Evidence for a Functional Monomeric Form of the Bacteriophage T4 Dda Helicase

Dda DOES NOT FORM STABLE OLIGOMERIC STRUCTURES*

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Patrick D. Morris, Alan J. Tackett, Kirk Babb, Bindu Nanduri, Chris Chick, Joseph Scott, and Kevin D. Raney‡
From the Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

The active form of many helicases is oligomeric, possibly because oligomerization provides multiple DNA binding sites needed for unwinding of DNA. In order to understand the mechanism of the bacteriophage T4 Dda helicase, the potential requirement for oligomerization was investigated. Chemical cross-linking and high pressure gel filtration chromatography provided little evidence for the formation of an oligomeric species. The specific activity for ssDNA stimulated ATPase activity was independent of Dda concentration. Dda was mutated to produce an ATPase-deficient protein (K38A Dda) by altering a residue within a conserved, nucleotide binding loop. The helicase activity of K38A Dda was inactivated, although DNA binding properties were similar to Dda. In the presence of limiting DNA substrate, the rate of unwinding by Dda was not changed; however, the amplitude of product formation was reduced in the presence of increasing concentrations of K38A Dda. The reduction was between that expected for a monomeric or dimeric helicase based on simple competition for substrate binding. When unwinding of DNA was measured in the presence of excess DNA substrate, addition of K38A Dda caused no reduction in the observed rate for strand separation. Taken together, these results indicate that oligomerization of Dda is not required for DNA unwinding.

When dsDNA is replicated, repaired, or recombined, the necessary ssDNA intermediates are provided by the activity of helicases (1–5). These enzymes appear to be ubiquitous, having been identified in viral, bacterial, and eukaryotic systems. Helicases hydrolyze nucleotide triphosphates, usually ATP, to obtain the energy needed to unwind dsDNA. They translocate on DNA often in a very processive manner, unwinding thousands of base pairs in a single binding event. Their processive nature implies that helicases have multiple DNA binding sites; indeed, a characteristic feature of many helicases is their propensity to form oligomeric structures, often dimers or hexamers.

Knowledge of the oligomeric form of a helicase is of fundamental concern in development of a mechanism for unwinding activity, and many approaches have been applied to determine oligomeric structure. The lack of evidence for oligomerization from biophysical experiments has led to the suggestion that some helicases function as monomers. PcrA helicase of Bacillus stearothermophilus is proposed to function as a monomer that contains two binding sites for DNA (6, 7). Evidence has been provided that suggests Escherichia coli Uvd helicase (helicase II) can be active as a monomer in vivo and in vitro (8). The E. coli Rep helicase has been proposed to function as a dimer (2), whereas other helicases such as T7 gene 4 helicase and T4 gp41 helicase appear to function as hexamers (9, 10).

Helicases have been classified according to sequence homology (11), and those enzymes in superfamily 1 and superfamily 2 have proven difficult to characterize in terms of oligomerization. The focus of this study is the bacteriophage T4 helicase, Dda, a 5′-to-3′ helicase classified in superfamily 1. Evidence suggests that Dda is involved in T4 replication initiation (12). T4 Dda mutants show a delayed DNA synthesis phenotype, consistent with this hypothesis. Dda also enhances the rate of branch migration owing to a specific interaction with the T4 recombinase (UvsX) and is therefore likely to play a role in recombination (13–15). Dda binds tightly to the T4 single-stranded DNA-binding protein, gp32, although the significance of this interaction has not been determined (15, 16).

Dda translocates on ssDNA with a strong directional bias in the 5′-to-3′ direction (17). It is capable of removing protein blocks placed in the path of the enzyme, including streptavidin bound to biotin-labeled oligonucleotides (18). Dda is not highly processive (19, 20). Unwinding of only a few hundred base pairs can be prevented by addition of ssDNA, even when Dda is incubated with the substrate prior to addition of the competitor DNA (19). In contrast, the replicative helicase from bacteriophage T4, gp41, can unwind thousands of base pairs in a single binding event (21). Like T7 gene 4 helicase, gp41 is a hexameric helicase that encircles ssDNA, resulting in very high processivity (18, 22). The oligomeric nature of Dda was investigated in order to determine whether its relatively low processivity might be related to the oligomeric structure, and to lay the foundation for future mechanistic studies.

EXPERIMENTAL PROCEDURES

Reagents—Phosphoenoxypruvate kinase/lactate dehydrogenase, NADH, peptatin A, phenylmethylsulfonyl fluoride, and lysozyme were from Sigma. ATP, Hepes, glycerol, EDTA, NaCl, isopropyl-1-thio-β-D-galactopyranoside, dextrose, and KOAc were from Fisher. T4 polynucleotide kinase was purchased from New England Biolabs. [γ-32P] ATP
was purchased from PerkinElmer Life Sciences. DSP was purchased from Pierce. All oligonucleotides were purchased from Operon Technologies. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis on a SE 410 Sturdier Vertical Slab Unit (Amersham Pharmacia Biotech). Oligonucleotides were visualized by UV shadowing. DNA strands were denatured with 0.5 M NaOH and was eluted from the acrylamide gel by electrophoresis by using an Elutrap apparatus (Schleicher & Schuell). Eluted DNA was loaded onto a Waters C_{18} Sep-Pak cartridge, de-salted with H_{2}O, then eluted with 60% methanol. DNA was dried in a Speed Vac and then redissolved in 10 mM Hepes (pH 7.5) and 1 mM EDTA. DNA was quantified by UV absorbance at 260 nm in 0.2 M KOH, and the concentration was determined by using a calculated extinction coefficient of 59,010 cm^{-1} M^{-1}.

**WT Dda Expression and Purification—**Recombinant plasmid pET26b-Dda was kindly provided by Dr. Craig Cameron (Pennsylvania State University). Dda was expressed in *E. coli* BL21/D3 cells. Expression was induced at 15 °C overnight by addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and 0.2% dextrose. Cells (30 g) were collected by centrifugation and suspended in 175 ml of lysis buffer (25 mM Tris acetate (pH 7.6), 500 mM NaCl, 1 mM EDTA, 5 mM BME, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 4 μg/ml pepstatin A, and 0.2 mg/ml lysozyme). Cell lysis was assisted by one cycle of freezing and thawing followed by sonication. The lysate was cleared by centrifugation at 20,000 rpm in a JA-25.50 rotor (Beckman) for 20 min at 4 °C. The supernatant was further clarified by ultrafiltration using a YM-10 filter (Amicon, Millipore), and dialyzed into storage buffer in the absence of BME for chemical cross-linking experiments. Protein concentration was measured by UV absorbance at 280 nm. A calculated extinction coefficient (59,010 cm^{-1} M^{-1}) based on the amino acid sequence was used to quantify the concentration of Dda in solution.

**WT Dda Expression and Purification—**Recombinant plasmid pET26b-Dda was kindly provided by Dr. Craig Cameron (Pennsylvania State University). Dda was expressed in *E. coli* BL21/D3 cells. Expression was induced at 15 °C overnight by addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and 0.2% dextrose. Cells (30 g) were collected by centrifugation and suspended in 175 ml of lysis buffer (25 mM Tris acetate (pH 7.6), 500 mM NaCl, 1 mM EDTA, 5 mM BME, 10% glycerol) containing 1.5 mM 30-mer and 15-mer. DNA was quantified by UV absorbance at 260 nm in 0.2 M KOH, and the concentration was determined by using a Bio-Select SEC 250–5 column (Bio-Rad). A flow cell was placed into an SLM Amino-Bowman fluorescence spectrometer so that elution from the column could be monitored by fluorescence of the K38A Dda. An excitation length of 280 nm and an emission wavelength of 340 nm. The proteins that were used to prepare a calibration curve were: thyroglobulin (bovine), 670 kDa; γ-globulin (bovine), 443 kDa; apoferritin, 158 kDa; bovine serum albumin, 66 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa. Gel filtration chromatography was performed using a standard elution buffer (50 mM Tris-Cl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA) with 1 mM ATP and 1 mM Mg(OAc)_{2}. 5 μM Dda was incubated in the elution buffer with ATP and MgCl_{2}, for 2 min prior to injection onto the column.

**Protein-DNA Binding Assay—**Binding of Dda to DNA was analyzed by fluorescence polarization using a Beacon fluorescence polarization spectrophotometer (PanVera). A 30-mer oligonucleotide was labeled with fluorescein on the 5’ end during DNA synthesis (IDT). The 30-mer and a complementary 15-mer were purified by denaturing polyacrylamide gel electrophoresis (Fig. 4 for sequence). A 12:1 ratio of the 30-mer and 15-mer, respectively, was heated to 95 °C, then slow-cooled to form a 30:15-mer partial duplex, which was purified by native polyacrylamide gel electrophoresis. Varying amounts of Dda or K38A Dda were added to a 1-ml aliquot of binding buffer (25 mM Hepes (pH 7.5), 0.1 mM EDTA, 150 mM KAc, 0.1 mg/ml bovine serum albumin, and 2 mM BME) containing 1 mM of the fluorescein-labeled 30:15-mer. Each sample was allowed to equilibrate in solution for 5 min, after which fluorescence polarization was measured. A second reading was taken after 30 min, in order to ensure that the mixture had equilibrated. Less than 5% change was observed between the 30-min measurement and the 5-min measurement indicating that equilibrium was reached. ATPohy (0.7 mM) and Mg(OAc)_{2} (10 mM) were then added to the samples, and polarization was measured after 5 min and again after 30 min. Measurements varied by less than 5% between the 30-min and 5-min measurements. The equilibrium dissociation constant was determined by plotting polarization as a function of protein concentration and fitting the data to the equation for a hyperbola by using the program KaleidaGraph (Synergy Software).

**Oligonucleotides—**ATPohy (0.7 mM) was determined using a coupled spectrophotometric assay (24). The reaction mixture contained 25 mM Hepes (pH 7.5), 150 mM KOAc, 10 mM Mg(OAc)_{2}, 5 mM ATP, 4 mM phosphoenolpyruvate, 21.6 units/ml phosphoenolpyruvate kinase, 33.2 units/ml lactate dehydrogenase, 0.9 mM NADH, 2 mM BME, and ssDNA. The source for ssDNA was either poly(dT) or denatured salmon testes (st) DNA. Final volume for each reaction mixture was 700 μl. A 30-mer oligonucleotide was labeled by fluorescence polarization using a Beacon fluorescence polarization spectrophotometer (PanVera). Oligonucleotides were visualized by UV shadowing. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. Functional Monomeric Form of Bacteriophage T4 Dda Helicase

**Protein-Protein Cross-linking—**DSP was dissolved in Me_{2}SO up to concentration of 100 and 50 mM. DSP (3 μM, stored in the absence of BME) was incubated in buffer (25 mM Hepes, pH 8.2) for 5 min prior to addition of other components such as 10 mM Mg(OAc)_{2}, 1.1 μM 30-mer oligonucleotide, or 2 mM ATP. After addition of the other components, incubation continued for 1 min after which the cross-linking agent (2 or 4 mM DSP) was added to the mixture. The cross-linking reaction proceeded for 3 min followed by addition of glycine (1.0 M) to quench the reaction. Samples were then analyzed by SDS-PAGE on a 4–20% pre-cast acrylamide gel (Jule). DNA was visualized by fluorescence of the acrylamide gel by electrophoresis by using an Elutrap apparatus (Schleicher & Schuell). Eluted DNA was loaded onto a Waters C_{18} Sep-Pak cartridge, de-salted with H_{2}O, then eluted with 60% methanol. DNA was dried in a Speed Vac and then re-dissolved in 10 mM Hepes (pH 7.5) and 1 mM EDTA. DNA was quantified by UV absorbance at 260 nm in 0.2 M KOH, and the concentration was determined by using a calculated extinction coefficient of 59,010 cm^{-1} M^{-1}.

**High Pressure Gel Filtration Chromatography—**The oligomeric nature of Dda was examined by high pressure gel filtration chromatography by using a Bio-Select SEC 250–5 column (Bio-Rad). A flow cell was placed into an SLM Amino-Bowman fluorescence spectrometer so that elution from the column could be monitored by fluorescence of the K38A Dda. An excitation length of 280 nm and an emission wavelength of 340 nm. The proteins that were used to prepare a calibration curve were: thyroglobulin (bovine), 670 kDa; γ-globulin (bovine), 443 kDa; apoferritin, 158 kDa; bovine serum albumin, 66 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa. Gel filtration chromatography was performed using a standard elution buffer (50 mM Tris-Cl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA) with 1 mM ATP and 1 mM Mg(OAc)_{2}. Protein concentration was measured by UV absorbance at 280 nm. A calculated extinction coefficient (59,010 cm^{-1} M^{-1}) based on the amino acid sequence was used to quantify the concentration of Dda in solution.

**Functional Monomeric Form of Bacteriophage T4 Dda Helicase**
the receiving vial at a concentration 30-fold above that of the substrate. An aliquot (20 μl) of each sample was mixed with gel loading buffer (0.1% xylene cyanol, 0.1% bromphenol blue, and 10% glycerol) for analysis by native 15% polyacrylamide gel electrophoresis. The fraction of duplex substrate and ssDNA product in each sample was determined using a Molecular Dynamics 445-SI PhosphorImager with ImageQuant software. The fraction of product was determined as described (25).

**DNA Unwinding under Conditions of Excess DNA Substrate**—20 pmol of freshly radiolaabeled strand (30-mer) was mixed with 20 pmol of displaced strand (15-mer), passed through two Sephadex G-25 spin columns, and heated to 95 °C, followed by slow cooling to room temperature. The radiolaabeled substrate was added to a concentrated stock solution (10 μM) of unlabeled 30:15-mer. The resulting solution was used for steady-state unwinding experiments. All concentrations are after initiating the reaction. WT Dda (5 nM) and various concentrations of mutant Dda ranging from 10 to 40 nM, were incubated for 2 min at 25 °C in unwinding buffer plus an ATP regenerating system which was made up of 4 mM phosphoenolpyruvate, 21.6 units/ml phosphoenolpyruvate kinase, and 33.2 units/ml lactate dehydrogenase. 400 nM 30-mer oligonucleotide was 1.1 μM. Samples were analyzed by SDS-PAGE on a 4-20% gradient pre-cast gel (Jule, Inc.), and visualized by silver staining.

**Results**

**Protein-Protein Cross-linking**—The oligomeric nature of many different helicases has been studied using protein-protein cross-linking. In many cases, ligands such as ATP, Mg2+, or DNA induce oligomerization. In the presence of a variety of ligands, chemical cross-linking with DSP revealed only a small amount of dimeric protein under all conditions tested (Fig. 1). Longer reaction times gave similar results, as did other cross-linking agents such as dimethyl suberimidate or bis(sulfosuccinimidylsuberate) (data not shown). Under similar conditions in the presence of ATP, gp41 helicase is readily cross-linked by DSP, giving rise to hexamers and larger species (10, 18). The results from cross-linking of Dda may be due to nonspecific protein-protein interactions of a monomeric species or may indicate weak, protein-protein interactions.

**High Pressure Gel Filtration Chromatography**—A Bio-Select column (Bio-Rad) was utilized for gel filtration chromatography studies. Dda elutes from this column corresponding to a molecular mass of ~28 kDa, although the actual molecular mass of Dda is ~50 kDa (Fig. 2). The late elution time indicates that Dda may interact with the column material. Alternatively, the molecular shape of Dda may allow the protein to occupy smaller volumes within the column matrix than would be expected for a 50-kDa protein. In either case, the results from these studies are inconclusive because of the unusual retention time of Dda. Other groups have reported the unusual behavior of Dda on gel filtration columns (19).

**Dda Does Not Exhibit Concentration-dependent ATPase Activity**—The ssDNA-stimulated ATPase activity of several helicases has been studied as a function of enzyme concentration. In the case of UvrD and NS3h (24, 27), an increase in specific activity at increasing protein concentration provided evidence for oligomerization. Dda was incubated in assay buffer along with 20 μM poly(dt) (nt), which is at least 10-fold greater than the apparent Kd, which is ~1-2 μM (17). Little change in the specific activity for ATP hydrolysis was observed from 4 to 100 nM Dda (Fig. 3). The lack of a change does not preclude formation of oligomeric species, it simply indicates that if Dda self-assembles, the monomeric units exhibit ATPase activity that is independent of one another under these conditions.

**The ATPase-deficient Mutant Dda (K38A) Is Capable of Binding DNA**—One of the conserved sequences among all members of the various helicase families is a nucleotide-binding loop of the amino acid sequence (G/A)XXGXXG(K/T) (11). Mutations in this region have been made in several helicases to create ATPase-deficient mutants that were incapable of unwinding duplex DNA substrates, but were still able to form oligomers and/or bind to ssDNA. For example, the first X residue of the conserved sequence was mutated from arginine to cysteine in the E. coli DnaB helicase (28), and the lysine residue was mutated to alanine in the T7 bacteriophage gene 4A helicase (29). In Dda, we have used site-directed mutagenesis to change the DNA sequence corresponding to amino acid 38 from AAG to GCG, which converts the lysine residue in the
sequence GPAGTGTKT to an alanine (Fig. 4A). A series of experiments have been performed to determine the effect of this K38A Dda mutant protein on the activity of WT Dda.

Equilibrium binding assays were performed to ensure that the K38A mutation did not compromise the ability of the mutant Dda to bind DNA. A 30:15-mer oligonucleotide (Fig. 4B) containing a fluorescein label on the 5'-end of the 30-mer was titrated with either WT Dda or K38A Dda, and the resulting change in fluorescence polarization was measured (Fig. 4C). The WT enzyme bound to the 30:15-mer with a KD of 31 ± 1 nM, whereas K38A Dda had a KD of 14 ± 1 nM (Fig. 4C). In the presence of 0.7 mM ATP and 10 mM MgOAc, WT Dda bound with a KD of 318 ± 26 nM and the mutant Dda bound with a KD of 107 ± 6 nM (Fig. 4C). Thus, the single mutation resulted in only a small affect on the affinity of K38A Dda for the 30:15-mer relative to WT Dda.

The Presence of Mutant Dda Does Not Decrease the Rate of ATP Hydrolysis by WT Dda—After generating the Dda mutant, its effect on WT Dda ATP hydrolysis activity was studied using the coupled spectrophotometric ATPase assay. 20 nM WT Dda did not hydrolyze ATP to a measurable extent in the absence of stDNA, but had a hydrolysis rate of 1652 nM s⁻¹ in the presence of 0.2 mM stDNA (concentration in nucleotides). 20 nM K38A Dda was incapable of hydrolyzing ATP in the absence of presence of stDNA (Fig. 5A). Various ratios of WT Dda and K38A Dda protein were mixed to determine the effect of the mutant on WT Dda ATP hydrolysis. In each experiment, the total protein concentration (WT + K38A) was held constant at 20 nM. The ATPase activity of 10 nM WT Dda is shown in Fig. 5A. 10 nM WT Dda hydrolyzed ATP at a rate of 705 nM s⁻¹ in the presence of stDNA, while in the absence of ATP no ADP hydrolysis was observed. In the presence of 10 nM K38A Dda, 10 nM WT Dda hydrolyzed ATP at a rate of 28 nM s⁻¹. Thus, addition of the K38A Dda did not substantially change the rate of ATP hydrolysis by the WT enzyme (Fig. 5B). This outcome supports the previous result (Fig. 3) and suggests that, if Dda forms oligomers, the ATPase activity of each subunit is independent of oligomerization.

Unwinding by Dda in the Presence of K38A Dda under Conditions of Excess Enzyme—Unwinding experiments were performed using a Kintel rapid chemical quench-flow apparatus to investigate the effect of K38A Dda on DNA unwinding by WT Dda. A 30:15-mer substrate (2 nM) was incubated with 250 nM WT Dda and varying concentrations of K38A Dda. The unwinding reaction was initiated by addition of 5 mM ATP, 10 mM Mg(OAc)₂, and 5 μM oligo(dT)₁₅. At varying times, the reaction was quenched by addition of 400 mM EDTA. Samples were analyzed by native polyacrylamide gel electrophoresis (Molecular Dynamics). Under these conditions, helicase that dissociates from the substrate is trapped by the oligo(dT)₁₅, thereby allowing only the first cycle of unwinding to be observed during the short time frame. The fraction of substrate unwound during the single cycle is shown over time (Fig. 6B). Dda unwound the substrate with a pseudo-first-order rate of 24 ± 5 s⁻¹ and an amplitude of 0.44 ± 0.04 (Fig. 6B), whereas K38A Dda did not unwind the duplex (data not shown).

Dda and varying amounts of K38A Dda were incubated in reaction buffer for 3 min, followed by incubation with substrate for an additional 5 min. The unwinding reaction was initiated upon addition of 5 mM ATP, 10 mM Mg(OAc)₂, and 5 μM oligo(dT)₁₅. Under single cycle conditions, a change in the rate of unwinding would be expected if hetero-oligomers were capable of unwinding the substrate at rates different than the WT enzyme. The rate of unwinding of substrate by 250 nM WT Dda in the presence of varying concentrations of K38 A Dda was similar to that in the presence of only WT Dda (Fig. 7A). The fact that no trend in the rates for unwinding was observed suggests that hetero-oligomers do not participate in the unwinding process. However, the amplitude for product formation is reduced with the addition of the mutant helicase (Fig. 6B). This result is expected if the mutant enzyme competes for binding to the substrate, but the WT Dda that remains productively bound is capable of unwinding the substrate in a single cycle.

The approximate reduction in the amplitude of product formation in the presence of mutant Dda can be analyzed assuming a simple competition for binding to substrate between WT Dda and K38A Dda. The amplitudes were plotted as a function of the concentration of mutant Dda (Fig. 7B). For a simple competition, the quantity of productively bound WT Dda can be calculated according to Equation 1 (27).

\[
A = A_{WT} \times \frac{[E_{WT}]K_{WT}^{MT}}{[E_{WT}]K_{WT}^{MT} + [E_{MUT}]K_{MUT}^{WT}} \tag{Eq. 1}
\]

A is the amplitude for unwinding by helicase under single cycle conditions. \(A_{WT}\) is the amplitude of unwinding in the presence of WT Dda only (0.44, Fig. 6B). \([E_{WT}]\) and \([E_{MUT}]\) are the concentrations of WT and mutant helicase, respectively. \(K_{WT}^{MT}\) is the equilibrium binding constant between WT Dda and 30:15-mer (31 nM, Fig. 4C). \(K_{MUT}^{WT}\) is the equilibrium binding constants between K38A Dda and 30:15-mer (14 nM, Fig. 4C). If Dda functions as a monomer, then the value for \(n\) will be 1, whereas if Dda functions as an oligomer, then \(n\) will be 2 or greater (27). The best fit of the data is shown, and provides a value for \(n\) of 1.3 ± 0.05 (Fig. 7B). For comparison, the dashed line below the data points in Fig. 7B shows the expected reduction in amplitude if Dda functions as a dimer, whereas the dotted line above the data points represents the expected reduction in amplitude if Dda functions as a monomer. Clearly, the data fall between these expected values. However, the difference of only 2-fold between the expected results for a monomer or dimer makes the observed data difficult to interpret for Dda. For comparison, the rate and amplitude for unwinding was dramatically reduced in an experiment reported
for the NS3 helicase domain (27).

Unwinding by Dda in the Presence of K38A Dda under Steady-state Conditions—It is possible that more than one Dda monomer can bind to the substrate under conditions of excess enzyme reported in Fig. 6. In this case, the presence of K38A Dda might lead to a reduction in unwinding due to steric interactions between Dda and K38A Dda, rather than specific protein-protein interactions. To avoid this possibility, unwinding reactions were performed in the presence of excess substrate, in order to greatly reduce the likelihood that two proteins would bind to the same substrate molecule, unless specific, protein-protein interactions were involved.

Dda was incubated with 30:15-mer and the unwinding reaction was initiated upon the addition of 5 mM ATP and 10 mM Mg(OAc)$_2$. A 15-mer oligonucleotide that was complementary to the displaced strand of the 30:15-mer substrate was introduced along with the ATP to prevent reannealing of products. Observed unwinding rates increased proportionally with 2.5, 5, and 7.5 nM Dda (Fig. 8A). Although the trapping strand is likely to sequester Dda, the observed rates of strand separation reflect the enzyme’s activity. Therefore, if K38A Dda binds to WT Dda and lowers the effective activity of the WT enzyme, then the observed rate for strand separation should reflect this occurrence. Dda (5 nM) was incubated with varying concentrations of K38A Dda, ranging from 0 to 40 nM for 2 min, followed by incubation with 30:15-mer (400 nM) for an additional 5 min. The reaction was initiated upon addition of ATP and Mg(OAc)$_2$. The substrate was unwound with a rate of 1.42 nM zM$^{-1}$s$^{-1}$ in the presence of 5 nM WT Dda (Fig. 8B). A comparison of unwinding rates at each concentration of K38A Dda is shown in Fig. 8C. The rates of unwinding by 5 nM WT Dda in the presence of 10, 20, and 40 nM K38A Dda were 1.42, 1.68, and 1.66 nM zM$^{-1}$s$^{-1}$, respectively. Thus, the presence of K38A Dda at concentrations 8-fold higher than that of the WT enzyme, did not decrease the observed rate of formation of ssDNA. Additionally, 5 nM WT Dda alone or in the presence of 40 nM K38A Dda was incubated...
with 5 mM ATP and the reaction was initiated by addition of 400 nM 30:15-mer. Under these conditions WT Dda alone unwound the substrate at a rate of 1.35 nM s⁻¹, and WT Dda plus K38A Dda unwound the substrate at a rate of 1.46 nM s⁻¹ (Fig. 8C). Thus, no decrease in the rate of unwinding was observed under a variety of conditions when competition for substrate was greatly reduced. These results suggest that protein-protein interactions are not required for unwinding to occur.

**DISCUSSION**

The search for a functional oligomeric structure of Dda has proven elusive. Experimental approaches such as chemical cross-linking and gel filtration did not strongly support nor eliminate formation of oligomeric species (Figs. 1 and 2). Biochemical assays in which the ATPase activity of Dda was measured at varying enzyme concentration did not provide any evidence for oligomerization (Fig. 3). Hence, methods that have provided support for oligomerization of other helicases gave negative results with Dda. Transient, protein-protein interactions that might be required for unwinding activity were not eliminated by these experiments.

To further investigate this, an ATPase-deficient mutant enzyme was prepared that was unable to unwind DNA. If oligomerization were required for function, then the mutant enzyme would be expected to lower the activity of the wild type due to formation of hetero-oligomers. The effects of an ATPase-deficient mutant Dda protein (K38A Dda) on the activities of WT Dda were examined. The presence of K38A Dda protein, which is inactive as a helicase, but is still capable of binding DNA (Fig. 4), fails to decrease the ATPase activity of WT Dda (Fig. 5). The rate of unwinding under single cycle conditions was not reduced by the addition of the mutant enzyme, indicating that hetero-oligomers do not likely participate in the unwinding reaction. The amplitude for unwinding by WT Dda helicase was reduced upon addition of K38A Dda (Fig. 6). The data fall between that expected for a monomeric or dimeric helicase when analyzed as though a simple competition for substrate binding existed between wild type Dda and mutant or hetero-oligomeric Dda (Fig. 7B). It is possible that more than one Dda monomer can bind to the substrate without invoking protein-protein interactions, which may lead to a greater than expected reduction in amplitude under conditions of excess enzyme and in the presence of the inactive K38A Dda.

The effect of mixing K38A Dda and WT Dda was further investigated by conducting unwinding experiments in the presence of excess substrate. Under these conditions, the competition for DNA binding between the two proteins is effectively eliminated, because the protein will be distributed among the substrate molecules. If Dda is acting as a monomer, the addition of mutant protein should have no effect on WT Dda unwinding activity under these conditions because the mutant protein will bind to DNA, but will not prevent WT Dda binding and subsequent unwinding activity. However, if protein-pro
tein interactions are required for unwinding, then such interactions should occur, despite the presence of excess DNA substrate. The results indicate that the presence of mutant Dda does not reduce the rate of unwinding (Fig. 8C), suggesting that Dda may not require oligomerization in order to function. Even when WT Dda alone or in the presence of K38A Dda is incubated with ATP prior to initiating the reaction with substrate, there is no reduction in the unwinding rate (Fig. 8C). Thus, Dda appears to be capable of functioning as a monomer. This may explain the fact that Dda is known to act in a distributive manner, cycling on and off of DNA during unwinding of long substrates (19). Perhaps this distributive mode of action is the result of a monomeric structure that does not allow Dda to encircle ssDNA in a manner similar to that of many hexameric helicases (4, 18, 22).

In order to unwind a region of dsDNA, and translocate to a new site along the nucleic acid, helicases need two DNA binding sites so that the enzyme does not dissociate during unwinding. Therefore, Dda is expected to contain more than one site that is capable of binding to DNA. An inchworm mechanism has been suggested for unwinding and translocation by monomeric helicases. Evidence for multiple DNA binding sites on a monomeric helicase has been provided in the case of the PcrA helicase. The crystal structure of PcrA in the presence of ssDNA and an adjacent binding site for dsDNA (7). Dda is classified as a super family 1 helicase, like PcrA, based on sequence comparison (11), although Dda is a 5’-to-3’ helicase (17), whereas PcrA translocates 3’-to-5’ (30). Another possible arrangement for “two” binding sites has been proposed for the NS3h helicase. The co-crystal structure of this enzyme in the presence of ssDNA indicates a binding site for ssDNA and an adjacent binding site for dsDNA (7). Dda is classified as a super family 1 helicase, like PcrA, based on sequence comparison (11), although Dda is a 5’-to-3’ helicase (17), whereas PcrA translocates 3’-to-5’ (30).

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In order to unwind a region of dsDNA, and translocate to a new site along the nucleic acid, helicases need two DNA binding sites so that the enzyme does not dissociate during unwinding. Therefore, Dda is expected to contain more than one site that is capable of binding to DNA. An inchworm mechanism has been suggested for unwinding and translocation by monomeric helicases. Evidence for multiple DNA binding sites on a monomeric helicase has been provided in the case of the PcrA helicase. The crystal structure of PcrA in the presence of a partial duplex DNA substrate indicates a binding site for ssDNA and an adjacent binding site for dsDNA (7). Dda is classified as a super family 1 helicase, like PcrA, based on sequence comparison (11), although Dda is a 5’-to-3’ helicase (17), whereas PcrA translocates 3’-to-5’ (30). Another possible arrangement for “two” binding sites has been proposed for the NS3h helicase. The co-crystal structure of this enzyme in the presence of ssDNA indicates a binding site for ssDNA and an adjacent binding site for dsDNA (7). Dda is classified as a super family 1 helicase, like PcrA, based on sequence comparison (11), although Dda is a 5’-to-3’ helicase (17), whereas PcrA translocates 3’-to-5’ (30).

Helicases in superfamily 1 and superfamily 2 have been difficult to characterize in terms of oligomeric structure, and much discussion has surrounded this issue. This is due to the fact that many biophysical approaches for studying oligomerization provide negative results when applied to these enzymes. Evidence has been presented that suggests that PcrA (7), UvrD (8), and NS3h (32) can function as monomers. An-
other helicase, PriA, was shown to exist as a monomer in solution, even when bound to ssDNA, although the functional form of the enzyme has not been determined (33). Others have provided evidence that suggests that oligomeric forms of UvrD (34) and NS3h (27) are required for optimal unwinding activity.

Previously, the ability of Dda to displace streptavidin from the 3' end of biotin-labeled oligonucleotides was found to exhibit a dependence on the length of the oligonucleotide. The streptavidin displacement reaction was found to proceed faster from longer oligonucleotides than from shorter ones (18). The results described here suggest that a monomeric form of Dda can function to unwind DNA. However, individual Dda monomers may align along the nucleic acid lattice to enhance overall activity in the streptavidin displacement assays, despite the lack of strong protein-protein interactions.

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