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A fluorescence-based assay for monitoring helicase activity

(dda helicase/stopped-flow spectroscopy/2-aminopurine)

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ABSTRACT **A continuous fluorescence-based assay is described for measuring helicase-mediated unwinding of duplex DNA. The assay utilizes an oligonucleotide substrate containing the fluorescent adenine analog, 2-aminopurine, at regular intervals. 2-Aminopurine forms a Watson–Crick-type base pair with thymine and does not distort normal B-form DNA. Fluorescence of the 2-aminopurines within this oligonucleotide is quenched 2-fold upon its hybridization to a complementary strand. Unwinding of this substrate by the T4 dda helicase restores the fluorescence of the 2-aminopurines and is easily followed using stopped-flow or steady-state fluorescence spectroscopy. The fluorescence-based assay provides rate data comparable to that obtained from conventional discontinuous assays using labeled substrates and additionally furnishes a means for following a single turnover. This assay should prove useful for defining the mechanism by which helicases unwind duplex DNA.**

Many cellular processes involving DNA such as replication, repair, and recombination utilize single-strand (ss) DNA intermediates derived from unwinding of double-strand DNA. Helicases are the enzymes that perform this function, presumably using the energy of nucleotide hydrolysis for breaking duplex hydrogen bonds (1). Much effort has recently been invested in determining the mechanism by which helicases carry out this function (2, 3). Lohman and coworkers (2, 4, 5) have provided strong evidence for a rolling mechanism for the *Escherichia coli* Rep helicase, which appears to function as a dimer (2–5). They propose that each monomer, initially bound to ssDNA, alternates in binding to the duplex region at the DNA fork. Hydrolysis of ATP provides energy to unpair the bound duplex giving rise to a new region of ssDNA. Their model predicts that the base pairs bound by the helicase are unwound simultaneously. von Hippel and coworkers (3, 6, 7) have provided a mechanism for unwinding by the *E. coli* transcription termination factor, Rho, which is a DNA·RNA helicase. This enzyme appears to function as a hexamer, and translocation along ssRNA is fueled by ATP hydrolysis that induces conformational changes in the enzyme and allows unidirectional movement along the bound RNA strand. This model does not invoke interactions between the enzyme and duplex region and suggests, instead, that the helicase movement might "unzipper" the DNA·RNA hybrid (3, 6, 7).

The standard assay for measuring helicase activity uses gel electrophoresis to observe unwinding of double-strand DNA by separating substrate (duplex strands) from product (single strands) on a native polyacrylamide or agarose gel (1). This assay is discontinuous and only measures substrate that has been completely unwound. It is not suitable for pre-steady-state analysis in the sense that unwinding cannot be observed prior to complete unwinding of the substrate. Thus, the models proposed for helicase-mediated unwinding of duplex DNA are difficult to test using the traditional assay.

We describe here, an assay that should prove valuable in probing helicase activity. The adenine analog, 2-aminopurine (2-AP), has fluorescent properties useful for studying DNA dynamics (8, 9) and also can form a Watson–Crick-type base pair, thus, maintaining the overall structural integrity of duplex DNA (10). Fluorescence of 2-AP has been used to measure the rate of nucleotide incorporation (11) and to monitor local melting of duplex termini (12) by the Klenow fragment of DNA polymerase I. We have prepared an oligonucleotide containing seven regularly spaced 2-AP residues that upon hybridization to a complementary oligonucleotide, quenches the 2-AP fluorescence 2-fold. This oligonucleotide has been used as a substrate for studying DNA unwinding by the T4 dda helicase, taking advantage of the restoration in fluorescence upon going from duplex DNA to ssDNA. We believe this is an informative technique for studying the mechanism by which helicases unwind DNA.

MATERIALS AND METHODS

dda Protein. The dda helicase clone (pKHdda in *E. coli* SG934) was kindly provided by Kevin Hacker and Bruce Alberts (University of California, San Francisco). The protein was purified by the described procedure (13) with one exception. The DNase I treatment was eliminated and additional sonication was applied to fully disrupt the cell mass. Protein concentration was determined by UV absorbance in 6 M urea and the extinction coefficient calculated from the reported amino acid sequence (ε_{280} = 59,060 $M^{-1}\cdot cm^{-1}$).

Oligonucleotides. Oligonucleotides were synthesized using the phosphoramidite method. Preparation of the 2-aminopurine 2′-deoxynucleoside phosphoramidite will be described elsewhere. Oligonucleotides were purified by preparative gel electrophoresis as described (14). Purified oligonucleotides were quantitated by using UV absorbance at 260 nm in 0.1 M NaOH and calculated extinction coefficients. The extinction coefficient for 2-AP at 260 nm is 1000 $M^{-1}\cdot cm^{-1}$ (15) and thus contributes very little to the overall absorbance of the oligonucleotide.

Fluorescence Titration. Experiments were performed using an SLM Aminco 8000 spectrofluorometer. The oligonucleotide containing the 2-APs (250 nM, referred to as 2-AP leading strand; Fig. 1*B*) was added to helicase unwinding buffer (1 ml) in a temperature-controlled cuvette at 25°C. Helicase unwinding buffer contained 25 mM Tris acetate (pH 7.4), 10 mM potassium acetate, 10 mM magnesium acetate, 3 mM ATP, 4 mM phospho*enol*pyruvate, pyruvate kinase (10 units/ml), 1 mM 2-mercaptoethanol, and bovine serum albumin (0.1 mg/ml). The partially complimentary oligonucleotide (referred to as the lagging strand in Fig. 1*B*) was titrated into the solution in 50-pmol aliquots. The two strands were allowed to anneal

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Abbreviations: 2-AP, 2-aminopurine, ss, single strand.

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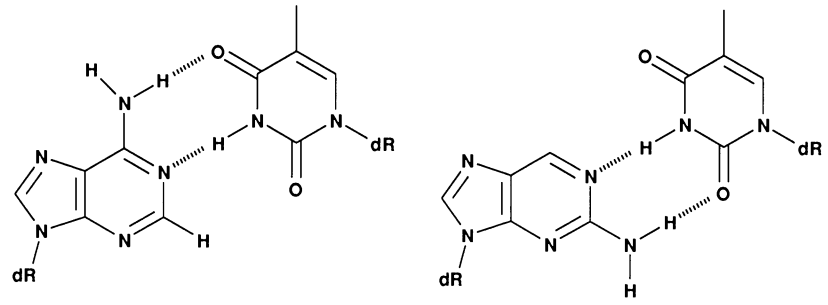
A



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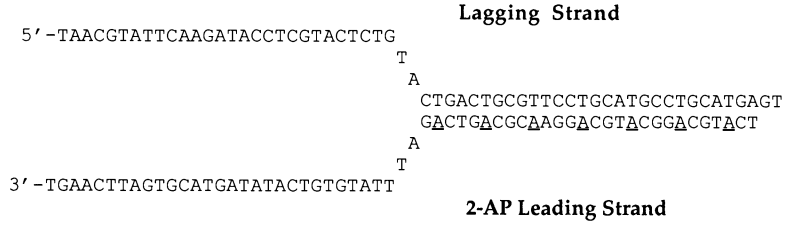


FIG. 1. (*A*) Base-pairing scheme for 2-aminopurine 2′-deoxynucleoside with thymidine. Normal base pairing for deoxyadenosine and thymidine are also shown. The overall structure of B-form DNA is maintained with 2-AP. (*B*) Oligonucleotide fork containing seven 2-AP residues (underlined) regularly spaced in the duplex region. The nomenclature of the two strands of the DNA fork is based on their roles in DNA replication.

for ≈5 min before fluorescence spectra were obtained. The excitation wavelength was set at 310 nm.

DNA Unwinding Measured by Gel Electrophoresis. The leading strand (10 pmol) of the DNA fork was 5′-end-labeled with ^{32}P. The labeled 2-AP leading strand was annealed to an equivalent amount of lagging strand and the labeled 2-AP DNA fork was then added to a stock solution of unlabeled 2-AP DNA fork. A second leading strand identical to the 2-AP strand except containing adenine instead of 2-AP was also end-labeled, annealed to lagging strand, and added to a stock solution of unlabeled DNA fork substrate. Either of the two DNA substrates was added to helicase assay buffer up to a final concentration of 250 nM. Dda helicase was added to the reaction mixture at levels up to 50 nM and incubated for 3 min at 25°C. The unwinding reaction was initiated by adding 10 μl of a solution of ATP and MgOAc to give final concentrations of 3 mM and 10 mM, respectively. Total volume for the reaction mixture was 60 μl. Aliquots (10 μl) of the unwinding reaction mixture were taken at 10-s intervals and added to 10 μl of quench solution. Quench solution contained EDTA (0.5 M) and a 30-mer oligonucleotide (10 μM) that was complimentary to the duplex region of the lagging strand. The 30-mer served as a trap to prevent reannealing of the unpaired strands after the reaction was quenched. Final trap concentration in quenched samples was 5 μM (40-fold in excess of substrate concentration). Nondenaturing load buffer (5 μl) consisting of 70% (vol/vol) glycerol was added to each solution and samples were analyzed by electrophoresis on a native 15% polyacrylamide gel. Gel images were obtained and analyzed using a Molecular Dynamics PhosphorImager. The quantity of ssDNA product was determined by comparing the relative amounts of ssDNA and duplex DNA and correcting for trap efficiency and ssDNA in control samples.

DNA Unwinding Measured by Fluorescence. Experiments were carried out using a stopped-flow spectrometer from Applied Photophysics (Leatherhead, Surrey, U.K.). Dda helicase was incubated with the 2-AP DNA fork in helicase unwinding buffer at 25°C in one syringe of the instrument, and ATP and MgOAc were incubated in the second syringe. The unwinding reaction was initiated by rapid mixing of the two solutions. Concentrations after mixing were as follows: 50 nM dda helicase, 250 nM 2-AP DNA fork, 3 mM ATP, and 10 mM MgOAc. Excitation wavelength was set at 310 nm with a bandpass of 30 nm. A 330-nm cutoff filter was used for observing fluorescence emission of 2-AP. Kinetic simulations were performed using the KINSIM program as described (14).

RESULTS

Fluorescence of the 2-AP DNA Strand Is Quenched Upon Annealing to a Complementary Strand. The adenosine analog, 2-aminopurine 2′-deoxyribonucleoside, can form a Watson–Crick-type base pair with thymidine (Fig. 1*A*) and maintain the overall structure of duplex DNA (10). An oligonucleotide was prepared in which seven 2-AP residues were incorporated at regular intervals by using phosphoramidite DNA synthesis methods (Fig. 1*B*, 2-AP leading strand). A partially complementary oligonucleotide (Fig. 1*B*, lagging strand) was also prepared, and upon annealing to the 2-AP leading strand, a 2-AP DNA fork species was formed (Fig. 1*B*).

Fluorescence titration of the 2-AP leading strand with the lagging strand was carried out to illustrate the change in fluorescence of the 2-AP residues upon going from a ssDNA environment to a duplex DNA environment (Fig. 2). The 2-AP fluorescence was quenched ≈2-fold upon hybridization to the partially complementary strand. Addition of 50 nM dda helicase to the 2-AP substrate (250 nM) changed the fluorescence by <5% (data not shown).

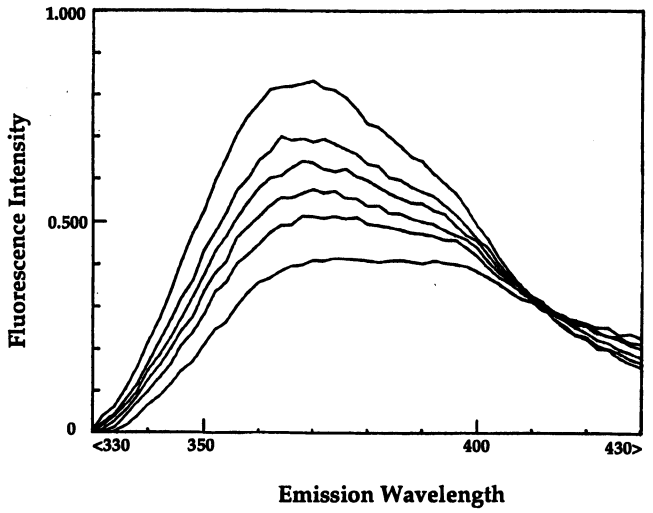


FIG. 2. Fluorescence titration of 2-AP leading strand (250 nM) with 50 nM aliquots of the partially complementary lagging strand. Fluorescence is quenched ≈2-fold upon hybridization of the two strands. Excitation wavelength was set at 310 nm.

2-AP Does Not Inhibit dda Helicase Activity. Although 2-AP forms a Watson–Crick-type base pair, we carried out unwinding experiments with an identical substrate in which adenine was substituted for 2-AP to determine whether the 2-AP inhibits T4 dda helicase activity. DNA fork substrates (250 nM) were incubated with T4 dda helicase (50 nM) in helicase unwinding buffer and the reaction was initiated by adding ATP and MgOAc up to final concentrations of 3 mM and 10 mM, respectively. Reactions were quenched using 0.5 M EDTA and an oligonucleotide trapping strand was added at the same time to prevent reannealing of ssDNA product. Products were separated by gel electrophoresis (Fig. 3*A*) and bands were visualized and quantitated using a Molecular Dynamics PhosphorImager. Unwinding was identical for each of the DNA fork substrates under the conditions of the assay (Fig. 3*B*).

The dda helicase was inhibited by DNA adducts covalently bound to the lagging strand, which is the strand to which the dda helicase binds (16). The 2-AP residues are located in the leading strand in the substrate described here, and it remains to be determined whether or not placement of the 2-AP residues in the lagging strand would inhibit the helicase. Other helicases have been shown to primarily recognize the DNA backbone (17), and therefore, 2-AP may not affect unwinding activity regardless of the strand in which it is placed.

DNA Unwinding Followed by Fluorescence. Stopped-flow fluorescence spectroscopy was used to determine whether helicase activity could be followed by fluorescence. The unwinding reaction was carried out under conditions identical to the gel electrophoresis assay and followed by observing the change in fluorescence of the 2-AP DNA fork substrate. DNA fork and dda helicase were incubated in one syringe in helicase assay buffer while ATP and MgOAc were placed in the second syringe. Rapid mixing led to initiation of the unwinding reaction. The progress of the reaction was clearly followed by observing the increase in fluorescence of the substrate, measured as a decrease in signal voltage by the instrument (Fig. 4). To compare the two methods for measuring helicase unwinding, the fluorescence data were converted to DNA concentration. A conversion factor was obtained using the stopped-flow spectrometer by observing spontaneous annealing of known concentrations of the 2-AP leading strand and the complementary lagging strand. The total fluorescence change obtained in this experiment corresponded to the concentration of oligonucleotide, thus giving the appropriate conversion factor (data not shown). Fluorescence data in Fig. 4 was converted to concentration and compared with the results from the gel electrophoresis experiment (Fig. 5). The results from the fluorescence assay

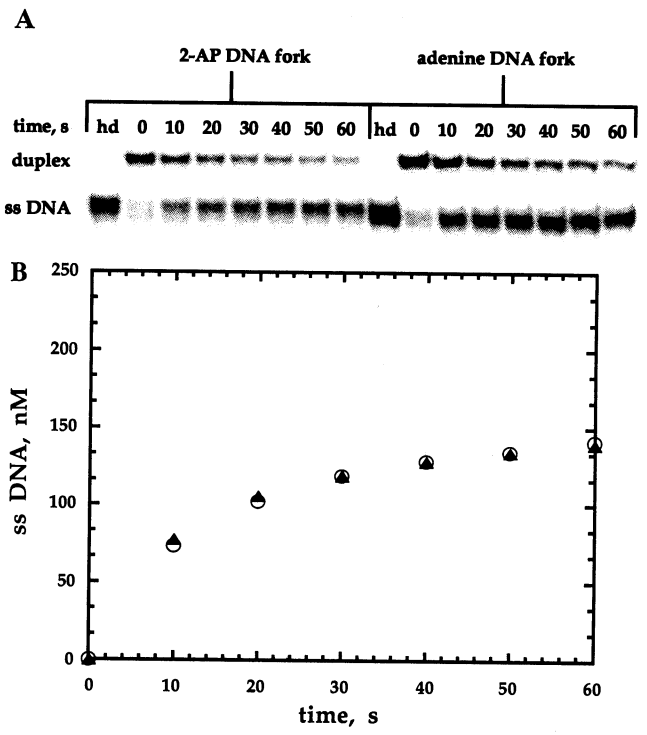


FIG. 3. DNA fork unwinding mediated by T4 dda helicase. dda helicase (50 nM) was incubated with 250 nM DNA fork, and unwinding was initiated by addition of ATP (3 mM) and MgOAc (10 mM). Reactions were stopped at various times by adding 0.5 M EDTA. A complementary 30-mer oligonucleotide was added along with the quench to prevent reannealing of product strands after the reaction. (*A*) Unwound strands due to helicase activity or heat denaturation (lane hd) were separated by gel electrophoresis using a native 15% polyacrylamide gel. DNA bands were analyzed using a Molecular Dynamics PhosphorImager. (*B*) The DNA fork containing 2-AP (▲) was unwound to the same degree as the DNA fork containing adenine (○), indicating that the 2-AP residues did not inhibit the dda helicase activity.

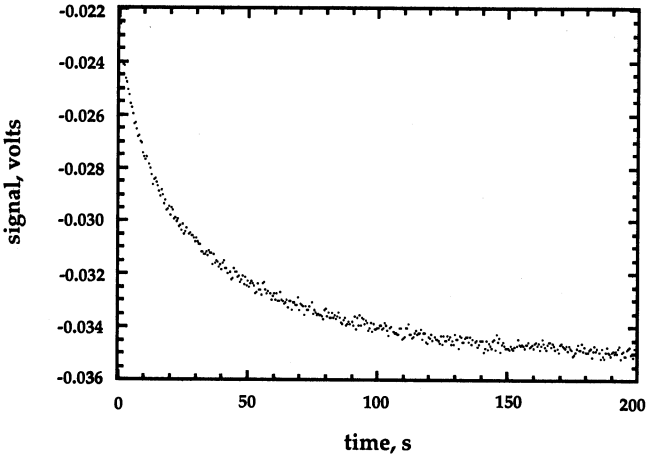


FIG. 4. DNA unwinding followed by fluorescence. Helicase-mediated unwinding of the 2-AP DNA fork was easily observable using stopped-flow fluorescence spectroscopy. Dda helicase (50 nM) was incubated with 2-AP DNA fork (250 nM) and the reaction was initiated by rapid mixing with ATP (3 mM) and MgOAc (10 mM). The increase in fluorescence is measured as a decrease in signal voltage.

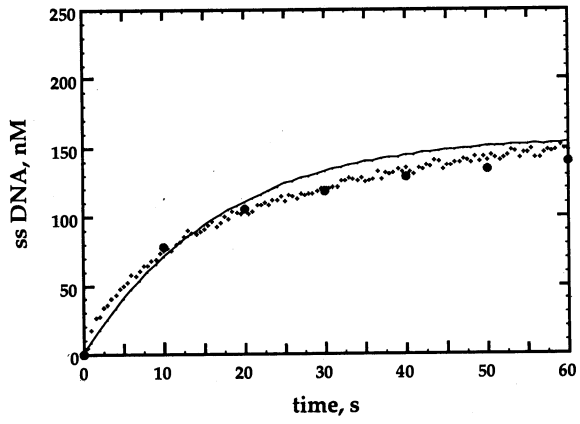
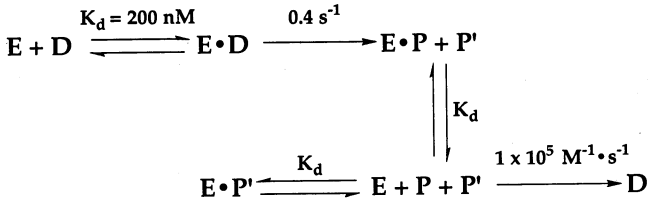


FIG. 5. Comparison of DNA unwinding as measured by fluorescence and gel electrophoresis. Fluorescence data (crosses) from Fig. 4 were converted to DNA concentration and plotted with data (circles) from Fig. 3. The fluorescence data correlated very closely with data from the gel electrophoresis experiment. The solid curve was generated by the HOPKINSIM program for the Macintosh by using the kinetic mechanism described in Scheme I.

match the data from the electrophoresis experiments very closely.

Kinetic Simulations of Unwinding Data. The unwinding reactions shown in Figs. 3 and 4 were carried out in the absence of a DNA trapping strand to prevent reannealing of product ssDNA. A trap and the quencher were added at the same time in the gel electrophoresis experiments to prevent reannealing after the reaction and during the electrophoresis analysis. Thus, reannealing did occur over the time frame of the unwinding reaction, and the observed unwinding curves in Figs. 3 and 4 represent a composite of several rates including helicase dissociation, duplex unwinding, and reannealing. A simple kinetic mechanism for the helicase reaction is shown in Scheme I. The first step in the mechanism



Scheme I

consists of association of the helicase, E, with the DNA fork substrate, D. This rate was assumed to be close to diffusion-controlled and the association rate constant was set at 1×10^8 $M^{-1} \cdot s^{-1}$. The dissociation rate of helicase from DNA was determined from the DNA dissociation constant (K_d), which was found to be 200 nM (K.D.R., unpublished observations), thus, giving a dissociation rate constant of 20 s^{-1}. The unwinding reaction is the next step represented in Scheme I. The helicase–substrate complex (E·D) forms products, P and P', where P is the lagging strand and P' is the leading strand. The helicase is shown bound to one of the single-strand products (E·P) after unwinding has occurred. The next step in the mechanism shows the product dissociating from the helicase (E·P forming E + P) in a step that is governed once again by the K_d.

The product strands P and P' can anneal over the time frame of the reaction to reform the DNA fork substrate, D, as mentioned above. The rate at which reannealing occurs was measured independently by following the UV absorbance change that occurs when the leading and lagging DNA strands hybridize. This rate constant was determined to be 1×10^5 $M^{-1} \cdot s^{-1}$ (data not shown). The rate for spontaneous dissociation of the 2-AP DNA fork and for annealing of DNA strands that were bound by the helicase were presumed to be negligible. Thus, the actual unwinding rate constant was the only remaining unknown in the kinetic mechanism that could contribute to the observed unwinding curve. This rate constant was varied to obtain the best fit to the actual data, and a value of 0.4 s^{-1} was obtained (Fig. 5).¶

DISCUSSION

The mechanism(s) by which helicase enzymes unwind duplex DNA has been the subject of intensive investigation (2–7). An important feature of any helicase mechanism is the means by which the energy of ATP hydrolysis is used, i.e., for breaking hydrogen bonds, translocating the enzyme along DNA, or both. Conventional helicase assays cannot observe base-pair melting in a continuous fashion and are thus inadequate. We have devised an assay that should be useful in addressing these questions. The DNA substrate described herein contains the fluorescent nucleotide analog, 2-aminopurine 2'-deoxyribonucleoside incorporated at regular intervals. This nucleoside maintains the overall topology of duplex DNA due to the Watson–Crick base-pairing capability of the 2-AP (Fig. 1). The fluorescence properties of 2-AP are sensitive to the surrounding environment (8, 9). Fluorescence of this oligonucleotide is quenched two-fold upon hybridizing to its complementary strand (Fig. 2), and this change serves as the basis for the continuous helicase assay. Comparison of the 2-AP DNA fork with one containing adenine confirms that the 2-AP residues do not inhibit the activity of the dda helicase (Fig. 3). Unwinding of the 2-AP DNA fork was easily monitored using stopped-flow fluorescence spectroscopy (Fig. 4). The fluorescence assay and the standard assay based on gel electrophoresis gave virtually identical results (Fig. 5), but the fluorescence assay was continuous and observable in real time.

The unwinding curve in Fig. 5 reaches a plateau that occurs prior to complete unwinding of the substrate. This plateau represents an equilibrium between unwinding of the substrate and reannealing of ssDNA product to reform duplex substrate. A working model for the kinetic mechanism of the dda helicase was devised to describe this unwinding curve (Scheme I) and kinetic simulations were used to define the actual unwinding rate constant, which cannot be extracted directly from the data. The unwinding rate constant determined from this approach was 0.4 s^{-1}, which corresponds to 12 base pairs per s (30 base pairs per substrate). The dda helicase was assumed to act as a monomer in this simulation; however, all helicases studied carefully to date actually adopt a multimeric form (1, 2). The dda helicase has a fast off-rate (20 s^{-1}) that prevents observation of a large burst of product due to the relatively slow unwinding rate (0.4 s^{-1}). Experiments in which the dda concentration was raised above the

¶Second-order rate constants determined for annealing of the 2-AP DNA fork, the adenine DNA fork, and a third DNA fork were 2×10^4, 1×10^4, and 1×10^5 $M^{-1} \cdot s^{-1}$, respectively. Sequence analysis indicated that the leading and lagging strands of the adenine fork (and presumably the 2-AP fork) had a significantly greater propensity for forming partially duplex structures with themselves (i.e., leading strand·leading strand duplexes) than the single strands from the third DNA fork. The slower annealing rates are most likely due to these structures that would not be expected to be present in the helicase unwinding reaction. Thus, the fastest rate constant for annealing was used in the kinetic simulations. Separate unwinding experiments were performed in which a trapping strand was included in the reaction mixture that prevents reannealing. Kinetic simulation of these experiments also gave an unwinding rate constant of 0.4 s^{-1}, which supported the mechanism and rate constant determined from Scheme I.

DNA concentration gave rise to slower unwinding and revealed that nonproductive DNA–protein or protein–protein complexes formed (K.D.R., unpublished observations).

Another fluorescence-based continuous helicase assay has been reported by Roman and Kowalczykowski (18). This assay utilizes the intrinsic fluorescence change of the *E. coli* single-strand binding (SSB) protein, which occurs upon binding of this protein to ssDNA. After DNA unwinding has occurred due to helicase activity, the SSB binds to product and a change in fluorescence is observed that corresponds to the amount of ssDNA covered by the protein. This assay was very useful in determining kinetic parameters for the RecBCD helicase. The assay described here should be suitable for addressing additional questions regarding helicase activity such as the number of base pairs that are unwound per translocation event (i.e., the number of base pairs unwound simultaneously). The unwinding rate can be determined on a pre-steady-state time scale, thus avoiding reannealing and other complicating factors.

The 2-AP assay should also be adaptable to existing fluorescence-based drug screening protocols. Selective inhibitors of virally encoded helicases may be obtained using a high-through-put fluorescence-based assay and the 2-AP DNA fork described here. The utility of this type of assay is not limited to helicases. Virtually any process that produces ssDNA from duplex DNA, or vice versa, should be amenable for study by this method.

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