Increasing the Length of the Single-Stranded Overhang Enhances Unwinding of Duplex DNA by Bacteriophage T4 Dda Helicase†

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ABSTRACT: Dda has been shown previously to be active as a monomer for DNA unwinding [Nanduri et al. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 14722] and streptavidin displacement [Byrd and Raney (2004) *Nat. Struct. Mol. Biol. 11*, 531]. However, its activity for streptavidin displacement increased as a function of the length of single-stranded DNA. We investigated whether Dda exhibited enhanced DNA unwinding of partially duplex DNA substrates as a function of increasing the length of the single-stranded overhangs. DNA substrates were prepared containing 16 base pairs and single-stranded overhangs of 4, 6, 8, 12, 16, 20, and 24 nucleotides. Under single turnover conditions in the presence of excess enzyme, the quantity of DNA unwound increased significantly as the length of the single strand overhang increased. Increased processivity was observed when the DNA substrate contained longer single-stranded overhangs. Equilibrium binding studies indicated that Dda bound to the substrates containing the longer overhangs significantly better than the shorter overhangs. To determine whether the increased processivity for unwinding was due to multiple molecules of Dda or due to the increased binding affinity to the longer overhangs, DNA unwinding was conducted under pre-steady-state conditions, which favor binding of monomeric Dda. Under pre-steady-state conditions, the quantity of product decreased somewhat as the single-stranded length increased, from 12 to 24 nucleotides. Thus, when monomeric Dda is required to translocate longer distances prior to unwinding, processivity is lowered. Taken together, these results indicate that enhanced binding to the longer single-stranded overhangs was not responsible for enhanced processivity under conditions of excess enzyme. Rather, multiple molecules of Dda bound to the same substrate exhibit greater processivity for DNA unwinding.

Helicases are ubiquitous enzymes, found in viruses, bacteria, and eukaryotes, that separate the strands of duplex DNA using the energy of NTP[1] hydrolysis (*1*−*5*). This provides the ssDNA intermediates necessary for virtually all aspects of nucleic acid metabolism. Despite their importance in the cell, the mechanism(s) by which DNA unwinding occurs are only beginning to be defined. Helicases have been suggested to contain multiple DNA binding sites, which allow for processive movement along DNA.

Helicases have been classified into five families based on conserved sequence motifs (*6*). SF3 and F4 helicases appear to function as hexamers or double hexamers (*3*). The oligomeric structure of several SF1 helicases has been well studied in the case of *Escherichia coli* Rep, *E. coli* UrvD, *E. coli* RecBCD, *Bacillus stearothermophilus* PcrA, and bacteriophage T4 Dda helicases. Rep, UvrD, and PcrA share considerable sequence and structural homology with each other while Dda shows sequence homology in the helicase motifs. It is possible that different enzymes within the same family exhibit different mechanisms in terms of the oligomeric structure of the enzyme.

Experiments investigating Rep's ability to unwind DNA suggest that oligomerization is required for both initiation of and continuation of the unwinding reaction (*7*, *8*). UvrD helicase has also been proposed to function as a dimer during DNA unwinding (*9*−*11*) but can translocate as a monomer on ssDNA (*12*). RecBCD is a heterooligomeric enzyme containing two helicase molecules. One helicase, RecB, binds to one strand of the duplex while the second helicase, RecD, binds to the second strand of the duplex (*13*). PcrA has been suggested to function as a monomer based on X-ray crystallographic data (*14*) and biochemical experiments (*15*). It has also been shown to translocate as a monomer (*16*). On the basis of these results, PcrA has been proposed to function by an inchworm mechanism (*14*, *17*).

Recent work on the NS3 helicase domain, an SF2 helicase, has been interpreted in terms of a functional cooperative model for DNA unwinding (*18*). Multiple molecules of NS3h are proposed to enhance processive DNA unwinding by aligning along the same DNA strand and translocating in the same direction. When one molecule of NS3h dissociates from the substrate, another molecule can take its place and continue the unwinding process. Also, multiple molecules are proposed to prevent reannealing behind the helicase as the enzyme moves through the duplex. Dda is an SF1 helicase that can function as a monomer for unwinding short

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[1] Abbreviations: BSA, bovine serum albumin; BME, β-mercaptoethanol; dsDNA, double-stranded DNA; F, family; NTP, nucleoside triphosphate; ssDNA, single-stranded DNA; SF, superfamily.

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duplexes (*19*, *20*) and for displacement of streptavidin from 3′-biotinylated oligonucleotides (*21*). Little evidence for oligomerization was observed in biophysical studies (*19*), although Dda's streptavidin displacement activity was observed to increase as the number of Dda molecules bound to the substrate increased, indicating that multiple molecules of Dda aligned along the ssDNA result in faster displacement of streptavidin (*21*). The results with Dda are reminiscent of the results with NS3h. When the data for streptavidin displacement are considered with the data from unwinding experiments, a cooperative inchworm mechanism is suggested whereby Dda is capable of functioning as a monomer through an inchworm-type mechanism, but activity is increased when multiple monomers cooperate.

Dda's ability to displace streptavidin from 3′-biotinylated oligonucleotides may reflect displacement of DNA binding proteins in the path of the replication machinery (*22*−*24*). However, the assay may not reflect the DNA unwinding activity of the enzyme. We sought to determine whether Dda also exhibits functional cooperativity during DNA unwinding.

EXPERIMENTAL PROCEDURES

Reagents. BSA and Sephadex G-25 were purchased from Sigma. ATP, Hepes, KOAc, EDTA, and BME were from Fisher. Poly(dT) was obtained from Amersham Pharmacia Biotech. [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences, and T4 polynucleotide kinase was purchased from New England Biolabs. Fluorescein-labeled oligonucleotides were purchased from Operon Technologies. All other oligonucleotides were purchased from Integrated DNA Technologies. Dda was overexpressed and purified as described (*19*).

Oligonucleotides. All unwinding substrates contained a 16 base pair duplex with a varying number of thymidines as a 5′-single-stranded overhang.

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*5′-(T)nCGCTGATGTCGCCTGG-3′
    3′- GCGACTACAGCGGACC-5′
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Protein−DNA binding substrates were identical to unwinding substrates except they contained a 3′-fluorescein label on the bottom strand. All oligonucleotides and DNA duplexes were purified by PAGE (*19*) and quantitated by absorbance at 260 nm in 0.2 M KOH using calculated extinction coefficients (*25*). The loading strand (top strand) of unwinding substrates was radiolabeled as described (*19*).

DNA Unwinding. Unwinding reactions were performed using a Kintek rapid chemical quench-flow instrument maintained at 25 °C as described (*20*) in a buffer containing 25 mM Hepes, pH 7.5, 10 mM KOAc, 0.1 mM EDTA, 0.1 mg/mL BSA, and 2 mM BME. The indicated concentrations (final concentrations) of Dda and DNA were incubated prior to initiating the unwinding reaction by addition of ATP and $Mg(OAc)_2$. Poly(dT), at the indicated concentrations, was added at the initiation of the reaction as a protein trap. A 16mer oligonucleotide complementary to the displaced strand (the bottom strand) was added with the ATP to trap the displaced strand in pre-steady-state experiments. The sample was collected in a tube containing the annealing trap for experiments where the enzyme concentration was in excess of the DNA. Data were fit to the equation for a three-step sequential mechanism (eq 1) (*9*, *26*). In eq 1, A is the

$$y = A\{1 - (1 + k_{obs}t + ((k_{obs}t)^2)/2)e^{-k_{obs}t}\} \quad (1)$$

amplitude of product formation and k_{obs} is a composite rate constant reflecting the contribution of the unwinding and dissociation rates to the observed rate.

Protein−DNA Binding. Dda binding to 0.5 nM DNA in unwinding buffer was analyzed using a Beacon fluorescence polarization spectrophotometer (PanVera) as described (*19*). DNA substrates were identical in sequence to those used for unwinding, except the bottom strand contained a 3′-fluorescein label. Data were fit to the quadratic equation (eq 2) using Kaleidagraph software, where mP is millipolarization units, ΔmP is the total change in millipolarization, [DNA] is the concentration of fluorescently labeled DNA substrate, [E] is the concentration of Dda, and K_D is the apparent equilibrium dissociation constant.

$$mP = (\Delta mP/[DNA])\{[(K_D + [DNA]_T + [E]_T) - \sqrt{(K_D + [DNA]_T + [E]_T)^2 - 4[E]_T[DNA]_T}]/2[DNA]_T\} \quad (2)$$

RESULTS

Product Formation Increases under Single Turnover Conditions When Dda Is Present in Excess as the Length of the Single-Stranded Region of the Substrate Increases. Substrates with a varying length single strand containing a 16 base pair duplex were designed in order to compare Dda's ability to unwind when increasing numbers of Dda molecules were bound to the substrate. Dda sequesters approximately six nucleotides when bound to ssDNA (*21*). This suggests that a substrate with a four nucleotide single strand would have zero to one Dda molecules bound, depending on how much fraying occurred at the ssDNA−dsDNA junction. A substrate with a six nucleotide single strand should be able to accommodate 1 Dda molecule, 1−2 with an eight nucleotide single strand, 2 with a 12 nucleotide single strand, 2−3 with a 16 nucleotide single strand, 3−4 with a 20 nucleotide single strand, and 4 with a 24 nucleotide single strand. Dda-catalyzed unwinding of these substrates was measured under single turnover conditions to determine the rate of unwinding and the quantity of substrate that could be unwound in a single binding event. Excess enzyme was present to ensure that all available binding sites would be occupied so that unwinding of a duplex by varying numbers of Dda molecules could be compared.

Dda was preincubated with the substrate, and the reaction was initiated by addition of ATP and Mg^{2+}. Poly(dT) was added with the ATP as a trap to bind any protein that dissociated from the substrate such that only the Dda initially bound to the substrate could convert it to product. Figure 1A shows separation of the ssDNA product from dsDNA substrate (2 nM) containing a 24 nucleotide single-stranded overhang. The quantity of product formed was determined by phosphorimager analysis and is plotted in Figure 1B in the presence of 100 and 300 nM Dda. Data were fit to a three-step mechanism using KaleidaGraph software (*9*, *26*). The rate constants for unwinding were 51 ± 5 s^{-1} at 100 nM Dda and 44 ± 3 s^{-1} at 300 nM Dda. The early region of

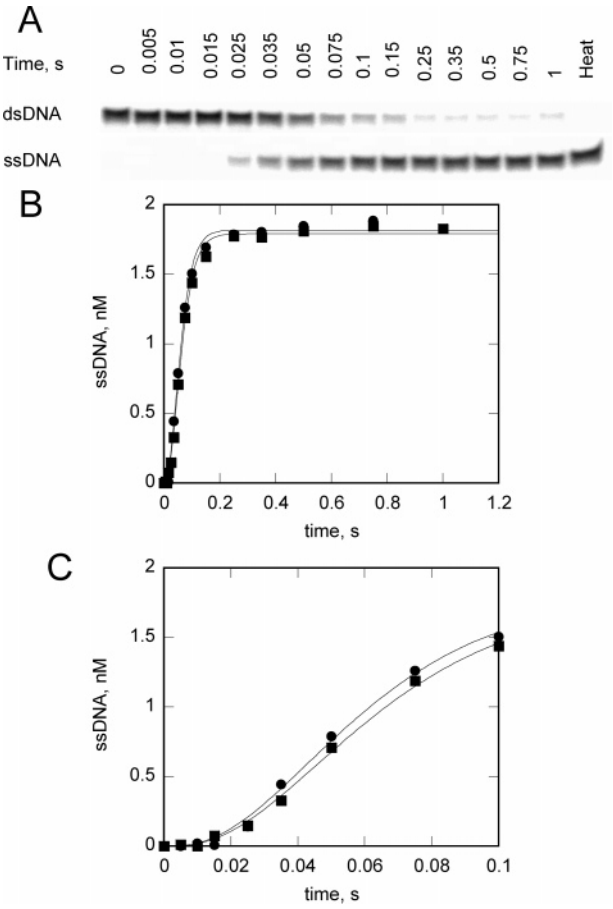

FIGURE 1: Unwinding under single turnover conditions with excess Dda to determine a saturating concentration of Dda. (A) Polyacrylamide gel showing product formation over time for unwinding of a substrate with a 24 nucleotide single strand (2 nM) by 100 nM Dda. (B) Product formation was plotted versus time for unwinding of 2 nM substrate with a 24 nucleotide single strand by 100 nM (●) and 300 nM (■) Dda. Data were fit to a three-step sequential mechanism. The observed rates were 51 ± 5 and 44 ± 3 s^{-1} for unwinding by 100 and 300 nM Dda, respectively. (C) Initial region of the plot in (B) illustrating the lag phase for DNA unwinding.

the unwinding reaction, shown in Figure 1C, shows the distinct lag phase, suggesting that multiple steps are required for unwinding of a 16 base pair duplex, as seen previously for unwinding a duplex of this length by Dda (*20*). The rate of unwinding by 100 nM Dda is not less than that obtained in the presence of 300 nM Dda, suggesting that 100 nM Dda is sufficient to saturate the rate; 100 nM Dda was also sufficient to saturate the rate with substrates containing single strands varying in length from 4 to 20 nucleotides.

Product formation curves by 100 nM Dda with a 2 nM amount of each substrate (single strand lengths: 4, 6, 8, 12, 16, 20, and 24 nucleotides) are shown in Figure 2A. The quantity of product formed increases as the length of the single-stranded overhang increases (Figure 2B). More ssDNA is formed from substrates that are able to bind more than one Dda molecule, suggesting that multiple Dda molecules exhibit higher processivity. However, tighter binding affinity might also explain the increase in DNA unwinding with increasing single-stranded overhang.

Dda Binds with Greater Affinity to Substrates with Longer Single Strands. The increase in the amplitude of ssDNA product as the length of the single strand increases from 4 to 24 nucleotides may be due to increased affinity of Dda to

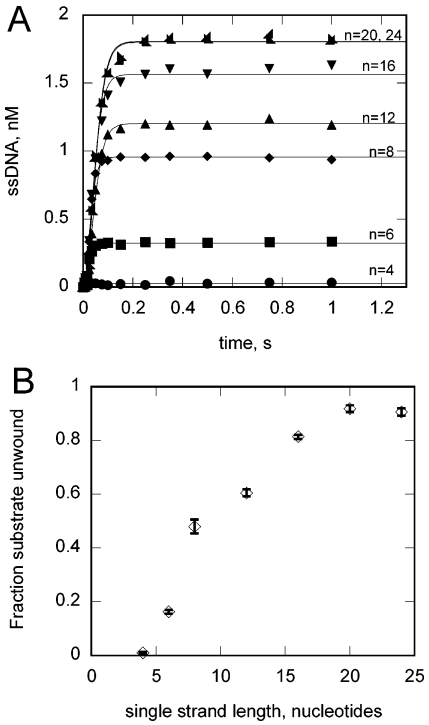

FIGURE 2: Unwinding of substrates with varying length single-stranded overhangs in the presence of excess Dda. (A) Unwinding of 2 nM DNA by 100 nM Dda. The length of the single-stranded overhang for each substrate is indicated in the figure. Data were fit to a three-step sequential mechanism. The observed rate constants for unwinding (k_{obs}) were 124 ± 7, 93 ± 4, 63 ± 7, 62 ± 1, 61 ± 9, and 51 ± 5 s^{-1} for substrates with 6, 8, 12, 16, 20, and 24 nucleotide single strands, respectively. No unwinding was observed with a 4 nucleotide single strand overhang. (B) Plot of the fraction of substrate converted to product as the length of the single strand increased from 4 to 24 nucleotides.

substrates with longer single strands. To determine whether this was the case, duplex substrates were prepared which were identical to the unwinding substrates, except the displaced strand contained a 3′-fluorescein label. The equilibrium dissociation constant for Dda with a blunt end 16 base pair duplex and duplexes containing 0, 4, 6, 8, 12, 16, 20, and 24 nucleotide single-stranded regions were determined using the change in polarization of the DNA upon binding of Dda (Figure 3). The K_D values obtained were 108 ± 42, 135 ± 32, 37 ± 29, 18 ± 15, 11 ± 7, 12 ± 9, 8 ± 5, and 6 ± 4 nM for binding to a blunt end duplex and duplexes containing 0, 4, 6, 8, 12, 16, 20, and 24 nucleotides of single strand, respectively. The results indicate that Dda does bind with greater affinity to substrates with long single strands compared to those with short, or no, single strand. This suggests that the increase in unwinding seen in Figure 2 as the length of the single strand increased from 4 to 24 nucleotides may be due to increased affinity for the substrate. The K_D values for Dda binding to duplexes with zero and four nucleotide single strands were nearly identical. This suggests that the lack of unwinding of the substrate containing the four nucleotide single-stranded overhang (Figure 2) may be due to the inability of Dda to bind productively to the short single-stranded region. Therefore, the lack of product in the case of substrates with very short overhangs cannot be used to draw conclusions regarding the effect of one or more Dda molecules. However, these results do indicate that Dda does not bind well to such substrates and

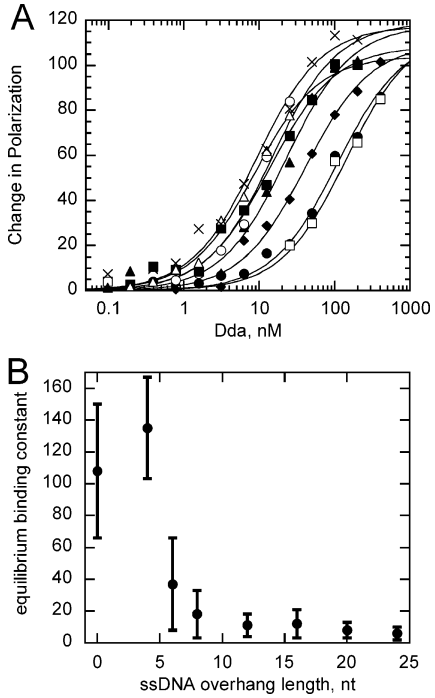

FIGURE 3: Measurement of the equilibrium binding constant for Dda binding. (A) The change in the fluorescence polarization was measured to monitor binding of Dda to 0.5 nM DNA. Dda bound to a blunt end duplex (●) and duplexes with 4 (□), 6 (◆), 8 (▲), 12 (○), 16 (■), 20 (△), and 24 (×) nucleotide single strands were measured. Data were fit to the quadratic equation resulting in values for the equilibrium dissociation constant of 108 ± 42, 135 ± 32, 37 ± 29, 18 ± 15, 11 ± 7, 12 ± 9, 8 ± 5, and 6 ± 4 nM for the substrates containing 0, 4, 6, 8, 12, 16, 20, and 24 nucleotide overhangs, respectively. (B) Plot of the equilibrium binding constants as a function of the length of the single-stranded overhang. The error bars represent the standard error for each fit.

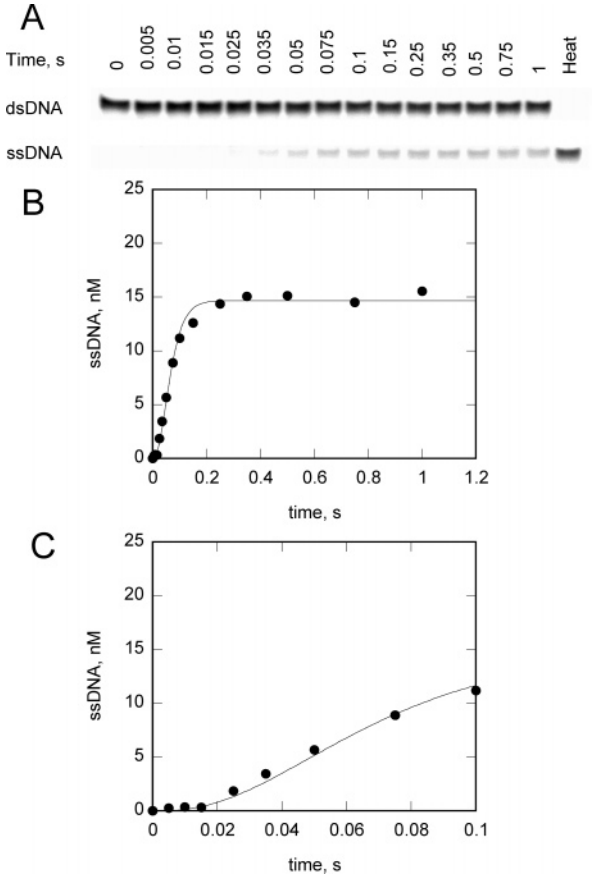

FIGURE 4: Pre-steady-state unwinding shows a burst in product formation. (A) Polyacrylamide gel showing unwinding by 25 nM Dda in the presence of 100 nM substrate containing a 24 nucleotide single-stranded overhang. (B) Plot of product formation over time for the reaction shown in (A). The observed rate from a fit to a three-step mechanism was 43 ± 2 s^{-1}. (C) Plot of the initial region of (B), indicating the lag phase in product formation.

does not appear to interact specifically with a ss/dsDNA junction in the absence of ATP binding.

Under Pre-Steady-State Conditions, in the Presence of a Protein Trap, Product Formation Does Not Increase as the Length of the Substrate Increases. To determine if tighter binding of the longer single-stranded overhangs is responsible for greater processivity, we conducted DNA unwinding under pre-steady-state conditions. Pre-steady-state kinetic analyses, which measure product formation during the first cycle in the presence of excess substrate, have demonstrated that Dda can unwind a 12 and a 16 base pair duplex as a monomer (*20*, *27*). When the substrate is in excess of the enzyme concentration, there is very little chance for multiple helicase molecules to bind to the same substrate molecule in the absence of cooperative DNA binding. Therefore, these conditions provide a test of the possible explanations for the increase in product formation seen in Figure 2. Under pre-steady-state conditions, each substrate will have the same number of Dda molecules bound, regardless of the length of the substrate.

Dda (25 nM) was incubated with substrate (100 nM), and the unwinding reaction was initiated by addition of ATP and Mg^{2+}. Poly(dT) was included as a protein trap, and an annealing trap was included to prevent the ssDNA products from reannealing. A gel showing product formation with the substrate containing the 24 nucleotide single strand is shown in Figure 4A, and a plot of the data is shown in Figure 4B. Data were fit to a three-step sequential mechanism. The first phase of the reaction provides a clear burst amplitude in product formation of ~15 nM for the 24 nucleotide substrate. The steady-state phase is eliminated by the presence of the protein trap. The initial region of the product formation curve is shown in Figure 4C to illustrate the lag phase, which is similar to that observed in Figure 2. Thus, the pre-steady-state conditions provide a kinetic progress curve that is very similar to the curves produced under conditions of excess enzyme, even when the ssDNA overhang is 24 nucleotides in length.

Similar pre-steady-state experiments were performed with substrates containing ssDNA overhangs varying in length from 6 to 24 nucleotides (Figure 5A). Since the burst amplitude can be an indicator of the active enzyme concentration, the ratio of the burst amplitude to the Dda concentration is shown in Figure 5B. Little product is observed when the single-stranded overhang is only 6 nucleotides in length, consistent with low affinity of Dda for this substrate. The fact that the ratio of the burst amplitude to the Dda concentration approaches 1 with the substrates containing 8 and 12 nucleotide ssDNA overhangs indicates that monomeric Dda is active and capable of unwinding a 16 base pair duplex, as shown earlier by Nanduri et al. (*20*). The quantity of product decreases steadily as the length of the ssDNA overhang increases from 12 to 24 nucleotides (Figure 5B). This result indicates that tighter binding is not responsible

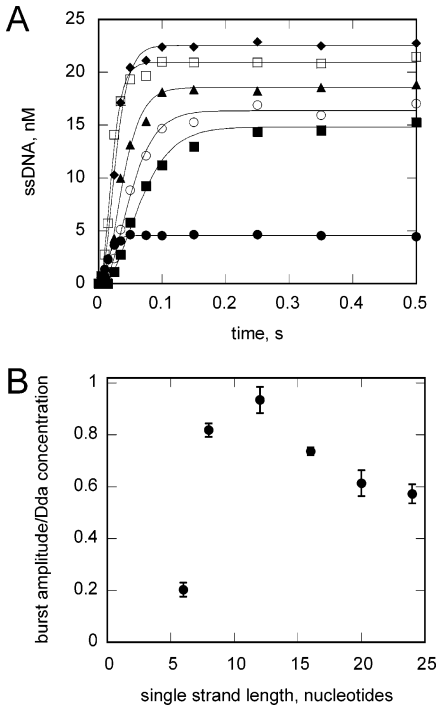

FIGURE 5: Pre-steady-state unwinding of substrates with varying length single-stranded overhangs. (A) Plot of DNA unwinding by 25 nM Dda in the presence of 100 nM DNA. Substrates contained 6 (●), 8 (□), 12 (◆), 16 (▲), 20 (○), and 24 (■) nucleotide single-stranded overhangs. The rate contants for unwinding obtained from fits to a three-step mechanism were 201 ± 12, 132 ± 4, 84 ± 4, 66 ± 5, 57 ± 4, and 43 ± 2 s^{-1} for substrates with 6, 8, 12, 16, 20, and 24 nucleotide single strands, respectively. (B) Plot of the ratio of the burst amplitude to the Dda concentration as a function of the length of the single-stranded overhang.

for the increased amplitudes for unwinding of the substrates containing longer ssDNA overhangs. The decrease in the burst amplitude/Dda concentration ratio can be explained by the lack of processivity of Dda. If Dda binds randomly to the single-stranded region of each substrate, then the average distance that it must translocate to completely unwind the duplex increases as the ssDNA overhang increases. Nearly all of the substrate was unwound for substrates containing 20 and 24 nucleotides of ssDNA in the presence of excess enzyme (Figure 2). However, only around 60% of the expected amplitude was observed for these same substrates under pre-steady-state conditions, which favors monomeric Dda. The pre-steady-state experiments strongly indicate that the increased amplitude observed under excess enzyme conditions in Figure 2 results from the binding of multiple Dda molecules to those substrates that contain sufficient ssDNA to bind multiple helicases.

Excess Dda Can Inhibit the Unwinding Reaction. For the single turnover unwinding experiments (Figures 1 and 2), the fraction of substrate converted to product was actually slightly higher at 100 nM Dda than 300 nM Dda. The difference in the amplitudes increased as the length of the single-stranded overhang decreased. Since 300 nM Dda is well above the K_D for Dda binding to duplex DNA, it is possible that, at 300 nM Dda, binding to the substrate occurs along the single-stranded region as well as the duplex region. Dda does not readily unwind blunt end duplexes; therefore, binding to the duplex region may actually inhibit unwinding somewhat. This possibility was tested by measuring the amplitude of product formation at increasing concentrations

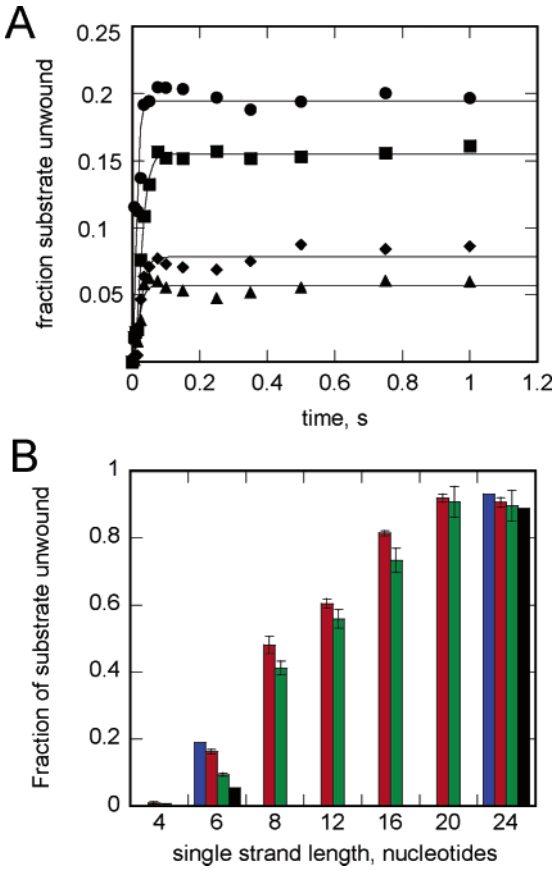

FIGURE 6: Dda binding to the duplex can inhibit unwinding by Dda. (A) Unwinding of the duplex with a 6 nucleotide single-stranded overhang by 50 nM (●), 100 nM (■), 300 nM (◆), and 600 nM (▲) Dda. (B) Plot showing the fraction of each substrate (2 nM) converted to product by varying concentrations of Dda: 50 nM (blue), 100 nM (red), 300 nM (green), and 600 nM (black).

of Dda (Figure 6). Indeed, as the Dda concentration is increased from 50 nM (below the K_D for duplex binding) to 600 nM (above the K_D for duplex binding), the quantity of product decreases for the substrate containing a 6 nucleotide ssDNA overhang (Figure 6A). The difference in the quantity of substrate unwound decreases as the length of the single strand increases from 6 to 24 nucleotides (Figure 6B). The quantity of product formed remains constant as the Dda concentration is varied when the single strand is 20 nucleotides or more. This suggests that Dda bound to the duplex may inhibit unwinding by Dda, but as the number of Dda molecules bound to ssDNA portion of the substrate increases, the inhibition is overcome. This result is similar to that observed previously for unwinding of modified DNA substrates. Monomeric Dda was not capable of unwinding a substrate containing a single peptide nucleic acid placed in the translocation strand; however, multiple Dda molecules were able to unwind the modified DNA substrate (*27*).

DISCUSSION

Monomeric Dda has been shown to be active for unwinding of short duplexes, and the results shown here support that conclusion. Unwinding of a substrate with a single-stranded region short enough to only accommodate one Dda molecule (six nucleotides) was observed (Figures 2, 5, and 6). Unwinding was also observed under pre-steady-state conditions in which the substrate concentration is greater than

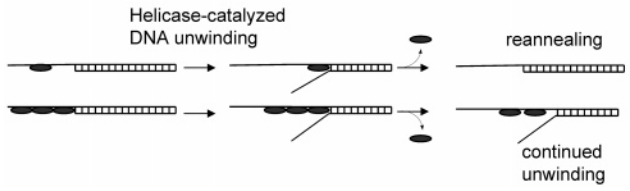

FIGURE 7: Model for DNA unwinding by a nonprocessive DNA helicase. A DNA substrate containing a 20 nt ssDNA overhang is shown when bound to a single Dda monomer as occurs under pre-steady-state conditions and when bound by three molecules of Dda as occurs under excess enzyme conditions. During DNA unwinding, the single monomer can dissociate from the duplex prior to complete unwinding, leading to reannealing so that no product is observed. When the substrate is bound by multiple helicase monomers, DNA unwinding can continue when one helicase monomer dissociates due to the activity of the additional helicase monomers.

the enzyme concentration (Figures 4 and 5). In this case, multiple Dda molecules should not be bound to each substrate since the substrate concentration is in excess and Dda does not exhibit strong interactions with itself (*19*). The quantity of substrate converted to product decreases as the length of the ssDNA overhang increases under pre-steady-state conditions (Figure 5B). This is probably due to the low processivity exhibited by Dda. The reduced amplitudes observed from 12 to 24 nucleotide ssDNA overhang in Figure 5 indicate that Dda binds randomly along the ssDNA portion of the substrate.

The amplitude for product formation increases as the length of ssDNA overhang increases when Dda concentration is in excess, supporting the role for multiple Dda molecules during unwinding of substrates containing longer ssDNA overhangs (Figure 2). This result is similar to results reported previously for streptavidin displacement from biotinylated oligonucleotides by Dda (*21*). Streptavidin displacement was found to occur much faster from substrates that were capable of binding multiple molecules of Dda. A simple model explaining these results is illustrated in Figure 7. For a nonprocessive helicase such as Dda, unwinding proceeds for a number of base pairs before the enzyme dissociates from the substrate. If sufficient base pairs remain, then reannealing will occur and no product will form. If more than one molecule of Dda is bound to the substrate, then additional helicase molecules can continue to unwind the remaining duplex after dissociation occurs. Although little evidence has been found for formation of a dimeric species for Dda, it is possible that the lead molecule interacts with the trailing molecule in a transient manner forming a functional dimer.

The model in Figure 7 is very similar to that proposed for DNA unwinding by the NS3 helicase domain (*18*). The model for NS3h does not require protein−protein interactions to occur to observe greater processivity. The increase in activity of NS3h was proposed to occur through functional cooperativity rather than structural interactions between helicase monomers, such as those reported for the dimeric form of UvrD helicase (*10*, *11*). For Dda, protein−protein interactions were proposed to explain the enhanced function of multiple molecules during streptavidin displacement. However, the interactions between Dda monomers may be due to the fact that the streptavidin blocked translocation of the lead helicase monomer, leading to a "pile-up" of trailing Dda molecules along the ssDNA. The data presented here do not support nor eliminate protein−protein interactions between Dda molecules for the enhanced unwinding activity. Interestingly, the rate constants obtained for DNA unwinding are consistently lower as a function of the increasing ssDNA overhang, even in the presence of excess enzyme (Figure 2). This could reflect the fact that multiple molecules of Dda interact, leading to a slower moving complex of helicase molecules such as a dimer. It is also possible that the slower rate constants are a result of the lead molecule of Dda dissociating earlier than the trailing molecules, thereby requiring the trailing molecules to cover the same stretch of DNA as the lead molecule prior to completion of unwinding. Hence, it remains unclear as to whether Dda adopts a functional oligomeric form when unwinding longer stretches of duplex DNA, but oligomerization is not required to explain the current data.

Oligomerization of helicases can result in multiple DNA binding sites, which can lead to higher processivity. Processivity appears to be most closely correlated with the affinity of the DNA for the enzyme, rather than the oligomeric structure per se. An example of a helicase that is very highly processive but clearly does not form hexamers is the RecBCD helicase. The recent crystal structure reveals two helicase motors, each capable of binding and unwinding DNA with opposite polarity (*28*). The third subunit also adopts a structure similar to that of a helicase, albeit with no activity of its own. The structure of RecBCD clearly shows that each strand of ssDNA is encircled by the enzyme. Therefore, the high processivity is likely due to the manner in which DNA is threaded through channels in the enzyme. Many helicases function as oligomers such as the hexameric helicases including DnaB (*29*), bacteriophage T7 gene 4 helicase (*30*), and bacteriophage T4 gp41 helicase (*31*). In these systems, protein−protein interactions between helicase monomers are relatively strong. Hexameric helicases generally exhibit higher processivity than monomeric enzymes because the DNA passes through a central channel formed by the hexamer (*32*, *33*). There appears to be a positive correlation between helicase oligomerization and overall processivity of the particular helicase; however, the nature of the DNA binding, i.e., threading of the enzyme through protein channels, appears to be most important for maintaining high processivity. It is possible that a monomeric helicase might be capable of binding to DNA in a manner that leads to very high processivity. Indeed, the type 1 restriction enzymes contain helicase motifs and translocate on dsDNA as monomers in a highly processive manner (*34*).

When the concentration of Dda is high enough to allow binding to the duplex region of the substrate in addition to the ssDNA tail, the quantity of substrate converted to product is decreased (Figure 6). However, when the single-stranded region of the substrate is long enough to accommodate three to four Dda molecules, this inhibition is no longer observed, suggesting that proteins (Dda) bound to the duplex do not affect unwinding by three or four Dda molecules. Unwinding is impeded when fewer Dda molecules are bound to the single strand, suggesting that multiple Dda monomers are more effective at displacing a protein while unwinding than a single Dda monomer. This is consistent with the increasing streptavidin activity as the number of Dda molecules bound to the substrate previously reported (*21*). This report shows that multiple Dda molecules bound to the same ssDNA overhang function together to produce more DNA unwinding

than monomeric Dda. In vitro, a potential role of the additional molecules during unwinding of short duplexes could be to replace the lead molecule when it dissociates from the substrate. Another role for multiple molecules might be to enhance processivity due to transient protein−protein interactions that reduce the probability for dissociation. In the case of displacement of streptavidin, protein−protein interactions appear to enhance the activity of the assembly of Dda molecules.

It should be noted that Dda is known to interact with other proteins from bacteriophage T4 including gp32, a single-stranded binding protein, and UvsX, a recombinase enzyme (*35*). The specific role of protein−protein interaction on Dda helicase activity has not been adequately addressed. Jongeneel et al. found that the effect of gp32 on Dda helicase activity was dependent on the order of addition and on the relative concentrations of the proteins (*36*). Dda was found to accelerate branch migration when added to UvsX-catalyzed strand exchange reactions (*37*). It is possible that interaction with these or other proteins precludes Dda from functioning in a manner whereby multiple helicase molecules are present on the same substrate molecule. However, it is also interesting to note that gp32 (*38*) and UvsX (*39*) are capable of binding to DNA in a cooperative manner such that filaments are formed along the nucleic acid. Such filaments may predispose Dda molecules to align along the nucleic acid or perhaps oligomerize as suggested in a recent study from the Morrical laboratory (*40*).

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