duplexes can readily be melted by the monomeric form of this enzyme.

A cartoon depicting a possible mechanism by which two helicases can function together to displace the *trp* repressor is shown in Figure 7. Monomeric Dda is shown in Figure 7A. Of the helicases for which structures are available, Dda is most similar to RecD (45). RecD contains two domains that include the known helicase motifs found in super family 1 helicases, and like Dda, translocates in a 5′-to-3′ direction. Based on the reported structure of RecD, and by making analogous comparison with the inchworm model proposed for PcrA helicase (19), the two domains in RecD are thought to adopt open and closed conformations as a function of binding ATP. Dda is also thought to contain two major 'helicase domains' based on limited proteolyis experiments (L. Blair and K. D. Raney, unpublished observations). The two domains of Dda are depicted as a leading domain and a trailing domain in Figure 7. Further, these two domains can adopt an open conformation in the absence of ATP binding and closed conformation in the presence of ATP binding. Movement of the helicase along nucleic acid is proposed to be driven by cycling between these conformations as a function of ATP binding and hydrolysis. When monomeric Dda encounters the *trp* repressor, movement of the helicase is impeded by the presence of the repressor, which serves as a protein block. Monomeric Dda can continue to hydrolyze ATP upon encountering a protein block, without immediately displacing the protein block (46). Therefore, it is likely that Dda is able to 'slip' backwards, which is indicated by the small reverse arrow in Figure 7A. Slipping by Dda occurs as a result of the trailing domain losing its grip on the nucleic acid, thereby re-establishing the open conformation without forward movement. When two molecules of Dda are bound to the same substrate, *trp* repressor can be displaced as a result of the second Dda molecule acting as a brake or an anchor for the

first molecule, thereby preventing the backwards slipping of the first molecule (Figure 7B). This model for Dda is similar to a model recently reported for the interaction between T7 polymerase and the T7 helicase. DNA synthesis by the T7 polymerase provides the driving force that speeds up the DNA unwinding rate for T7 helicase by ∼10-fold (47). The enhanced rate of the helicase has been interpreted in terms of a 'push' by the polymerase or as a 'brake' by the polymerase to prevent the helicase from sliding backwards.

The fact that multiple molecules of Dda are required for protein displacement may illustrate an additional level of regulation available for determining the outcome of collisions between translocases and DNA binding proteins. The number of helicase molecules that can be loaded onto a particular DNA sequence is likely to be dependent on whether DNA repair, replication or recombination or transcription is being catalyzed. Dda helicase has been implicated in playing some role in disrupting biological significant protein–nucleic acid interactions. The first step in the homologous pairing reaction catalyzed by the T4 uvsX recombinase is inhibited by Dda helicase, presumably by disruption of the uvsX protein–DNA filament (48). However, Dda is known to bind to uvsX and stimulate branch migration activity (49). The protein displacment activity of Dda is believed to allow branch migration through a DNA–protein complex containing an RNA polymerase promoter complex (50). The number of Dda molecules required for these processes *in vivo* remains to be determined. In addition to binding to uvsX, Dda is also known to bind to the T4 single-stranded binding protein, gp32. Each of these proteins is known to form filaments along nucleic acid. If these proteins play a role in loading Dda onto ssDNA, then it is possible that multiple Dda molecules are loaded onto specific DNA sites *in vivo* as a result of the interaction of Dda with multiprotein filaments such as uvsX and gp32.

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