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# Kinetics of *Escherichia coli* Helicase II-Catalyzed Unwinding of Fully Duplex and Nicked Circular DNA<sup>†</sup>

Gregory T. Runyon<sup>‡</sup> and Timothy M. Lohman<sup>\*</sup>

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, 660 South Euclid Avenue, St. Louis, Missouri 63110

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**ABSTRACT:** *Escherichia coli* helicase II (UvrD) protein can initiate unwinding of duplex DNA at blunt ends or nicks, although these reactions require excess protein. We have undertaken kinetic studies of these reactions in order to probe the mechanism of initiation of unwinding. DNA unwinding was monitored directly by using agarose gel electrophoresis and indirectly through the rate of ATP hydrolysis by helicase II in the presence of an ATP-regenerating system. In the presence of fully duplex DNA and excess helicase II, the rate of ATP hydrolysis displays a distinct lag phase before the final steady-state rate of hydrolysis is reached. This reflects the fact that ATP hydrolysis under these conditions results from helicase II binding to the ssDNA products of the unwinding reaction, rather than from an intrinsic duplex DNA-dependent ATPase activity. Unwinding of short blunt-ended duplex DNA (341 and 849 base pairs) occurs in an "all-or-none" reaction, indicating that initiation of unwinding by helicase II is rate-limiting. We propose a minimal mechanism for the initiation of DNA unwinding by helicase II which includes a binding step followed by the rate-limiting formation of an initiation complex, possibly involving protein dimerization, and we have determined the phenomenological kinetic parameters describing this mechanism. Unwinding of a series of DNA substrates containing different initiation sites (e.g., blunt ends, internal nicks, and four-nucleotide 3' vs 5' ssDNA flanking regions) indicates that the rate of initiation is slowest at nicks and, surprisingly, at ends possessing a four-nucleotide 3' ssDNA flanking region.

*Escherichia coli* helicase II, the product of the *uvrD* gene (Hickson et al., 1983; Taucher-Scholz & Hoffmann-Berling, 1983; Kumura & Sekiguchi, 1984; Maples & Kushner, 1982), is a DNA helicase (Kuhn et al., 1979) that catalyzes the ATP-dependent unwinding of duplex DNA [for reviews, see Geider and Hoffman-Berling (1981), Matson and Kaiser-Rogers (1990), Matson (1991), and Lohman (1992, 1993)]. Helicase II has been implicated to function in replication (Klinkert et al., 1980) as well as recombination (Arthur & Lloyd, 1980; Howard-Flanders & Bardwell, 1981; Lloyd, 1983). However, it is known to function in methyl-directed mismatch repair (Lahue et al., 1989) and *uvrABC* excision repair (Caron et al., 1985; Husain et al., 1985; Kumara et al., 1985; Orren et al., 1992). Both of these repair pathways utilize a nicked DNA intermediate. At low helicase II to DNA ratios in vitro, initiation of unwinding requires DNA possessing a 3' single-stranded DNA (ssDNA) flanking the duplex (Matson, 1986; Runyon & Lohman, 1989). However, at higher protein concentrations in vitro, helicase II can initiate unwinding at either a blunt end (Runyon & Lohman, 1989; Runyon et al., 1990; Georgi-Geisberger & Hoffmann-Berling, 1990) or a nick (Runyon & Lohman, 1989; Runyon et al., 1990). In fact, helicase II can unwind long stretches of fully duplex DNA (at least 2700 base pairs (bp)) in the absence of flanking ssDNA (Runyon & Lohman, 1989; Runyon et al., 1990). These observations have led to the suggestion that helicase II may initiate unwinding at a nick in the duplex DNA introduced

by mut H or UvrB/UvrC enzymes during methyl-directed mismatch repair or UvrABC excision repair, respectively (Runyon et al., 1990). In order to probe the mechanism of helicase II-catalyzed DNA unwinding, we have carried out kinetic studies using DNA substrates that differ in the type of initiation site (e.g., nicks, blunt ends).

## MATERIALS AND METHODS

**Buffers, Helicase II Protein, and DNA.** Buffers were made with reagent grade chemicals and distilled-deionized water that was passed through a Milli-Q system (Millipore, Bedford, MA). TE buffer is 10 mM Tris-HCl (pH 8.1 at 25 °C) and 1 mM Na<sub>3</sub>EDTA. Helicase II storage buffer is 20 mM Tris-HCl (pH 8.3 at 25 °C), 0.2 M NaCl, 50% (v/v) glycerol (spectral grade, Aldrich), 1 mM Na<sub>3</sub>EDTA, 0.5 mM Na<sub>2</sub>-EGTA, and 25 mM 2-mercaptoethanol. Helicase II protein was purified, and its concentration was determined spectrophotometrically, using  $\epsilon_{280} = 1.05 \times 10^5 \text{ M}^{-1} (\text{monomer}) \text{ cm}^{-1}$  (Runyon et al., 1993). The 341-, 849-, and 1625-bp blunt-ended duplex DNAs were prepared as described (Runyon & Lohman, 1989), and DNA concentrations were determined spectrophotometrically in TE buffer ( $\epsilon_{260} = 6500 \text{ M}^{-1} (\text{nucleotide}) \text{ cm}^{-1}$ ). DNA was labeled with <sup>32</sup>P at the 5' end as described (Runyon & Lohman, 1989).

**Spectrophotometric ATPase Assay.** A spectrophotometric ATPase assay was used to monitor the kinetics of ATP hydrolysis catalyzed by helicase II (Runyon et al., 1993). Unless stated otherwise, reactions were carried out in 25 mM Tris-HCl (pH 7.5 at 37 °C), 2.5 mM MgCl<sub>2</sub>, 1.5 mM ATP (pH 7, 5.7 mM Na<sup>+</sup>), 10% (v/v) glycerol, 17 mM NaCl, 5 mM 2-mercaptoethanol, 25 units/mL pyruvate kinase (type II rabbit muscle, Sigma), 25 units/mL lactic dehydrogenase (type II rabbit muscle, Sigma), 8 mM phosphoenolpyruvate (PEP) (pH 7, 21.4 mM Na<sup>+</sup>, Boehringer Mannheim), and 0.9 mM NADH (grade I, disodium salt, Boehringer Mann-

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<sup>\*</sup> Address correspondence to this author at the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, 660 S. Euclid Ave., St. Louis, MO 63110-1093.

<sup>‡</sup> Present address: Department of Cell Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131.

heim). Most experiments were performed at DNA concentrations of  $0.125 \mu\text{M}$  (nucleotide). Under these conditions, the solubility of the helicase II protein is greater than  $4 \mu\text{M}$  (monomer) and the free protein is monomeric up to at least  $1.5 \mu\text{M}$  (monomer) (Runyon et al., 1993). The final pH of the solution was 7.5 at  $37^\circ\text{C}$  in a final volume of 1 mL. Positive-displacement pipettes (Microman, Gilson) were used to deliver all solutions containing greater than 5% (v/v) glycerol.

Reaction mixtures (without helicase II protein or DNA,  $915 \mu\text{L}$ ) were preincubated in 1-mL quartz cuvettes (Spectrasil; 1-cm path length) at  $37^\circ\text{C}$  for at least 5 min. Concentrated helicase II in storage buffer was added to the reaction mixture along with sufficient storage buffer so that the total added volume was  $80 \mu\text{L}$ , and the solution was incubated for 1 min at  $37^\circ\text{C}$ . Reactions were started by adding DNA ( $5 \mu\text{L}$  in TE buffer), and reaction solutions were mixed by repeated inversion of the stoppered cuvette. Reactions were monitored continuously at 380 nm and  $37^\circ\text{C}$  starting  $10 \pm 1$  s after mixing. The rate of initiation of DNA unwinding (lag time,  $\tau_{\text{obs}}$ ), although not the final steady-state rate, was dependent on the order of component addition (i.e., ATP,  $\text{MgCl}_2$ , DNA, or helicase II). The lag time was  $\sim 50\%$  longer when either ATP or  $\text{MgCl}_2$  was added last. Experiments reported here were initiated by adding DNA last.

No component of the spectrophotometric assay was inhibitory or rate-limiting under the solution conditions examined. However, to insure this at high pH ( $>\text{pH } 8.5$ ), high glycerol concentration ( $>20\%$  (v/v)), or high NaCl concentration ( $>250 \text{ mM}$ ), the concentrations of the coupling enzymes (pyruvate kinase and lactic dehydrogenase) were increased to 60 units/mL and the spectrophotometric reaction was monitored at 380 nm, rather than at 340 nm, in order to remain within the linear absorbance range of the spectrophotometer at these high NADH concentrations.

**Analysis of the Spectrophotometric Helicase Assay To Obtain the Pseudo-First-Order Rate Constant for DNA Unwinding,  $k_{\text{obs}}$ .** We utilized the intrinsic ssDNA-dependent ATPase activity of helicase II protein to monitor DNA unwinding catalyzed by excess helicase II, using the spectrophotometric ATPase assay in the presence of an ATP-regenerating system. In the presence of double-stranded DNA (dsDNA), the majority of ATP hydrolysis results from binding of helicase II to ssDNA resulting from duplex DNA unwinding. At any time, the absorbance change at 380 nm,  $\Delta A_{380}$ , is proportional to the total amount of ATP hydrolyzed ( $0.826 \text{ mM ATP hydrolyzed}/\Delta A_{380}$ ).

Our preparations of helicase II protein possess an apparent "DNA-independent" ATPase activity as previously described (Runyon et al., 1993). This activity represents only 0.03–0.13% of the enzyme's specific ssDNA-dependent ATPase activity ( $k_{\text{cat}} = 10\text{--}40 \text{ min}^{-1}$ ). However, in the studies reported here, this DNA-independent ATPase activity becomes significant (approaching  $\sim 25\%$  of the overall ATPase activity) at the high helicase II concentrations required to catalyze the unwinding of fully duplex DNA. Therefore, in order to obtain the ATPase activity resulting only from DNA unwinding, each DNA unwinding reaction was accompanied by a control reaction carried out in the absence of DNA. The time course of the control was subtracted from the time course in the presence of DNA using the data processing system of the HP 8450A spectrophotometer.

Estimates of  $k_{\text{obs}}$  and  $v_{\text{ss}}$  were obtained from the experimental time course of ADP production by comparison with a series of simulated curves generated using eq 7 (see below). In the

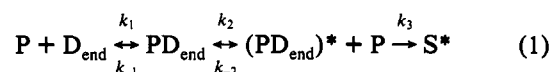
presence of high helicase II concentrations ( $\geq 0.55 \mu\text{M}$  monomer), optimal fits were obtained by constraining  $v_{\text{ss}} = 0.0050$  in eq 7 and varying only  $k_{\text{obs}}$  to obtain the best-fit value. This value of  $v_{\text{ss}}$  was equal to the steady-state rate of ATP hydrolysis when poly(dT) ( $0.125 \mu\text{M}$  nucleotide) is saturated with helicase II.

The true final steady-state rate of ATP hydrolysis could not always be achieved due to the limited range of the spectrophotometric signal available in our assays. In some experiments, particularly those with helicase II concentrations  $< 0.55 \mu\text{M}$ , the reaction reached only about one-half the final steady-state rate at an absorbance change of 0.5 AU, at which point all of the NADH had been oxidized. Therefore, curve fitting of the experimental time courses was only performed in the absorbance range between  $-0.05$  and  $-0.50$  so that the results from different experiments could be compared more accurately. For reactions performed at helicase II concentrations  $< 0.55 \mu\text{M}$  or in solution conditions that affect the  $k_{\text{cat}}$  for ATP hydrolysis, the time course was analyzed to obtain both  $k_{\text{obs}}^*$  (defined below) and  $v_{\text{ss}}$  by using eq 7. The values of  $v_{\text{ss}}$  obtained in these conditions represent the steady-state rate of hydrolysis for helicase II–ssDNA complexes that are not saturated with protein. Since the final states of the complexes under these conditions are unknown but certainly different from the fully saturated complexes described above, we refer to the apparent rate constant determined under these conditions as  $k_{\text{obs}}^*$ .

**Analysis To Obtain  $k_2$  and  $K_M$ .** Under conditions where helicase II is in excess over DNA ( $[\text{helicase II}] \geq 0.55 \mu\text{M}$  monomer), the kinetic parameters  $k_2$  and  $K_M$  were obtained from analysis of the dependence of  $k_{\text{obs}}$  on total helicase II monomer concentration,  $P_T$ , using eq 4. Nonlinear regression analysis was performed using RS 1 (BBN Software Products Corp., Cambridge, MA), with each value of  $(k_{\text{obs}})_i$  weighted by the coefficient of variability ( $1 - (\Delta_i/(k_{\text{obs}})_i)$ ), in which  $\Delta_i$  is the absolute value of the difference between  $k_{\text{obs}}$  and the confidence limit between  $k_{\text{obs}}$  in the  $i$ th experiment (Steel & Torrie, 1980).

**DNA Unwinding Monitored by Agarose Gel Electrophoresis.** Unwinding of fully duplex DNA molecules was monitored by using agarose gel electrophoresis and analyzed as previously described (Runyon & Lohman, 1989). Assay conditions were identical to those used in the spectrophotometric assay described above (including the ATP-regenerating system), and  $5'$   $^{32}\text{P}$  end-labeled blunt-ended DNAs ( $0.125 \mu\text{M}$  nucleotide) of varying lengths were used as DNA substrates. Reactions ( $40 \mu\text{L}$ ) were initiated as described above for the spectrophotometric assay and were terminated at the indicated times by the addition of  $10 \mu\text{L}$  of stop mixture ( $0.05 \text{ M Na}_3\text{-EDTA}$  and  $20\%$  (v/v) glycerol). In these reactions, the DNA was not deproteinized before electrophoresis.

**Model for the Initiation of Helicase II-Catalyzed DNA Unwinding.** Our studies suggest that a minimum of three steps (see eq 1) is required for a phenomenological description of the kinetics of helicase II-catalyzed initiation of unwinding of fully duplex DNA. In the first step, with apparent



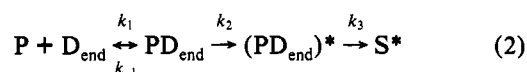
equilibrium constant  $K_1 = k_1/k_{-1}$ , one (or more) helicase II monomer, P, binds to a DNA end (or a nick),  $\text{D}_{\text{end}}$ , to form a protein–DNA complex,  $\text{PD}_{\text{end}}$ . The second step, with forward rate constant  $k_2$ , is rate-limiting and leads to formation of intermediate  $(\text{PD}_{\text{end}})^*$ . The third step, with rate constant

$k_3$ , represents the rapid (compared to initiation) propagation of DNA unwinding and incorporates all steps occurring after the rate-limiting step, including binding of additional helicase II to the unwound ssDNA products. Therefore, the rate of this propagation step cannot be measured in our steady-state assay. The products of the unwinding reaction,  $S^*$ , are the separated strands of helicase II-covered ssDNA.

The mechanism in eq 1 is based on the following observations: (i) Helicase II can completely unwind duplex (ds) DNA (Runyon & Lohman, 1989). (ii) Helicase II initiates unwinding of fully duplex DNA from a blunt end or a nick, but not from internal duplex regions (Runyon et al., 1990). (iii) High ratios of helicase II to DNA are required to completely unwind blunt-ended DNA in vitro (Runyon & Lohman, 1989). (iv) Helicase II does not bind detectably to dsDNA under our reaction conditions (Runyon et al., 1990). (v) *At least* one intermediate step occurs during the initiation of DNA unwinding, on the basis of the observation of a pronounced lag in the helicase II-catalyzed hydrolysis of ATP in the presence of fully duplex DNA (see below). (vi) Propagation of DNA unwinding is rapid compared to initiation, on the basis of the low concentration of partially unwound intermediates observed during unwinding (Runyon & Lohman, 1980; Runyon et al., 1990). (vii) In the presence of excess helicase II, the products of DNA unwinding are ssDNA saturated with helicase II (Runyon & Lohman, 1989; Runyon et al., 1990). Either step 1 or step 2 likely involves formation of a helicase II dimer or higher-order oligomer, on the basis of the fact that helicase II can self-assemble to form dimers which are stabilized upon binding to DNA (Runyon et al., 1993). However, since the active form of helicase II at an unwinding fork is not known, we have not explicitly included helicase II dimerization in eq 1. If dimerization occurs in step 1, then the apparent equilibrium constant  $K_1$  represents a composite of a DNA binding constant and a protein dimerization constant.

We make the following assumptions in our analysis of the unwinding kinetics: (i) All helicase II monomers are fully active. This is consistent with the facts that all of the protein binds ssDNA and that the specific ATPase and helicase activities for all of our protein stocks are constant (within 8%), but remains an assumption. (ii) The following relationships hold:  $k_{-2} \ll k_2$ , and  $k_{-3} \ll k_3$ . We are confident that  $k_{-3} \ll k_3$  since helicase II-saturated ssDNA is stable and does not dissociate under these conditions. (iii)  $PD_{\text{end}}$  is at steady-state. (iv) The free protein forms a complex with Mg-ATP. This is supported by the observed effects of  $Mg^{2+}$  and ATP on the assembly state of helicase II (Runyon et al., 1993).

With the assumption that  $k_{-2} \sim 0$ , the scheme in eq 1 simplifies to that given in eq 2.



With the assumptions that  $k_2$  is the rate-limiting step and that  $PD_{\text{end}}$  is in steady state, the rate of ssDNA formation is given by eq 3 (Strickland et al., 1975),

$$\frac{d(S^*)}{dt} = [D_{\text{end}}]_T (1 - \exp(-k_{\text{obs}}t)) \quad (3)$$

where  $[D_{\text{end}}]_T$  is the total concentration of DNA ends and  $k_{\text{obs}}$  is the pseudo-first-order rate constant for DNA unwinding as

defined in eq 4, with  $K_M = (k_{-1} + k_2)/k_1$ .

$$k_{\text{obs}} = \frac{k_2[P_T]}{[P_T] + K_M} \quad (4)$$

Under conditions of excess helicase II, the total helicase II monomer concentration,  $[P_T]$ , is equal to the free monomer concentration,  $[P]$ . Therefore, the kinetics of initiation of unwinding can be described by two phenomenological parameters,  $k_2$  and  $K_M$ .

Integration of eq 3 yields eq 5, which is the time dependence of ssDNA, or  $S^*$ , formation. Since the rate of ATP hydrolysis

$$[S^*] = [D_{\text{end}}]_T \left[ t + \frac{(\exp(-k_{\text{obs}}t) - 1)}{k_{\text{obs}}} \right] \quad (5)$$

is proportional to the concentration of helicase II bound to ssDNA, then, under conditions such that all ssDNA is saturated with helicase II protein, the final steady-state rate of ATP hydrolysis,  $v_{\text{ss}}$ , is proportional to the total DNA concentration,  $[D_T]$ , which is related to the concentration of DNA ends as in eq 6, where  $N$  is the length of the ssDNA in nucleotides.

$$[D_T] = N[D_{\text{end}}]_T \quad (6)$$

Equation 5 can be rewritten to yield an expression for the time dependence of the production of ADP, which is given in eq 7.

$$[ADP] = v_{\text{ss}} \left[ t + \frac{(\exp(-k_{\text{obs}}t) - 1)}{k_{\text{obs}}} \right] \quad (7)$$

Note that when  $\tau_{\text{obs}} = (1/k_{\text{obs}}) = 0$ , eq 7 reduces to  $[ADP] = v_{\text{ss}}t$ , which describes the constant rate of ATP hydrolysis by helicase II observed in the presence of ssDNA.

## RESULTS

**Helicase II-Catalyzed Unwinding of Fully Duplex DNA Monitored by Agarose Gel Electrophoresis.** We have shown that helicase II-catalyzed unwinding of fully duplex DNA can be monitored by agarose gel electrophoresis (Runyon & Lohman, 1989). In the experiments reported here, unwinding is performed in the presence of excess helicase II and an ATP-regenerating system. Reactions are quenched at various times by the addition of EDTA to a final concentration of 10 mM, and samples are applied directly to an agarose gel *without* removal of the helicase II protein. Therefore, partially unwound DNA molecules have helicase II bound to the ssDNA regions and will migrate differently than fully duplex DNA or fully unwound ssDNA that is covered with helicase II. Figure 1 shows time courses for unwinding blunt-ended linear dsDNAs of different lengths (341, 849, and 1625 bp) at a ratio of 2 helicase II monomers/nucleotide. At each time point, only fully duplex and fully unwound DNA molecules are apparent, suggesting that unwinding occurs in a nearly "all-or-none" fashion and indicating that initiation is rate-limiting compared to propagation.

The data in Figure 1 were analyzed, and the results are plotted in Figure 2 as both the percentage of fully unwound DNA (closed circles) and the disappearance of the fully native DNA (open circles). We expect that the disappearance of fully native DNA overestimates the extent of unwinding since this population includes DNA molecules that are only partially unwound. On the other hand, the appearance of fully unwound DNA likely underestimates the extent of unwinding since it includes only fully unwound DNA. Although DNA that migrates with intermediate mobility most likely represents

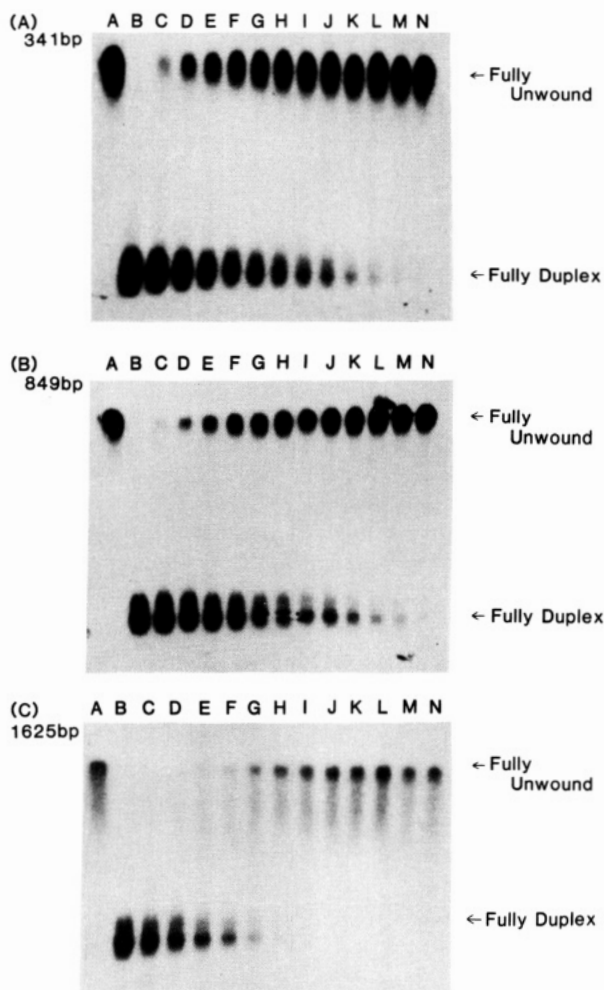


FIGURE 1: Kinetics of helicase II-catalyzed unwinding of blunt-ended DNAs of different lengths at constant helicase II protein concentration ( $0.25 \mu\text{M}$  monomer) and DNA (nucleotide) concentration ( $0.125 \mu\text{M}$  nucleotide). Reactions were performed in the presence of an ATP-regenerating system (containing 10% (v/v) glycerol) under the standard reaction conditions (see Materials and Methods) and quenched at the indicated time,  $t$ . The products were separated by agarose gel electrophoresis, and the gels were visualized by autoradiography. Panel A: 341-bp *HaeIII* M13mp11 fragment (1.25% agarose). Lanes: A, alkaline-denatured DNA plus helicase II; B,  $t = 0$ ; C, 30 sec; D, 1 min.; E, 1.5 min.; F, 2 min.; G, 2.5 min.; H, 3 min.; I, 4 min.; J, 5 min.; K, 6 min.; L, 7 min.; M, 8 min.; N, 9 min. Panel B: 849-bp *HaeIII* M13mp11 fragment (0.6% agarose). Lanes are as in panel A. Panel C: 1625-bp *HaeIII* M13mp11 fragment (0.5% agarose). Lanes: A, alkaline-denatured DNA plus helicase II; B,  $t = 0$ ; C, 30 sec; D, 1 min.; E, 1.5 min.; F, 2 min.; G, 2.5 min.; H, 3 min.; I, 3.5 min.; J, 4 min.; K, 5 min.; L, 6 min.; M, 7 min.; N, 8 min.

partially unwound DNA, fully unwound DNA that is not saturated with helicase II may also migrate with intermediate mobility.

Figure 2 demonstrates that for each DNA substrate, 80–90% of the DNA population is unwound completely within  $\sim 8$  min. The time courses for the 341-bp duplex, as calculated by the two methods described above, are the same within experimental error, indicating the absence of significant amounts of partially unwound intermediates during unwinding. However, differences in the time courses obtained by the two methods are evident at short times for the 849-bp duplex and over the entire time course for the 1625-bp duplex (Figure 2B,C). Therefore, some partially unwound DNA is detectable during unwinding of both the 849- and the 1625-bp duplex.

When unwinding experiments are performed at higher helicase II concentrations ( $>2.5$  helicase II monomers/DNA

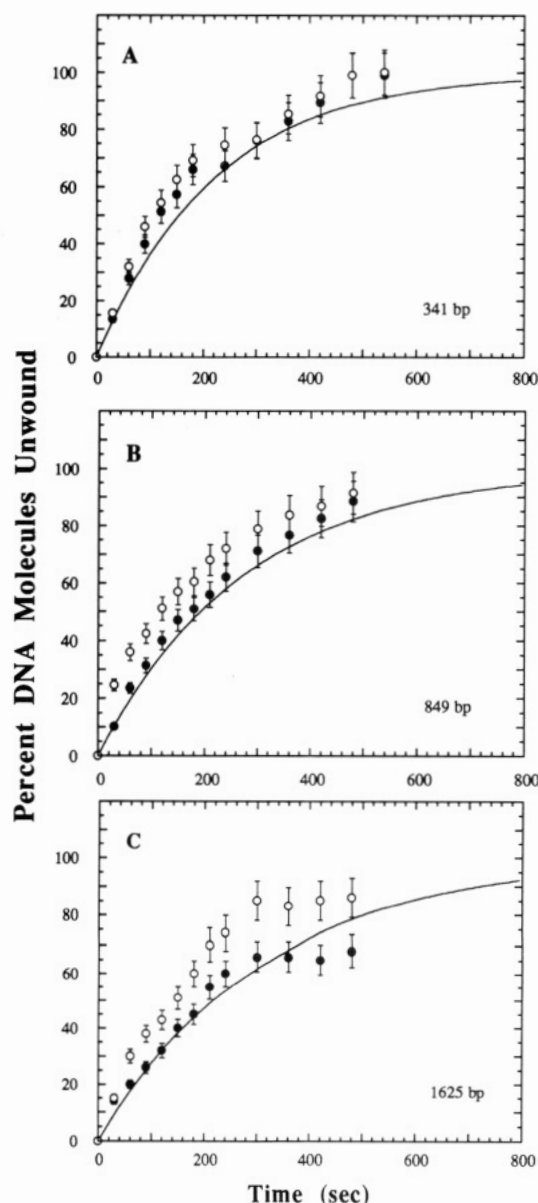


FIGURE 2: Kinetics of helicase II-catalyzed unwinding of blunt-ended DNA monitored by both agarose gel electrophoresis and ATP hydrolysis at constant helicase II concentration ( $0.25 \mu\text{M}$  monomer) and DNA concentration ( $0.125 \mu\text{M}$  nucleotide). The gels in Figure 1 were analyzed to determine the time course of disappearance of fully duplex DNA ( $\bullet$ ) and the time course of appearance of completely unwound ssDNA ( $\circ$ ). Separate experiments, identical to those described in Figure 1, were performed, but ATP hydrolysis was monitored by using the spectrophotometric ATPase assay. The time courses were analyzed by using eq 7 to obtain  $k_{\text{obs}}$  and  $v_{\text{ss}}$ ; eq 8 was used to generate the solid curves. Panel A, 341-bp blunt-ended *HaeIII* M13mp11 DNA fragment; panel B, 849-bp blunt-ended *HaeIII* M13mp11 DNA fragment; panel C, 1625-bp blunt-ended *HaeIII* M13mp11 DNA fragment.

nucleotide) or with DNA substrates longer than 1625 base pairs, a significant fraction of the DNA remains in the well, possibly due to formation of helicase II–DNA aggregates or precipitated complexes. This appears to be due to the fact that helicase II solubility is lower in the electrophoresis running buffer; hence contact with the buffer at these higher helicase II concentrations may induce precipitation of the complexes.

**Kinetics of DNA Unwinding Monitored by ssDNA-Dependent ATPase Activity of Helicase II.** DNA unwinding cannot be monitored continuously by using the agarose gel electrophoresis method. Therefore, we have used an indirect, spectrophotometric assay which reflects the rate of ATP

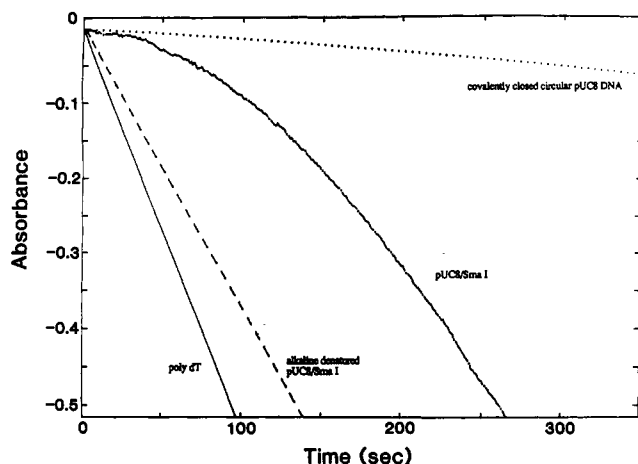


FIGURE 3: Kinetics of helicase II-catalyzed hydrolysis of ATP in the presence of single-stranded and duplex DNA effectors monitored in the presence of an ATP-regenerating system. Helicase II ( $1.39 \mu\text{M}$  monomer) and DNA ( $0.125 \mu\text{M}$  nucleotide) were mixed under our standard conditions, and ATP hydrolysis was measured using the spectrophotometric assay. The DNA substrates were covalently closed circular, supercoiled pUC8 DNA (dotted line), linear, blunt-ended pUC8 DNA (dash-dot line), alkaline-denatured linear pUC8 DNA (dashed line), and poly(dT) (solid line).

hydrolysis catalyzed by helicase II in order to monitor DNA unwinding continuously. This assay is based on the fact that in the presence of *excess* helicase II the rate of ATP hydrolysis is directly proportional to the amount of ssDNA produced from unwinding. The use of *excess* helicase II (over the final concentration of ssDNA) ensures that all ssDNA sites will be saturated with helicase II; hence the specific ATPase activity of helicase II will reach a constant value after unwinding is complete (see below). Therefore, the instantaneous rate of ATP hydrolysis is proportional to the amount of DNA unwound at any time during the reaction.

Figure 3 shows the time courses for ATP hydrolysis in the presence of excess helicase II and the following DNA effectors at equal concentrations: poly(dT), single-stranded (alkaline-denatured) pUC8 DNA, fully duplex linear pUC8 DNA, and covalently closed circular (ccc) duplex pUC8 DNA. In the presence of both types of ssDNA, the rates of ATP hydrolysis are constant with no lag time for the production of ADP, although the rate of ATP hydrolysis is higher with poly(dT) than with alkaline-denatured ssDNA. However, in the presence of linear duplex DNA, the time course exhibits a distinct lag phase, such that the rate of ATP hydrolysis increases with time, approaching a constant rate equal to that observed in the presence of ssDNA. As shown below, this time course reflects the increased rate of ATP hydrolysis that accompanies the production of ssDNA during the course of DNA unwinding.

Figure 3 also shows that the rate of ATP hydrolysis by helicase II is reduced significantly in the presence of duplex cccDNA. The low rate of ATP hydrolysis ( $8.5 \pm 4.6 \mu\text{M}$  ATP hydrolyzed  $\text{min}^{-1}$ ), corresponds to only  $\sim 3\text{--}4\%$  of the final steady-state rate observed in the presence of linear duplex DNA or poly(dT). This suggests that helicase II is not able to hydrolyze ATP when bound only to duplex DNA. The low but finite rate of ATP hydrolysis by helicase II in the presence of cccDNA may be due to the presence of a small amount of nicked circular DNA that contaminates our preparations of pUC8 cccDNA. Our cccDNA stocks contain  $\sim 5\text{--}7\%$  nicked circles, and the extent of ATP hydrolysis observed is consistent with the unwinding of these nicked circles by helicase II. Furthermore, the rate of cccDNA-dependent ATP hydrolysis

is independent of the concentration of excess helicase II ( $0.55\text{--}1.39 \mu\text{M}$  monomer), whereas it is proportional to the concentration of cccDNA. We conclude that helicase II does not possess an intrinsic duplex-DNA-dependent ATPase activity, but only hydrolyzes ATP when bound to ssDNA or during the course of unwinding duplex DNA. Therefore, monitoring ATP hydrolysis in the presence of excess helicase II should provide a means to continuously monitor helicase II-catalyzed unwinding of duplex DNA.

The time course of ATP hydrolysis during unwinding of fully duplex DNA was analyzed by using eq 7 to obtain the apparent first-order rate of unwinding,  $k_{\text{obs}}$ , and the final steady-state rate of ATP hydrolysis,  $v_{\text{ss}}$ . This was accomplished by visual comparison of the experimental time course with a series of simulated time courses generated using eq 7 (after subtraction of the DNA-independent ATPase activity). In the presence of excess helicase II ( $\geq 0.55 \mu\text{M}$  monomer), optimal fits were obtained, using a constant value of  $v_{\text{ss}}$ ,  $0.0050 \pm 0.0002 \text{ AU/s}$ , which equals the steady-state rate of ATP hydrolysis measured at the same concentration of poly(dT) ( $0.125 \mu\text{M}$  nucleotide) with excess helicase II (see below). Therefore, DNA unwinding experiments performed at these helicase II concentrations were analyzed by constraining  $v_{\text{ss}} = 0.0050 \text{ AU/s}$  in eq 7 and varying only  $k_{\text{obs}}$  to determine its best-fit value. The confidence limits of  $k_{\text{obs}}$  were obtained from comparisons of the minimum and maximum values of  $k_{\text{obs}}$  that still result in simulated time courses that closely overlap the experimental time course.

**Effect of Helicase II Concentration on the Final Steady-State Rate of ATP Hydrolysis.** In the presence of duplex DNA, the final steady-state rate of ATP hydrolysis,  $v_{\text{ss}}$ , approaches the rate determined in the presence of poly(dT), consistent with our conclusion that the final product of unwinding ssDNA saturated with helicase II. However, as discussed above, it is difficult to obtain an accurate estimate of  $v_{\text{ss}}$  from a curve-fitting analysis of the ATP hydrolysis kinetics. Furthermore, estimates of  $k_{\text{obs}}$  are less certain when  $k_{\text{obs}}$  and  $v_{\text{ss}}$  are both determined from the curve-fitting analysis. If the final value of  $v_{\text{ss}}$  were known, then an analysis of the DNA unwinding reaction would require only a single-parameter fit to determine  $k_{\text{obs}}$ . The final value of  $v_{\text{ss}}$  can nominally be obtained from the rate of hydrolysis in the presence of only ssDNA, at the same nucleotide concentration used in the unwinding reactions. However,  $v_{\text{ss}}$  is different and slightly greater in the presence of poly(dT) when compared to alkaline-denatured pUC8 DNA; hence it was not clear which value should be used to constrain  $v_{\text{ss}}$ .

In order to determine this, we measured the final steady-state rate of ATP hydrolysis as a function of helicase II concentration in the presence of poly(dT), alkaline-denatured pUC8 DNA, and linear duplex pUC8 DNA, all at the same DNA concentration ( $0.125 \mu\text{M}$  nucleotide), and the results are shown in Figure 4. In the presence of poly(dT), the steady-state rate of ATP hydrolysis reaches a plateau above  $\sim 0.3 \mu\text{M}$  helicase II (monomer), suggesting that the strands are fully saturated above this protein concentration. In the presence of linear duplex pUC8 DNA (2671 base pairs), the steady state rate of hydrolysis also reaches the same plateau value as observed with poly(dT), although higher helicase II concentrations are required ( $\geq 0.55 \mu\text{M}$  monomer). Therefore, we conclude that the ssDNA produced by the helicase II-catalyzed unwinding of duplex DNA is fully saturated with helicase II at these protein concentrations and that the same value of  $v_{\text{ss}}$  applies under these conditions as when helicase II is bound to poly(dT). Hence, we use a constant value of  $v_{\text{ss}}$



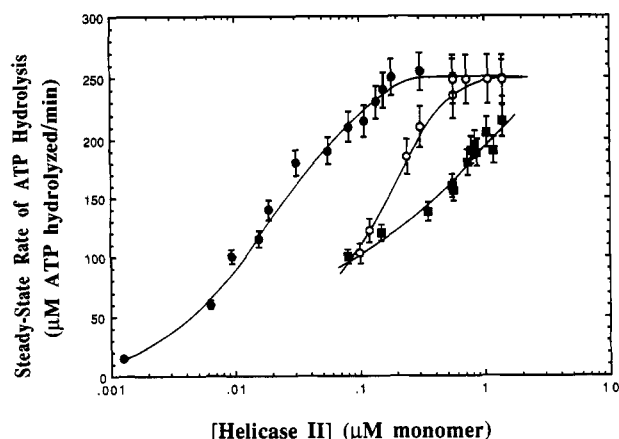


FIGURE 4: Steady-state rate of ATP hydrolysis measured as a function of helicase II concentration in the presence of single-stranded and duplex DNA using the spectrophotometric assay under standard conditions at DNA concentrations of 0.125  $\mu\text{M}$  (nucleotide). Steady-state rates ( $v_{ss}$ ) were determined at each helicase II concentration through use of eq 7 as described under Materials and Methods. DNA substrates are poly(dT) ( $\bullet$ ), alkaline-denatured linear pUC8 DNA ( $\blacksquare$ ), and blunt-ended duplex pUC8 DNA (*Sma*I cut) ( $\circ$ ).

= 0.0050 AU/s (248  $\mu\text{M}$  ATP hydrolyzed  $\text{min}^{-1}$ ) to analyze the time course of ATP hydrolysis during DNA unwinding for experiments performed with helicase II concentrations  $\geq 0.55$   $\mu\text{M}$  (monomer).

When alkaline-denatured DNA is used as the effector over the same range of helicase II concentrations, the rate of ATP hydrolysis is significantly lower than when either poly(dT) or dsDNA (final rate) is used (see Figure 4). Furthermore, the steady-state rate of ATP hydrolysis does not reach a plateau even at helicase II concentrations  $> 0.55$   $\mu\text{M}$  monomer. This suggests that the denatured pUC8 DNA still contains sufficient intramolecular base pairing that is not melted out by helicase II, thus reducing the number of helicase II binding sites and the steady-state hydrolysis rate. These base-paired regions likely form rapidly upon dilution of the DNA into the neutral-pH reaction solutions, but before helicase II protein can bind. If this interpretation is correct, it is curious that helicase II is apparently unable to unwind these base-paired regions. Presumably, higher helicase II concentrations are required to eventually attain the same plateau value for  $v_{ss}$ . The fact that the same value of  $v_{ss}$  is obtained with poly(dT) as with pUC8 ssDNA that has been unwound by helicase II suggests that, during the course of unwinding duplex DNA, helicase II is able to load onto the unwound ssDNA in an ordered manner such that it can saturate the ssDNA. Presumably, the same final saturated state is achieved (and hence the same steady-state rate of ATP hydrolysis) upon helicase II binding to poly(dT), since this DNA cannot form intramolecular base pairs. The observation that the same plateau value of  $v_{ss}$  is achieved at saturating helicase II concentrations for both poly(dT) and unwound pUC8 DNA also suggests that the intrinsic ATPase activity of helicase II is independent of base composition.

**Comparison of the Kinetics of Helicase II-Catalyzed Unwinding of Fully Duplex DNA As Monitored by the Spectrophotometric ATPase and Agarose Gel Electrophoresis Assays.** To determine whether the spectrophotometric ATPase assay can be used to monitor quantitatively the helicase II-catalyzed unwinding of duplex DNA, we compared the rates of unwinding obtained using the ATPase assay vs the gel assay. Both assays were performed in the same solution conditions in the presence of an ATP-regenerating system, and with the same DNA and helicase II stocks and concentrations ([helicase II] = 0.25  $\mu\text{M}$  monomer). Due to the

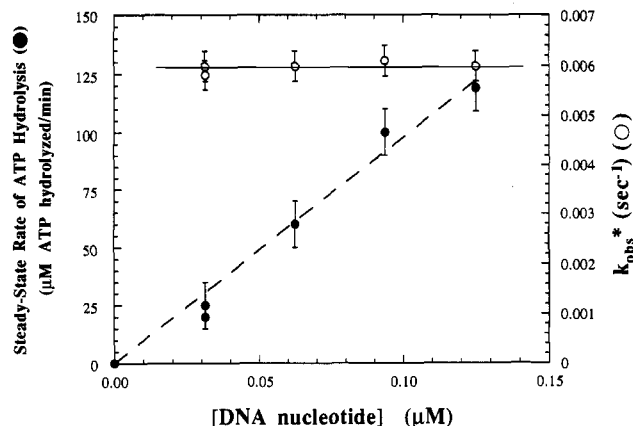


FIGURE 5: Dependence on DNA concentration of the steady-state rate of ATP hydrolysis. Steady-state kinetic parameters  $k_{obs}^*$  and  $v_{ss}$  for helicase II-catalyzed ATP hydrolysis were obtained as a function of DNA concentration for blunt-ended pUC8 DNA (*Sma*I cut). Reactions were performed at constant helicase II concentration (0.55  $\mu\text{M}$  monomer) and at DNA concentrations from 0.031 to 0.125  $\mu\text{M}$  (nucleotide) under standard conditions as described under Materials and Methods. Values of  $v_{ss}$  ( $\bullet$ ) and  $k_{obs}^*$  ( $\circ$ ) were obtained from analysis of each time course by using eq 7.

limitations mentioned above for the agarose gel assay, this comparison could not be made at a helicase II concentration  $\geq 0.55$   $\mu\text{M}$  (monomer). Therefore, both  $v_{ss}$  and  $k_{obs}^*$  (defined above) were obtained from fits of the spectrophotometric time course to eq 7. This value of  $k_{obs}^*$  was then used to calculate the percentage of DNA molecules unwound as a function of time, using eq 8. The results are shown as solid lines in Figure

$$\text{percentage of DNA molecules unwound} = \frac{100(1 - \exp(-k_{obs}^* t))}{(8)}$$

2 and indicate that the unwinding rate estimated from the ATPase assay is equivalent to the rate of appearance of fully unwound ssDNA, whereas it is slightly lower than the rate of disappearance of fully native DNA. Thus the spectrophotometric assay can be used as a continuous assay to monitor the kinetics of helicase II-catalyzed DNA unwinding.

The lag in ATP hydrolysis that is observed in the presence of linear duplex DNA is not dependent on the use of the spectrophotometric assay, since it is also observed when ATPase activity is monitored using thin-layer chromatography (TLC). However, the rate of ATP hydrolysis is faster, and the lag time is shorter by  $\sim 20\%$ , when measured using the spectrophotometric assay. This is likely due to the presence of the ATP-regenerating system in the spectrophotometric assay which maintains a constant concentration of ATP during the reaction. ATP regeneration also ensures that helicase II remains soluble throughout the reaction and eliminates product (ADP) inhibition of the ATPase reaction. Similar effects of ATP regeneration have been observed on helicase II-catalyzed ssDNA-dependent ATPase activity (Runyon et al., 1993).

**Effects of DNA Concentration on Helicase II-Catalyzed DNA Unwinding.** Unwinding of fully duplex linear DNA was examined as a function of DNA concentration (from 0.031 to 0.125  $\mu\text{M}$  nucleotide) at a constant helicase II concentration (0.55  $\mu\text{M}$  monomer), using the spectrophotometric assay. Since linear blunt-ended pUC8 DNA was used in this study, the concentration of DNA ends also changes with the DNA concentration. The time courses were fit to eq 7 by varying both  $v_{ss}$  and  $k_{obs}^*$ . Figure 5 indicates that the rate of DNA unwinding,  $k_{obs}^*$ , is independent of DNA concentration, whereas  $v_{ss}$  is proportional to DNA concen-

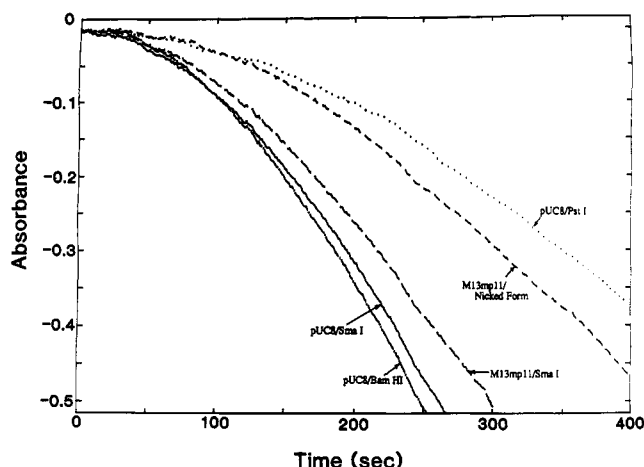


FIGURE 6: Time courses of ATP hydrolysis by helicase II (1.39  $\mu\text{M}$  monomer) monitored using the spectrophotometric ATPase assay under our standard conditions in the presence of different duplex DNA substrates (0.125  $\mu\text{M}$  nucleotide). DNA substrates were pUC8 (*SmaI* cut) (solid line), pUC8/*BamHI* (dash-dot line), pUC8/*PstI* DNA (dotted line); M13mp11/*SmaI* (long-dashed line); nicked circular M13mp11 (short-dashed line).

tration, as expected. These results provide support for the mechanism presented in eq 2.

**Rate of Initiation of DNA Unwinding is Influenced by the DNA Initiation Site.** Since helicase II initiates unwinding from the ends of linear duplex DNA (Runyon et al., 1990), we examined unwinding of DNAs possessing different types of ends. We examined linear DNA with blunt ends, linear DNA with four-nucleotide 5' single-stranded ends, linear DNA with four-nucleotide 3' single-stranded ends, and circular duplex DNA containing a single unique nick. The linear DNA molecules were prepared by treatment of covalently closed circular pUC8 DNA and/or M13mp11 RF DNA with different restriction enzymes, whereas the nicked circular DNA was prepared by treating supercoiled M13mp11 DNA with purified f1 gene II protein, which generates a unique nick in the plus strand within the origin of replication (Runyon et al., 1990; Georgi-Geisberger & Hoffmann-Berling, 1990).

Figure 6 compares the kinetics of ATP hydrolysis, reflecting unwinding of these DNA substrates at identical DNA (0.125  $\mu\text{M}$  nucleotide) and helicase II concentrations (1.39  $\mu\text{M}$  monomer). Definite lag times for ATP hydrolysis are apparent with each DNA. Equivalent kinetics, with the shortest lag times, were observed for each blunt-ended pUC8 DNA (*SmaI* or *HincII* treated) as well as for the pUC8 DNA possessing a four-nucleotide 5' ssDNA flanking region (*HindIII* or *BamHI* treated). However, we were surprised to observe a significantly slower rate of unwinding for DNA possessing a four-nucleotide 3' ssDNA flanking region (*PstI* treated). Although it is not surprising that a four-nucleotide 3' ssDNA flanking region does not facilitate initiation, it is surprising that initiation is slower, since helicase II prefers unwinding duplex DNA with a 3' single-stranded flanking region, although this has previously been tested only with ssDNA that is significantly longer than four nucleotides (Matson, 1986). Initiation of unwinding was also slower with the nicked circular M13 DNA than with the blunt-ended DNA; however, even initiation on the nicked circular DNA was faster than on the *PstI*-treated pUC8 DNA. Identical results were obtained upon repeating these experiments with different protein and DNA stocks.

A series of experiments was performed with varying helicase II concentrations for each of these DNA substrates, and the

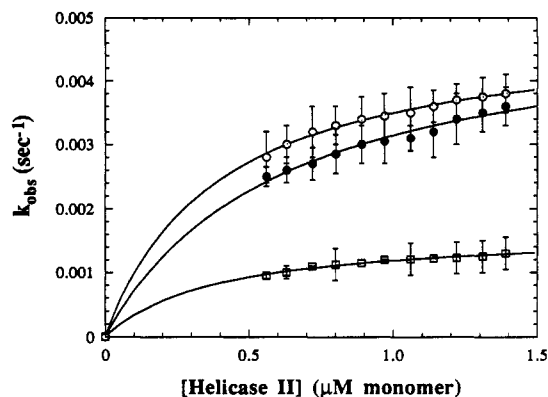


FIGURE 7: Dependence on helicase II concentration of  $k_{\text{obs}}$  for unwinding duplex pUC8 DNA with different types of DNA ends. Reactions were performed under standard conditions at constant DNA concentration (0.125  $\mu\text{M}$  nucleotide) using the spectrophotometric assay to monitor ATP hydrolysis. Values of  $k_{\text{obs}}$  were obtained from analysis of each time course using eq 7. Values of  $k_2$  and  $K_M$  listed in Table I were determined by nonlinear least squares analysis, and the solid curves were generated using eq 4; blunt ends (*SmaI* cut) (●); four-nucleotide 5' single-stranded ends (*BamHI* cut) (○); four-nucleotide 3' single-stranded ends (*PstI* cut) (□).

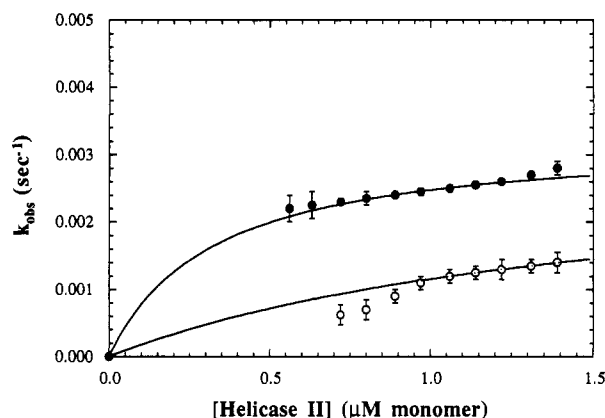


FIGURE 8: Dependence on helicase II concentration of  $k_{\text{obs}}$  for unwinding of blunt-ended and nicked circular M13mp11 DNA. Reactions were performed at constant DNA concentration (0.125  $\mu\text{M}$  nucleotide) and analyzed as described in the caption to Figure 7 with blunt-ended M13mp11 DNA (*SmaI* cut) (●) and M13mp11 DNA that was nicked at a single unique site by treatment with f1 gene II protein (○) (Runyon et al., 1990). Solid curves were generated using eq 4, and the best-fit values of  $k_2$  and  $K_M$  are listed in Table I, which were determined by nonlinear least squares analysis.

values of  $k_{\text{obs}}$  obtained are plotted in Figures 7 and 8. These data were analyzed by using eq 4 to obtain  $k_2$  and  $K_M$ , and the results are given in Table I. The smooth curves describing the data were simulated by using eq 4 and the kinetic parameters in Table I. All of the experiments reported in Figures 7 and 8 were performed in a single day and with the same solutions and protein stocks in order to minimize experimental variability.

The values of  $K_M$  for all of the blunt-ended pUC8 DNA substrates are identical, although  $k_2$  is slightly lower for *SmaI*- vs *HincII*-treated DNA (Table I). The lower  $k_2$  may result from the higher G + C content that exists at the end of the *SmaI*-treated DNA, although we have not examined this possibility further. However, the  $K_M$  values for the blunt-ended DNA substrates (*SmaI* and *HincII*) are nearly a factor of 2 greater than those of the four-nucleotide 5' flanking DNA (*BamHI* and *HindIII*), although the values of  $k_2$  are similar for all of these substrates (see Table I and Figure 7). The values of  $K_M$  for DNA substrates possessing either a 3' or a



Table I: Kinetic Parameters for the Helicase II-Catalyzed Unwinding of dsDNA<sup>a</sup>

DNA substrate	$K_M$ ( $\mu$ M)	$k_2$ ( $s^{-1}$ )
Blunt-Ended DNA		
pUC8/ <i>Sma</i> I	$0.61 \pm .06$	$0.0051 \pm 0.0019$
pUC8/ <i>Hinc</i> II	$0.67 \pm .04$	$0.0061 \pm 0.0002$
Linear DNA with a Four-Base 5' Overhang		
pUC8/ <i>Hind</i> III	$0.33 \pm 0.04$	$0.0052 \pm 0.0018$
pUC8/ <i>Bam</i> HI	$0.40 \pm 0.04$	$0.0049 \pm 0.0001$
Linear DNA with a Four-Base 3' Overhang		
pUC8/ <i>Pst</i> I	$0.39 \pm 0.04$	$0.0017 \pm 0.0001$
Different M13mp11 RF Forms		
M13mp11/ <i>Sma</i> I	$0.32 \pm 0.06$	$0.0033 \pm 0.0002$
M13mp11/nicked form <sup>b</sup>	$1.6 \pm 0.1$	$0.0030 \pm 0.0007$

<sup>a</sup> Data evaluated over a helicase II protein concentration range from 0.56–1.39  $\mu$ M in the presence of 0.125  $\mu$ M DNA (nucleotide). The experiments for each set of data were all performed in a single day and in the same assay solution. <sup>b</sup> Evaluated between 1.12 and 1.39  $\mu$ M concentration of helicase II protein.

5' four-nucleotide flanking ssDNA are the same within experimental error ( $0.37 \pm 0.04$   $\mu$ M). However, the rate-limiting step, with rate constant  $k_2 = 1.7 \times 10^{-3} s^{-1}$ , is a factor of 3 lower for the DNA with the 3' ssDNA flanking region than for the DNA possessing the 5' ssDNA flanking region. Therefore, the slower rate of initiation of unwinding for the *Pst*I-treated DNA (3' four-nucleotide single-stranded flanking region) reflects a lower value of  $k_2$ .

We also compared the unwinding of linear blunt-ended vs circular M13mp11 DNA possessing a unique nick introduced by the *f1* gene II protein. These results are shown in Figure 8, and the kinetic parameters  $k_2$  and  $K_M$  are given in Table I. It is obvious from Figure 8 that the values of  $k_{obs}$  determined at low helicase II concentrations are not described well by eq 4. Therefore, the kinetic parameters  $k_2$  and  $K_M$  for this set of experiments were determined by fitting only the values of  $k_{obs}$  determined at the five highest helicase II concentrations (1.12–1.39  $\mu$ M monomer). The value of  $K_M$  determined with the blunt-ended M13 DNA is  $0.32 \pm 0.06$   $\mu$ M, which is the same within experimental error as the  $K_M$  determined with the blunt-ended pUC8 DNA (see Table I). However, the value of  $K_M$  for the nicked circular M13 DNA (1.6  $\mu$ M) is 5 times larger than for the blunt-ended M13 DNA, suggesting that helicase II binds to the nicked site with reduced affinity relative to a blunt end. On the other hand, the values of  $k_2$  ( $\sim 3 \times 10^{-3} s^{-1}$ ) are identical for both the nicked circular and the blunt-ended M13 DNA substrate, although these are nearly a factor of 2 lower than those found for the shorter pUC8 DNA.

**Effects of Solution Conditions on Helicase II-Catalyzed DNA Unwinding.** We investigated the effects of NaCl, glycerol, MgCl<sub>2</sub>, and ATP concentrations and pH on the kinetics of DNA unwinding by helicase II, and the values of  $k_2$  and  $K_M$  are reported in Table II. However, a molecular interpretation of the changes in these kinetic parameters is not possible at this time since we do not yet understand how the oligomerization state or the affinity for DNA of the helicase II protein are affected by these solution variables. Therefore, the estimates of  $k_2$  and  $K_M$  are only reported to provide a phenomenological description of the kinetics. The rate of DNA unwinding decreases significantly upon increasing NaCl (from 0 to 50 mM) and glycerol concentrations (from 10 to 15% (v/v)). Interestingly, the rate of DNA unwinding in the presence of 0.55  $\mu$ M helicase II (monomer) is optimal near pH 7.0, although the optimum pH for the ssDNA-dependent

Table II: Effects of Solution Conditions on the Kinetic Parameters for the Helicase II-Catalyzed Unwinding of dsDNA<sup>a</sup>

condition	$K_M$ ( $\mu$ M)	$k_2$ ( $s^{-1}$ )	$v_{ss}$ ( $\mu$ M ATP/ min <sup>-1</sup> )
[NaCl] (mM)			
0	$1.1 \pm 0.14$	$0.0075 \pm 0.0005$	$250 \pm 17$
10	$0.68 \pm 0.43$	$0.0048 \pm 0.0012$	$250 \pm 17$
40	$0.65 \pm 0.54$	$0.0037 \pm 0.0011$	$250 \pm 17$
[glycerol] (v/v)			
10.0%	$1.6 \pm 0.2$	$0.0068 \pm 0.0006$	$250 \pm 17$
12.5%	$0.41 \pm 0.65$	$0.0024 \pm 0.0005$	$231 \pm 16$
15.0%	$0.15 \pm 0.10$	$0.0017 \pm 0.0002$	$188 \pm 13$

<sup>a</sup> Values of  $k_{obs}$  were evaluated over a helicase II concentration range from 0.56 to 1.39  $\mu$ M in the presence of 0.125  $\mu$ M (nucleotide) blunt-ended (*Sma*I) pUC8 DNA.

ATPase activity of helicase II with denatured ssDNA is pH  $7.8 \pm 0.3$  (Runyon, 1991), consistent with a previous report (Kushner & Maples, 1988). Optimal rates of unwinding are obtained at 2.5 mM MgCl<sub>2</sub> and >0.25 mM ATP at 0.55  $\mu$ M helicase II (monomer).

## DISCUSSION

We have investigated the kinetics of initiation of unwinding of fully duplex DNA catalyzed by the *E. coli* helicase II protein. The unwinding reaction was monitored directly by using agarose gel electrophoresis and indirectly by using a continuous assay that monitors the extent and rate of ATP hydrolysis by the helicase when bound to ssDNA. The ATPase assay provides a continuous measure of DNA unwinding in the presence of excess helicase II, which ensures that the product ssDNA is coated with helicase II. Therefore, the instantaneous rate of ATP hydrolysis is proportional to the amount of helicase II bound to the ssDNA products at all times during the reaction. In both assays, helicase II protein remains bound to the ssDNA products of the unwinding reaction, thus preventing strand reannealing.

**A Model for Initiation of Helicase II-Catalyzed Unwinding of Duplex DNA.** The time course of ATP hydrolysis catalyzed by helicase II in the presence of fully duplex DNA exhibits an initial lag phase in which there is little or no ATP hydrolysis, and the rate of hydrolysis increases with time until a final steady-state rate is achieved that equals the rate on single-stranded poly(dT). This behavior differs qualitatively from that observed in the presence of ssDNA, where helicase II displays a constant rate of ATP hydrolysis with no lag phase. We conclude that initiation of unwinding by helicase II on blunt-ended and nicked circular DNA is rate-limiting compared to propagation of unwinding and that initiation involves at least one intermediate prior to propagation (see eq 2 and Figure 9). In this model, the DNA unwinding reaction is described by three distinct steps: (1) binding of helicase II to the site of initiation (ends of the duplex), (2) the rate-limiting formation of an initiation complex, and (3) rapid unwinding of the duplex DNA.

**(1) Binding of Helicase II to the Ends of Duplex DNA.** An early step in the unwinding reaction involves binding of helicase II to the ends of the duplex DNA (or the nick), which we assume to be reversible and described by equilibrium constant  $K_1$ . However, it is possible that helicase II assembles to an oligomeric form as part of this step. Helicase II can form at least a dimeric species in the absence of DNA, and helicase II dimers are stabilized upon binding DNA (Runyon et al., 1993). However, under the conditions and helicase II concentrations used in our unwinding experiments (1.5 mM ATP and 2.5 mM Mg<sup>2+</sup>) helicase II exists as a monomer in

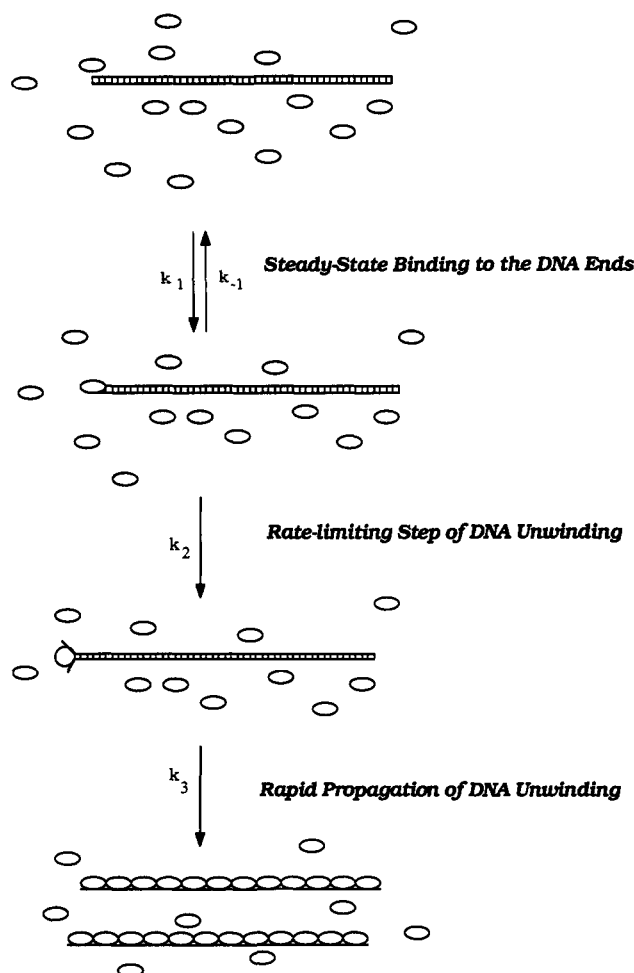


FIGURE 9: Schematic of the proposed mechanism for initiation of DNA unwinding by helicase II on fully duplex DNA (see also eq 2 in text).

solution (Runyon et al., 1993). Therefore, upon binding DNA, it is likely that helicase II assembles to form at least a dimer and that the dimer or possibly a higher-order oligomer is the active form of the helicase. This possibility is given more credence in light of the recent evidence that the *E. coli* Rep helicase, which shares significant homology with helicase II (Gilchrist & Denhardt, 1987; Daniels et al., 1992), is induced to dimerize upon binding either ss- or dsDNA, and the Rep dimer or a larger Rep oligomer appears to be the active form of the helicase since a chemically cross-linked Rep dimer retains ATPase and helicase activities (Chao & Lohman, 1991; Wong et al., 1992; Wong & Lohman, 1992). Furthermore, nearly all DNA helicases that have been examined self-assemble to form oligomeric structures, usually dimers or hexamers (Lohman, 1992, 1993). However, for the purposes of our phenomenological characterization of the unwinding kinetics, we have not considered a helicase II oligomerization step explicitly.

(2) *Formation of an Initiation Complex: The Rate-Limiting Step in DNA Unwinding.* The rate-limiting step in the DNA unwinding reaction is the formation of an initiation complex,  $(PD_{\text{end}})^*$ , which occurs with rate constant  $k_2$  (see eq 3 and Figure 9). This step may involve formation of helicase II oligomers or unwinding of the first base pair(s) of the DNA molecule. We do not know whether this initiation complex can hydrolyze ATP.

(3) *Rapid Propagation of DNA Unwinding.* The final propagation step, in which the dsDNA is unwound rapidly

and irreversibly with apparent rate constant  $k_3$ , incorporates all steps occurring after the rate-limiting step with rate constant  $k_2$  ( $k_3$  cannot be measured in our experiments since it occurs after the rate-limiting step). This final step is coupled to the binding of helicase II to the ssDNA products, yielding a final product of unwound ssDNA saturated with helicase II protein as indicated by electron microscopy studies (Runyon et al., 1990).

Since we have no independent estimates of the rate constants  $k_1$ ,  $k_{-1}$ , or  $k_2$ , we have made a steady-state assumption and describe the apparent DNA unwinding rate constant,  $k_{\text{obs}}$ , in terms of two kinetic parameters,  $k_2$  and  $K_M = (k_{-1} + k_2)/k_1$ . However, these parameters provide only a phenomenological framework for describing the kinetics and cannot be linked to precise molecular steps in the absence of additional information about the assembly state and DNA binding properties of the active helicase at the different steps in the reaction.

*Are High Helicase II Concentrations Required for Complete Unwinding of Fully Duplex DNA?* The experiments reported here require excess helicase II protein since all of the ssDNA that is produced must be saturated with helicase II to insure that ATP will be hydrolyzed in direct proportion to the concentration of ssDNA products, thereby enabling ATP hydrolysis to be used as a measure of DNA unwinding. In our reaction conditions, complete unwinding of all DNA molecules requires protein to DNA ratios greater than 1 monomer/DNA nucleotide (Runyon & Lohman, 1989; Runyon et al., 1990). However, it is not likely that all of the helicase II in these reactions is functioning to actively unwind the DNA. Part of the requirement for excess helicase II is almost certainly due to the fact that the protein is also functioning as its own helix-destabilizing protein to prevent reannealing of the unwound ssDNA, as well as to a low affinity of helicase II for nicks and blunt ends. It is possible that the level of helicase II required to unwind fully duplex DNA might be reduced if a helix-destabilizing protein, such as the *E. coli* SSB protein (Lohman et al., 1988; Sigal et al., 1972), were included in the reaction; however, we have not tested this under these conditions.

On the basis of helicase II-catalyzed DNA unwinding experiments analyzed by electron microscopy, Wessel et al. (1990) suggested that the active form of helicase II is a protein cluster that translocates along ssDNA in a 3'-to-5' direction. However, these experiments of Wessel et al. (1990) were performed in the presence of excess helicase II protein, and *E. coli* SSB protein was added after the reaction was stopped; hence it is impossible to determine whether all of the helicase II remaining on the DNA actually functioned in the unwinding reaction. At this point, one cannot rule out the possibility that unwinding requires the participation of helicase II that is bound to ssDNA at some distance from the unwinding fork, rather than only at the ssDNA/dsDNA junction. The fact that there appears to be a high basal level of helicase II in *E. coli* (Klinkert et al., 1980) and that this level increases upon SOS induction (Kumara & Sekiguchi, 1984; Nakayama et al., 1983; Siegel, 1983; Arthur & Eastlake, 1983) suggests that high concentrations of helicase II are required for at least one of its functions (Runyon & Lohman, 1989).

*The Type of DNA Ends Influences Initiation of DNA Unwinding by Helicase II.* In this study, we have examined the kinetics of initiation of unwinding and how this is influenced by the type of DNA at the initiation site. The rates of initiation of unwinding were essentially identical for DNA substrates containing blunt ends or four-nucleotide single strands flanking

the 5' ends of the duplex. However, the rates were reduced significantly when the initiation site was either a nick or a 3' four-nucleotide ssDNA flanking region. The slower rate of initiation of unwinding of the DNA substrate containing a 3' four-nucleotide ssDNA flanking region is surprising in light of the known preference of helicase II to unwind duplex DNA containing long 3' ssDNA flanking regions (Matson, 1986; Runyon & Lohman, 1989). Although it is not surprising that a four-nucleotide 3' ssDNA would be too short to facilitate initiation, we did not expect initiation to be slower relative to the four-nucleotide 5' ssDNA flanking region.

On the basis of the model described in eq 2, the slower rate of unwinding of the DNA substrate containing the 3' ssDNA flanking region, as compared to the 5' ssDNA, reflects a lower value of  $k_2$ , with no effect on  $K_M$ . Compared to the unwinding of fully blunt-ended DNA, lower values of  $K_M$  were found for DNA substrates with a four-nucleotide ssDNA flanking region, independent of whether it is on the 3' or the 5' end. This lower  $K_M$  may reflect a higher binding affinity of helicase II for the flanking ssDNA. The flanking ssDNA may also provide a site on which helicase II can more easily assemble into an active oligomeric complex (dimer?) that may be required for the propagation of DNA unwinding. The assembly of such an oligomeric complex may become rate-limiting if the flanking ssDNA is not long enough to facilitate assembly.

The highest value of  $K_M$  was measured for helicase II-catalyzed unwinding of the nicked circular M13 DNA. Again, this may reflect a lower affinity of helicase II for a nick. However, we also observed that unwinding of the nicked circular DNA is severely inhibited below helicase II concentrations of  $\sim 1 \mu\text{M}$  monomer (see Figure 8). Although the basis for this inhibition is not known, it is possible that different types of helicase II complexes are needed for initiation of unwinding at nicks vs ends.

**Implications for the Role of Helicase II in Vivo.** Helicase II is involved in UvrABC-catalyzed excision repair and methyl-directed mismatch repair of DNA (Van Houten, 1990; Modrich, 1989, 1991). The DNA to be repaired is nicked at an intermediate stage during both of these processes. Since helicase II can initiate unwinding of duplex DNA at blunt ends and internal nicks at high protein to DNA ratios in vitro (Runyon et al., 1990) and can unwind regions of duplex DNA several thousand base pairs long, it has been suggested that helicase II initiates unwinding at the nick(s) during these repair processes (Runyon & Lohman, 1989; Runyon et al., 1990). However, unwinding of fully duplex DNA in vitro requires helicase II concentrations that are considerably higher than those needed to function in methyl-directed mismatch repair in vitro (Lahue et al., 1989). It appears that high helicase II concentrations are induced during the SOS response in vivo, and it remains a possibility that these high helicase II concentrations are utilized to initiate DNA unwinding from DNA nicks or blunt ends in vivo. Blunt-ended duplex DNA is also an intermediate in a number of recombination pathways (Smith, 1988, 1991), and it is possible that helicase II may function to unwind these DNA intermediates. For instance, the "short-chunk" integration observed during bacterial conjugation (Smith, 1991) may require DNA unwinding by helicase II. The hypo-rec phenotype observed with some *uvrD* mutants (Hori & Clark, 1973; Zieg et al., 1978) as well as the hyper-rec phenotype (Feinstein & Low, 1986; Lundblad & Kleckner, 1982, 1984) may also reflect the ability of helicase II to unwind these sorts of DNA substrates in vivo (Ehrlich et al., 1990).

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