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DNA Helicase II of *Escherichia coli*

CHARACTERIZATION OF THE SINGLE-STRANDED DNA-DEPENDENT NTPase AND HELICASE ACTIVITIES*

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***Escherichia coli* helicase II has been purified to near homogeneity from cells harboring a multicopy plasmid containing the structural gene for helicase II, *uvrD*. In this paper a detailed description of the single-stranded DNA-dependent nucleoside 5′-triphosphatase and helicase reactions catalyzed by helicase II is presented. The results of this study suggest that nucleoside 5′-triphosphate hydrolysis provides the energy required for translocation of the enzyme along single-stranded DNA. Measurements of the rate of ATP hydrolysis using a variety of single-stranded DNAs of known structure and length suggest a processive translocation mechanism for helicase II. Single-stranded DNA coated with either *Escherichia coli* single-stranded DNA binding protein (SSB) or bacteriophage T4 gene 32 protein fails to support helicase II ATPase activity. Moreover, helicase II is apparently unable to displace a molecule of bound SSB protein from single-stranded DNA when it is encountered in the process of translocation along a single-stranded DNA effector.**

The helicase reaction has been characterized using an *in vitro* strand displacement helicase assay. The helicase reaction requires concomitant nucleoside 5′-triphosphatase hydrolysis that is satisfied by the hydrolysis of either rATP or dATP. As the length of duplex DNA present in the partial duplex helicase substrate is increased from 71 base pairs to 343 base pairs, the fraction of duplex DNA molecules that are unwound by helicase II decreases in the absence of any accessory proteins. However, the total number of *base pairs* of duplex DNA unwound depends primarily on the amount of enzyme added to the helicase reaction and not on the length of the duplex DNA present in the partial duplex DNA substrate. These data suggest the number of base pairs of duplex DNA unwound is directly proportional with the concentration of helicase II in the reaction mixture. In addition, the rate of the unwinding reaction is independent of the length of the duplex DNA available for unwinding. Helicase II has been shown to dissociate from single-stranded DNA molecules infrequently acting as an ATPase. However, the enzyme dissociates from partial duplex helicase substrates more frequently. This suggests a more distributive reaction mechanism on duplex DNA than was observed on single-stranded DNA substrates. The fraction of 343-base pair partial duplex DNA molecules unwound by helicase II can be increased by the addition of appropriate concentrations of *E. coli* SSB to the reaction. This suggests that helicase II and SSB may act in a concerted reaction to unwind duplex DNA.

The double-stranded structure of DNA mandates the existence of a mechanism capable of unwinding duplex DNA to provide single-stranded templates for the processes of DNA replication, repair, and perhaps recombination. A class of enzymes capable of catalyzing this reaction, the helicases, has been described in bacteriophage-infected cells (1, 2), bacterial cells (3–5), and in lower eukaryotes (6). The helicases encoded by bacteriophage T4 and phage T7 have well-documented roles in DNA replication, both in unwinding duplex DNA ahead of the advancing DNA polymerase and as part of the enzymatic mechanism responsible for synthesis of RNA primers on the lagging strand at the replication fork (1, 7). The specific role(s) of the helicases isolated from *Escherichia coli* is less clear. The *E. coli* chromosome encodes at least four helicases; helicase II (8–11), helicase III (12), rep protein (13) and the dnaB protein (14). In addition, the *E. coli* F factor encodes another helicase, helicase I (15), which may be involved in bacterial conjugation. Recently, a helicase capable of stimulating yeast DNA polymerase I was isolated from yeast cells (6). The precise physiological role of this enzyme in yeast has yet to be determined. Although a number of helicases have been identified in both prokaryotes and eukaryotes, a great deal remains to be learned regarding the role of these enzymes in DNA metabolism.

Recently a great deal of knowledge has been gained regarding *E. coli* helicase II, the product of the *uvrD* gene (8–11). Bacterial cells with mutations in this gene show several different phenotypes including increased sensitivity to ultraviolet light (16–20), an inability to carry out methyl-directed mismatch repair (21, 22), lower recombination frequencies (23), and improper transposition (24). The pleiotropic phenotype of *uvrD* mutants implicates helicase II in a number of different pathways of DNA metabolism in *E. coli*. In an effort to better understand the biochemical role of this enzyme in the cell, we have undertaken a detailed investigation of the helicase and DNA-dependent nucleoside 5′-triphosphatase (NTPase)[1] reactions catalyzed by helicase II.

Helicase II was originally purified by Richet and Kohiyama (25) as a DNA-dependent adenosine nucleoside 5′-triphosphatase (ATPase). Other rNTPs are not hydrolyzed to a

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[1] The abbreviations used are: NTPase, nucleoside 5′-triphosphatase; ATPase, adenosine nucleoside 5′-triphosphatase; NTP, nucleoside 5′-triphosphate; bp, base pair; SSB, *E. coli* single-stranded DNA binding protein; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate, ATP(γ)S, adenosine 5′-*O*-3′-(thiotriphosphate).

significant extent, and helicase II is considerably more active as an ATPase with a single-stranded DNA effector than it is in the absence of DNA or in the presence of duplex DNA (25, 26). Subsequent biochemical studies carried out by Hoffmann-Berling and colleagues (26, 27) showed the enzyme (i) to be capable of hydrolyzing both ATP and dATP and (ii) to catalyze a DNA unwinding (helicase) reaction dependent on the ATP hydrolysis reaction. The initial characterization of the helicase reaction relied on an indirect, coupled helicase assay (27, 28). For use in this assay, duplex DNA molecules were converted into substrates for helicase II by incubating with an exonuclease to generate a single-stranded DNA binding site for the enzyme. These substrate molecules were subsequently incubated with helicase II in the presence of ATP, and unwinding was detected by measuring the amount of DNA converted into a form susceptible to S1 nuclease. Using this assay, helicase II was reported to require concomitant ATP hydrolysis to accomplish the unwinding reaction and to unwind DNA nonprocessively (27, 28). A single-stranded DNA binding site is required to initiate the helicase reaction, and the amount of helicase II required for complete unwinding is a function of the length of the duplex DNA substrate (28, 29).

Recently, we and others (14, 30, 31) have developed a more direct helicase assay that measures the displacement of a radioactively labeled DNA fragment from a single-stranded circular DNA molecule. This assay for helicase activity relies on the unwinding of relatively short DNA duplexes and has been used to characterize several prokaryotic helicases (14, 30, 31). Using this direct helicase assay, it has recently been shown that helicase II unwinds DNA unidirectionally in the 3′ to 5′ direction (32). This is opposite to the direction of unwinding reported earlier (28, 29) which was determined using the indirect, coupled helicase assay. The direction of translocation exhibited by helicase II is opposite to that of helicase I (28),[2] helicase III (33), and dnaB protein (14); it is in the same direction as rep protein (34). The 3′ to 5′ direction of translocation exhibited by helicase II has been postulated to be important for its role in the excision repair of UV-irradiated DNA (32).

In this report the helicase reaction catalyzed by helicase II has been characterized using the *in vitro* strand displacement assay. The enzyme efficiently unwinds partial duplex DNA molecules with up to 340 base pairs (bp) of duplex DNA and requires concomitant ATP hydrolysis for activity as a helicase. The amount of helicase II required in the unwinding reaction is proportional with the length of duplex DNA unwound, and the rate of unwinding is independent of the length of the duplex region to be unwound. Furthermore, *E. coli* single-stranded DNA binding protein (SSB) stimulates the unwinding reaction catalyzed by helicase II. We present a model for the interaction between helicase II and duplex DNA and suggest a role for SSB in the helicase II reaction. In addition, the single-stranded DNA-dependent ATPase reaction catalyzed by helicase II has been characterized. The data presented suggest that helicase II migrates processively along single-stranded DNA using the energy provided by NTP hydrolysis to fuel translocation.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains and Bacteriophage—*E. coli* 71.18 (35), HB101 (36), HF4704rep3 (33), and KMBL3533 (8) have been described. Bacteriophage M13mp7 was grown on *E. coli* 71.18 as described (35). Phage T7 was obtained from C. Richardson (Harvard Medical School) and was grown on *E. coli* 011′ as described by Studier (37). *E. coli* B and bacteriophage T4 have been described (38). *E. coli* KMBL3533 or HF4704rep3 containing plasmid pGT26 were used for the isolation of helicase II. Plasmid pGT26 is a pBR328 derivative containing the structural gene for helicase II (8) and was kindly provided by H. Hoffmann-Berling (Max Plank Institute). *E. coli* containing plasmid RLM55, an overproducer of *E. coli* SSB, was kindly provided by Dr. R. McMacken (Johns Hopkins University).

Enzymes—Helicase II was isolated by a modification of the procedure of Richet and Kohiyama (25) as previously described (32). Helicase II used in the studies reported here is greater than 95% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and has a relative molecular mass of 76,000 g/mol. Our most active preparation of helicase II has a specific activity of 1×10^6 units/mg. One unit is defined as the amount of enzyme necessary to catalyze the production of 1 nmol of rADP in 10 min at 37 °C under the conditions of the standard assay (see below).

E. coli SSB was purified according to a procedure provided by Dr. R. McMacken (Johns Hopkins University) and was greater than 95% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Bacteriophage T4 gene 32 protein was the generous gift of Dr. B. Alberts (University of California at San Francisco). Restriction endonucleases were purchased from New England Biolabs; the reaction conditions used were those suggested by the supplier. DNA polymerase I large fragment was purchased from New England Biolabs.

DNA and Nucleotides—M13mp7 single-stranded DNA and replicative form I DNA were purified as described (39). The purification of phage T7 DNA and phage T4 DNA has been described (40). All unlabeled nucleotides and β,γ-methylene rATP were from P-L Biochemicals; ATP(γ) S and AMP-PNP were purchased from Boehringer Mannheim. [α-^{32}P]dCTP was from ICN Biomedical, Inc. and [^{3}H] NTPs were from ICN Biomedical, Inc. or New England Nuclear.

Methods

Helicase Substrate Preparation—The DNA substrate used in helicase assays consists of the ^{32}P-labeled complementary strand of a restriction fragment that has been annealed to single-stranded M13 phage DNA to create a partial duplex. The DNA restriction fragments used to construct partial duplex substrates were the products of digesting M13mp7 replicative form I DNA with *Hae*III. The resulting DNA fragments were resolved on a 6% nondenaturing polyacrylamide gel. Gel slices containing the desired DNA fragments were excised from the gel and the DNA was electroeluted. To prepare the helicase substrate, the desired restriction fragment (100 ng) and single-stranded M13 phage DNA (2 μg) were combined in a solution containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol and heated at 95 °C for 5 min to denature the DNA. The mixture was then allowed to anneal at 65 °C for 20 min. The resulting partial duplex was labeled at the 3′-OH terminus in a reaction mixture containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 40–80 μCi [α-^{32}P]dCTP, and 5 units of DNA polymerase I (large fragment). Incubation was carried out at 23 °C for 30 min. The concentration of dCTP was subsequently increased to 50 μM using unlabeled dCTP, and incubation was continued for an additional 20 min at 23 °C. Unreacted radioactive nucleotide and the complementary strand of the restriction fragment (which does not anneal to M13mp7 single-stranded DNA) were removed by gel filtration through a 1 ml Sepharose 6B or agarose A5M (Bio-Rad) column eluted with 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 100 mM NaCl. The void volume, containing the 3′-end-labeled substrate, is pooled and used directly in helicase assays. This DNA substrate is contaminated to some degree by single-stranded M13 DNA and to a lesser degree by the complementary strand of the restriction fragment.

Helicase Assay—The helicase assay measures the displacement of a ^{32}P-labeled DNA fragment from a single-stranded circular partial duplex DNA molecule catalyzed by helicase II. Reaction mixtures (20 μl) contained 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 1.8 mM ATP (unless otherwise indicated), and approximately 2 μM substrate DNA. Incubation was at 37 °C for 10 min (unless otherwise indicated), and the reaction was terminated by the addition of 10 μl of 50 mM EDTA, 40% glycerol, 0.3% sodium dodecyl sulfate, 0.03% xylene cyanol, 0.03% bromphenol blue. The products of the reaction were loaded directly onto an 8% nondenaturing polyacrylamide gel. The electrophoresis buffer was 50 mM Tris, 50 mM borate, 1 mM EDTA; electrophoresis was carried out

[2] E. E. Lahue and S. W. Matson, unpublished results.

at 70–200 V overnight depending on the length of the 3'-end-labeled fragment. The polyacrylamide gel was analyzed by film autoradiography or by slicing the gel into 1-cm sections and counting in a liquid scintillation counter.

Assay of Nucleoside 5'-Triphosphate Hydrolysis—The hydrolysis of ATP catalyzed by helicase II was assayed by measuring the formation of [^{3}H]ADP from [^{3}H]ATP as described (40). Reaction mixtures (20 μl) were identical to those for helicase reactions except that [^{3}H]ATP (6–20 cpm/pmol) was substituted for ATP at a concentration of 0.55 mM and the DNA effector concentration was varied from 16 nM to 1.6 μM. The linear partial duplex helicase substrate employed as a DNA effector in ATP hydrolysis reactions was obtained by *Bam*HI digestion of the circular 343-bp partial duplex helicase substrate. The circular helicase substrate (0.12 μg) was incubated at 65 °C for 10 min prior to the addition of $MgCl_2$ to 6 mM and 1 unit of *Bam*HI restriction endonuclease. Reaction was continued at 37 °C for 60 min, and the restriction enzyme was denatured by incubation at 65 °C for 15 min. The linear DNA was used directly in ATP hydrolysis reactions.

Other Methods—DNA concentrations were determined by directly measuring the absorbance at 260 nm and are expressed in nucleotide equivalents. The concentration of the helicase substrate has been estimated based on the known concentration of DNA in the annealing reaction mixture and assuming a 75% recovery of the DNA from the gel filtration column. Protein concentrations were determined by the method of Lowry *et al.* (41) using bovine serum albumin as the standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was by the method of Laemmli (42).

RESULTS

Helicase II Hydrolyzes ATP and dATP Preferentially—Both ATP and dATP are hydrolyzed to ADP (dADP) and P_i by helicase II in the presence of single-stranded DNA (26). Previous studies have indicated slight (25) or no (26) hydrolysis of UTP, CTP and GTP. To determine the NTP specificity of helicase II in the hydrolysis reaction, apparent K_m and V_{max} values have been measured for each of the eight predominant naturally occurring NTPs in the DNA-dependent NTPase assay using single-stranded circular M13 DNA as the effector (Table I). Five of the eight NTPs can be hydrolyzed by helicase II to varying extents, although ATP and dATP are clearly preferred. The apparent K_m values for ATP and dATP are similar at 70 μM (dATP) and 110 μM (rATP). This result is in excellent agreement with those reported previously (25, 26). The V_{max} value for the ATP hydrolysis reaction is 1450 pmol of ADP formed/10 min/unit. This is essentially the same as the V_{max} value for the dATP hydrolysis reaction (Table I). Of the six remaining NTPs tested, rCTP does not saturate helicase II at the highest concentrations used, dTTP, dCTP, and rUTP are not hydrolyzed to a detectable extent and dGTP and rGTP have apparent K_m values that are more than an order of magnitude higher than the apparent K_m determined for rATP and dATP. ATP and dATP are also the preferred NTPs as cofactors of the helicase reaction.

TABLE I

K_m or K_I for nucleoside 5'-triphosphate hydrolysis

NTP hydrolysis reactions were as described under "Experimental Procedures" using 0.2 units of helicase II. The DNA effector was single-stranded circular M13 DNA at a concentration of 3 μM. K_m values were determined from double reciprocal plots of ATP hydrolysis initial rate *versus* ATP concentration experiments. K_I values were determined from double reciprocal plots of ATP hydrolysis initial rate *versus* ATP concentration experiments at two concentrations of inhibitor.

NTP	K_m	V_{max}	K_I
	mM	*pmols/10 min/ 0.2 unit*	*μM*
rATP	0.110	290	
rCTP	3.0	[a]	
rGTP	6.4	390	
rUTP	[b]	[b]	
dATP	0.070	240	
dCTP	[b]	[b]	
dGTP	4.8	480	
dTTP	[b]	[b]	
ATP(γ)S			2
AMP-PNP			38
β,γ-methylene ATP			[c]
β,γ-methylene TTP			[c]

[a] The enzyme was not saturated at the highest concentration of NTP used.

[b] No detectable hydrolysis of NTP.

[c] No inhibition of the ATP hydrolysis reaction was detected at the highest concentration (1.05 mM) of NTP analog used.

In conjunction with the studies to determine apparent K_m values for each NTP, four NTP analogs were evaluated as inhibitors of the DNA-dependent ATPase activity of helicase II (Table I). Both ATP(γ)S and AMP-PNP are effective inhibitors of the ATPase activity of helicase II with K_i values of 2 and 38 μM, respectively. As expected from the NTP specificity of helicase II, the nonhydrolyzable analog of dTTP, β,γ-methylene dTTP, is not an inhibitor of the ATPase reaction. However, β,γ-methylene rATP also fails to inhibit the ATPase reaction suggesting that this analog does not interact with helicase II. Both ATP(γ)S and AMP-PNP are competitive inhibitors of the DNA-dependent ATPase activity of helicase II. Double reciprocal plots of initial velocity *versus* ATP concentration experiments using several concentrations of the inhibitor ATP(γ)S yield a single intercept on the ordinate as expected for a competitive inhibitor (data not shown). Similar results were obtained using AMP-PNP as the nonhydrolyzable ATP analog.

Since both ATP and dATP are hydrolyzed with similar kinetics by helicase II, it was of interest to determine whether ATP(γ)S and AMP-PNP were also inhibitors of the dATP hydrolysis reaction. Inhibition curves obtained using either [^{3}H]ATP or [^{3}H]dATP as the hydrolysis substrate and either ATP(γ)S or AMP-PNP as the inhibitor are essentially identical (data not shown). In addition, the K_I value determined for each inhibitor is not dependent on the hydrolysis substrate used. These results suggest that both ATP and dATP fit into the same active site on the protein. This is consistent with the results of others (26) demonstrating an effective competition between ATP and dATP as hydrolysis substrates of helicase II.

Nucleoside 5'-Triphosphate Hydrolysis Reflects Processive Translocation of Helicase II—*E. coli* helicase II catalyzes two distinct reactions, (i) a DNA-dependent ATPase reaction (25, 26) and (ii) a helicase reaction (27–29, 32). The ATPase activity of helicase II has a requirement for single-stranded DNA as an effector. There is little or no activity in the absence of DNA or in the presence of duplex DNA (25, 26). In addition, it has been demonstrated that helicase II unwinds DNA unidirectionally in an ATP-dependent reaction (28, 29, 32). The role of ATP hydrolysis in the unwinding reaction is not known. One possibility is that ATP hydrolysis fuels processive translocation of helicase II along single-stranded DNA. This has been shown for the GTPase activity of the gene 41 helicase of bacteriophage T4 (43) and for the TTPase activity of the gene 4 helicase of phage T7 (40). If this is also the case for helicase II, then measurements of ATP hydrolysis reaction rates may provide a qualitative measure of translocation.

Since helicase II does not hydrolyze ATP in the absence of single-stranded DNA, presumably dissociation of the enzyme from the DNA effector forces the enzyme into a conformation which is not capable of ATP hydrolysis. If ATP hydrolysis reflects processive translocation of the enzyme along single-stranded DNA, then the length of the polynucleotide serving

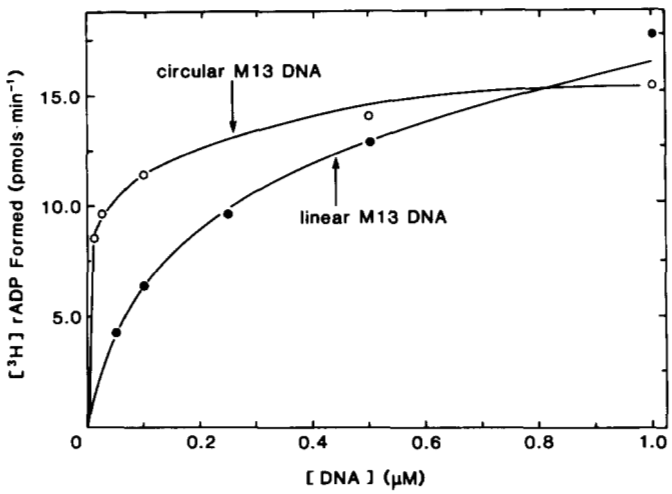

FIG. 1. **Helicase II translocates processively along single-stranded DNA.** DNA-dependent ATP hydrolysis reactions were as described under "Experimental Procedures" using 0.1 unit of helicase II. M13mp7 phage DNA was linearized using *Eco*RI which cuts the single-stranded circular DNA molecule at the hairpin formed by the polylinker sequence. ○, the DNA effector was circular single-stranded M13 DNA. ●, the DNA effector was linear single-stranded M13 DNA.

as the DNA effector is expected to have an effect on ATP hydrolysis reaction rates. This has been observed with the gene 41 protein of bacteriophage T4 (43) and the phage T7 gene 4 protein (40). In both cases, the amount of DNA required to achieve half-maximal reaction velocity decreased as the length of the DNA effector increased. This is most likely due to the fact that each enzyme migrates processively along a single-stranded DNA effector utilizing NTP hydrolysis to fuel translocation. The presence of fewer termini on longer DNA effector molecules necessitates fewer dissociation and rebinding events, and the enzyme is saturated with DNA effector at lower DNA nucleotide concentrations. If, on the other hand, the helicase II ATPase reaction reflects binding with subsequent dissociation of the enzyme from single-stranded DNA (a distributive interaction), then the amount of DNA required for half-maximal ATP hydrolysis should be the same with each DNA effector regardless of length. This has been demonstrated for the rep protein helicase (44, 45).[3]

In the following experiments, the initial rate of ATP hydrolysis was used to determine whether or not there is an effect of polynucleotide chain length on the helicase II ATP hydrolysis reaction (Fig. 1 and Table II). As the length of the polynucleotide chain serving as the DNA effector decreases, the concentration of DNA (nucleotide equivalents) required to achieve the half-maximal rate of hydrolysis (K_{eff}) increases. The DNA with the lowest K_{eff} (15 nM) is single-stranded circular M13 DNA which is, functionally, infinitely long. The K_{eff} value determined for linear M13 DNA is approximately 15-fold higher than for circular DNA although both DNA molecules contain the same number of nucleotides (Table II). The V_{max} value is not affected by the chain length (less than 20%) using naturally occurring DNAs. It is possible that the presence of the complementary strand in the preparation of linear single-stranded M13 DNA was influencing the result observed. To insure that this was not the case, the experiment shown in Fig. 1 was done using linear single-stranded DNA prepared by cleaving circular single-stranded DNA with *Eco*RI. In this experiment the K_{eff} value determined for linear M13 DNA is 16-fold higher than that determined for circular M13 DNA (Fig. 1).

[3] J. W. George and S. W. Matson, unpublished results.

TABLE II

Concentration of DNA nucleotide required for half-maximal ATP hydrolysis activity

ATP hydrolysis reactions were as described under "Experimental Procedures" using 0.2 units of helicase II and ATP at a concentration of 0.5 mM.

DNA	Length	K_{eff}	V_{max}
	nucleotides	*nM*	*pmols/min*
Oligo dT_4	4	1,000	15.0
Oligo dA_{40-60}	40–60	670	27.5
Linear[a], denatured[b] M13 DNA	7,000	250	31.2
Linear, denatured[b] T7 DNA	40,000	167	33.3
Linear, denatured[b] T4 DNA	160,000	97	35.2
Single-stranded, circular M13 DNA	∞	15	37.0
UV-irradiated single-stranded, circular M13 DNA	∞	26	ND[c]
Poly(rA)	1,000	[d]	[d]

[a] M13 RFI DNA was linearized by restricting with *Bam*HI to completion. The DNA was then dialyzed against 10 mM Tris-HCl, pH 8, 0.5 mM EDTA.

[b] Duplex DNA was denatured by heating at 95 °C for 5 min immediately prior to use in the hydrolysis assay.

[c] ND, not determined.

[d] No detectable hydrolysis.

The K_{eff} values determined for denatured phage T4 DNA and denatured phage T7 DNA are intermediate between those of circular and linear M13 DNA (Table II). This, presumably, reflects the intermediate length of these two DNA molecules. It is interesting to note that the T4 DNA used in these experiments is fully substituted with glucosylated, hydroxymethylcytosine in place of cytosine residues. The presence of this bulky sidegroup appears to have no effect on the ability of helicase II to translocate along the DNA chain as evidenced by a V_{max} value that is similar to that obtained with the other DNA molecules used.

The increase in the DNA concentration required to achieve half-maximal reaction velocity when shorter polynucleotides are used as effectors (*i.e.* linear M13 DNA, T7 DNA, and T4 DNA) might reflect an inhibition of the hydrolysis reaction caused by nonproductive binding to free ends in the reaction mixtures containing linear DNA effectors. To test this possibility, the K_{eff} value was determined using circular single-stranded M13 DNA in the presence of 1.9 μM oligo$(dA)_{40-60}$. These reactions contain approximately 38 nM 3′-OH and 38 nM 5′-PO_4 ends in addition to circular, single-stranded M13 DNA ranging in concentration from 6 nM to 3 μM. The presence of an excess of 3′-OH and 5′-PO_4 ends in the reaction mixture had no effect on K_{eff} (data not shown).

These data suggest that helicase II translocates along a single-stranded DNA molecule using the energy derived from ATP hydrolysis. The effect of chain length must be the result of a rate-limiting step involving the ends of linear DNA molecules. Either slow dissociation of the enzyme from DNA when an end is reached after completion of unidirectional translocation or slow binding to a new DNA effector are possibilities for this rate-limiting step. The low (when compared with other K_{eff} values) K_{eff} value determined with very long linear DNA molecules or circular DNA molecules must reflect the long distance over which a single helicase II molecule can translocate along a DNA molecule without dissociating. The fact that a difference in K_{eff} can be measured between T7 DNA and circular M13 DNA suggests that helicase II can processively translocate over a length of DNA greater than the unit length of T7 DNA.

Oligodeoxyribonucleotides of length 4–60, oligo(dT)$_4$ and oligo(dA)$_{40-60}$, can also effect the hydrolysis of ATP catalyzed by helicase II regardless of nucleotide sequence. However, the V_{max} value obtained using oligo(dT)$_4$ decreases significantly to less than 50% of that observed with circular M13 DNA. As shown in Table II, single-stranded RNA is not an effector of ATPase activity. Interestingly, single-stranded, circular M13 DNA that has been irradiated with ultraviolet light has a K_{eff} value essentially identical to that obtained with DNA that has not been irradiated. This result suggests that helicase II is able to translocate past a pyrimidine dimer encountered on the DNA molecule. This may be important for the role of helicase II in the repair of DNA containing pyrimidine dimers.

Single-stranded DNA Binding Proteins Inhibit Hydrolysis of ATP by Helicase II—Single-stranded DNA binding proteins have been shown to inhibit the DNA-dependent NTP hydrolysis activity of a number of helicases including helicase II (3, 5, 26, 40, 43, 46). However, the mechanism by which *E. coli* SSB inhibits the ATP hydrolysis activity of helicase II has not been determined. In addition, recent experiments indicate that *E. coli* SSB can stimulate the helicase reaction catalyzed by helicase II at appropriate concentrations (see Fig. 8). For these reasons further investigation of the inhibition of the helicase II ATPase reaction was carried out.

As the concentration of *E. coli* SSB added to the ATP hydrolysis reaction mixture is increased, the rate of ATP hydrolysis catalyzed by helicase II decreases to less than 10% of the rate observed in the absence of *E. coli* SSB (Fig. 2*A*). At SSB concentrations just sufficient to completely coat all the single-stranded M13 DNA present in the reaction mixture (0.2 μg of SSB) the rate of ATP hydrolysis has been reduced by more than 80%. These data suggest a competition between helicase II and *E. coli* SSB for binding sites on the single-stranded DNA molecule. This result is apparently due to a direct interaction between SSB and the single-stranded DNA since an increase in the DNA concentration and a commensurate increase in *E. coli* SSB concentration produces the same inhibition curve (data not shown).

In a similar experiment, helicase II was incubated with M13 DNA prior to the addition of *E. coli* SSB in order to allow a helicase II·DNA complex to become established in the absence of SSB. The DNA-binding protein was subsequently added, and the rate of ATP hydrolysis in the presence of increasing SSB concentrations was determined. As shown in Fig. 2*A*, the addition of *E. coli* SSB after helicase II has bound to single-stranded DNA also results in inhibition of the ATP hydrolysis reaction. In fact, the kinetics of inhibition are nearly identical to those observed when SSB is added to the DNA prior to the addition of helicase II. Since it is likely that helicase II migrates processively along a single-stranded DNA effector, these data suggest that the translocation of helicase II along single-stranded DNA is impeded by the presence of bound *E. coli* SSB molecules. Furthermore, helicase II is apparently unable to displace bound SSB molecules encountered in its path. These results are consistent with a previously published report (26) except that inhibition in this case is observed with a lower SSB/DNA weight ratio.

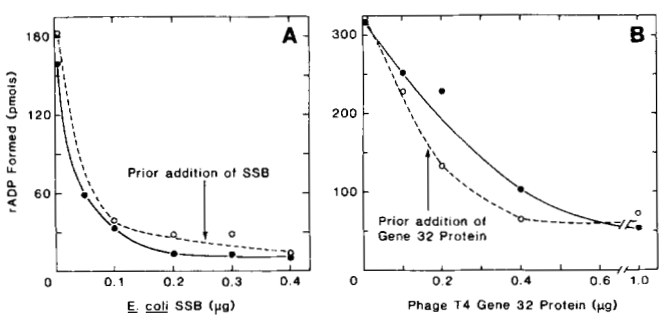

FIG. 2. **E. coli SSB and phage T4 gene 32 protein inhibit ATP hydrolysis catalyzed by helicase II.** *Panel A*, DNA-dependent ATP hydrolysis reactions were as described under "Experimental Procedures" using 0.1 unit of helicase II. ○, *E. coli* SSB, at the concentration indicated, was incubated in the reaction mixture for 5 min at 37 °C prior to the addition of helicase II; ●, helicase II was added to a 37 °C reaction mixture and 30 s later *E. coli* SSB was added to the concentration shown. *Panel B*, DNA-dependent ATP hydrolysis reactions were as described under "Experimental Procedures" using 0.2 units of helicase II. ○, phage T4 gene 32 protein, at the concentration indicated, was incubated in the reaction mixture for 5 min at 37 °C prior to the addition of helicase II; ●, helicase II was added to a 37 °C reaction mixture, and 30 s later phage T4 gene 32 protein was added to the concentration shown.

The results presented above suggest that inhibition of the helicase II ATPase activity by *E. coli* SSB results from an interaction between the DNA-binding protein and DNA. To determine whether or not there is also an interaction between *E. coli* SSB and helicase II, the effect of another single-stranded DNA binding protein, the bacteriophage T4 gene 32 protein, on the hydrolysis of ATP catalyzed by helicase II was determined (Fig. 2*B*). Increasing concentrations of gene 32 protein inhibit the hydrolysis of ATP catalyzed by helicase II. The kinetics of inhibition of the helicase II ATPase reaction are similar to those observed with *E. coli* SSB but not identical (see "Discussion"). Coating of the DNA with gene 32 protein results in approximately 60% inhibition of the helicase II ATPase reaction (0.24 μg T4 gene 32 protein). As before, a similar experiment was done in which helicase II was incubated with M13 DNA prior to the addition of gene 32 protein to allow a helicase II·DNA complex to form. The same kinetics of inhibition of the helicase II ATPase reaction were observed under these conditions as when gene 32 protein is added to the DNA prior to the addition of helicase II (Fig. 2*B*). Thus, helicase II is unable to displace bound gene 32 protein from single-stranded DNA as it is encountered during unidirectional translocation.

Taken together, these two sets of experiments strongly suggest that the inhibition of helicase II ATPase activity observed when either *E. coli* SSB or gene 32 protein are added to an ATPase reaction results from an interaction between the binding protein and DNA. There is no evidence for a specific interaction between helicase II and *E. coli* SSB. Furthermore, helicase II is unable to displace bound *E. coli* SSB or bound gene 32 protein when these molecules are encountered by helicase II as it translocates along single-stranded DNA.

An in vitro Assay for Measuring Helicase Activity—The helicase reaction catalyzed by *E. coli* helicase II was originally demonstrated using a coupled assay that measured the release of acid-soluble radioactivity by S1 nuclease. Radioactively labeled duplex DNA was converted into S1 nuclease sensitive single-stranded DNA by the action of the helicase (27–29). This assay is thought to detect the complete unwinding of a duplex DNA substrate; presumably partial unwinding reactions will not be observed due to renaturation of the substrate DNA. The DNA substrates used in this assay have been relatively long duplex DNA molecules such as bacteriophage λ DNA and linear phage fd replicative form I DNA. Since helicase II requires a single-stranded region of DNA for binding (27, 28), these duplex DNAs were partially digested with an exonuclease to provide the required single-stranded binding site prior to their use as substrate in a helicase reaction.

Recently, we and others (14, 30–32), have developed a direct assay for measuring helicase activity that utilizes a partial

duplex DNA molecule with a short region of duplex DNA. Such a DNA substrate is illustrated in Fig. 3. It consists of the complementary strand of a ^{32}P-labeled DNA restriction fragment annealed to single-stranded M13mp7 phage DNA. The length of the partial duplex is determined by the specific restriction fragment chosen and can be varied to a large extent. After incubation with a helicase, the products of the reaction are resolved on a nondenaturing polyacrylamide gel (Fig. 3). Labeled DNA fragment which has been unwound (and therefore displaced from the single-stranded M13 DNA molecule) during the helicase reaction will migrate faster in the gel than fragment which remains annealed to the single-stranded M13 DNA molecule. The helicase reaction can be quantitated by determining the percentage of labeled fragment displaced from the single-stranded M13 DNA molecule. A decided advantage of this assay is the use of a circular DNA substrate. Such a molecule can be used with helicases that translocate along single-stranded DNA in either the 5′ to 3′ direction or the 3′ to 5′ direction without biasing the result of the assay in favor of an enzyme that migrates in a specific direction. It is important to note that, in this case, the ^{32}P-DNA fragment is completely base-paired on the M13mp7 single-stranded DNA molecule; there is no single-stranded tail present on the fragment to be displaced. Such a single-stranded tail has been shown to be required to observe the helicase reaction catalyzed by either the T7 gene 4 protein or the *E. coli* dnaB protein (14, 30). There is no requirement for a DNA substrate containing a single-stranded tail in helicase reactions catalyzed by *E. coli* helicase II. However, it is not known if a substrate with a single-stranded tail would be unwound by helicase II at a greater rate than a substrate without a single-stranded tail.

The Helicase Reaction Requires Concomitant ATP Hydrolysis—Using a partial duplex DNA substrate containing 71 bp of duplex DNA (see Fig. 4), the dependence of the helicase II unwinding reaction on ATP hydrolysis has been determined (data not shown). Under the conditions of the standard helicase assay, approximately 60% of the duplex DNA substrate is unwound by 0.85 μg/ml helicase II in the presence of 1.8 mM ATP. When ATP is omitted from the reaction mixture helicase II is unable to catalyze the unwinding of the duplex DNA indicating a requirement for ATP as a cofactor for the helicase reaction. To test whether or not the hydrolysis of ATP is required, a poorly hydrolyzed ATP analog, ATP(γ)S was substituted for ATP in the helicase reaction. ATP(γ)S is a potent competitive inhibitor of the single-stranded DNA-dependent ATPase activity of helicase II, and it fails to satisfy

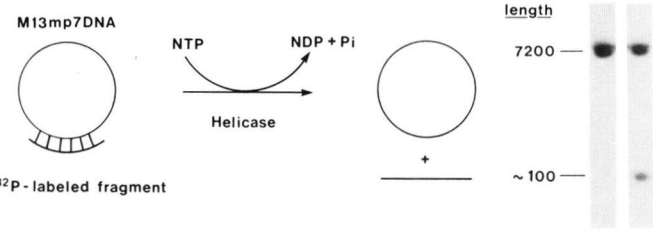

FIG. 3. **Scheme for measuring helicase activity.** The DNA substrate consists of the complementary strand of a restriction fragment that has been annealed to M13mp7 single-stranded DNA and labeled at the 3′-end. Construction of the DNA substrate is detailed under "Experimental Procedures." The products of the helicase reaction can he separated on a nondenaturing polyacrylamide gel that resolves labeled DNA fragment remaining annealed to M13mp7 DNA from labeled DNA fragment that is unwound (displaced) in the helicase reaction. The polyacrylamide gel is analyzed by film autoradiography or by slicing the gel and quantitating the fraction of displaced fragment.

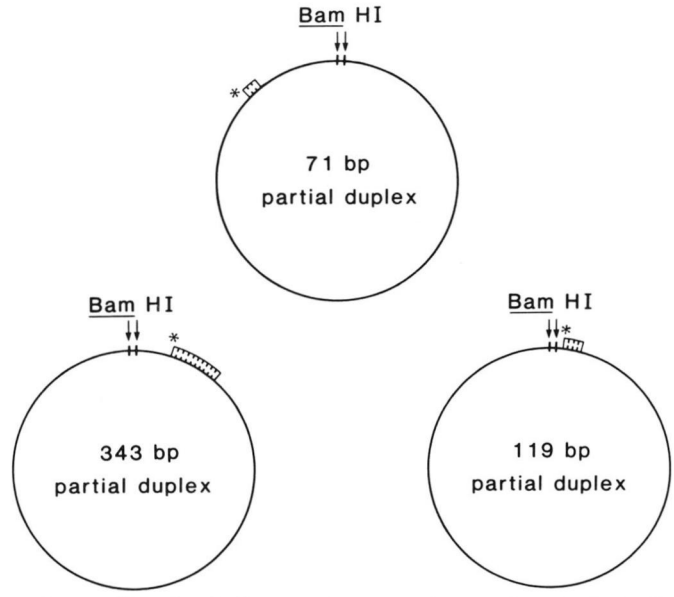

FIG. 4. **Circular helicase substrates.** The 71-bp circular helicase substrate, the 119-bp circular helicase substrate, and the 343-bp circular helicase substrate were constructed as described under "Experimental Procedures." The DNA restriction fragment was labeled at its 3′ terminus on each substrate.

the ATP requirement in the helicase reaction. In addition, when ATP(γ)S is added to a helicase reaction mixture containing ATP there is an inhibition of the helicase reaction. Taken together, these results suggest that helicase II requires concomitant ATP hydrolysis to catalyze unwinding of the partial duplex DNA substrate.

ATP and dATP are Preferred Hydrolysis Substrates in the Helicase Reaction—The helicase reaction catalyzed by helicase II requires the concomitant hydrolysis of an NTP. To determine which of the eight predominant naturally occurring NTPs would satisfy this requirement, each NTP has been tested in a helicase reaction using a 71-bp partial duplex DNA molecule as the DNA substrate (data not shown). The apparent K_m value was determined for each NTP and has been taken as a measure of the NTP preference of helicase II. Both ATP and dATP serve as NTP hydrolysis substrates in the helicase reaction with apparent K_m values of 150 and 200 μM, respectively. Of the remaining six NTPs tested, CTP, dCTP, GTP, and dGTP were able to substitute for ATP as the required NTP cofactor in the helicase reaction albeit poorly. When using any of these NTPs in place of ATP less than 15% of the labeled DNA fragment was unwound by helicase II in the presence of 3 mM NTP. Even at the highest concentrations tested (greater than 3 mM), the two remaining NTPs, UTP and dTTP, failed to satisfy the requirement for a hydrolyzable NTP cofactor as evidenced by no detectable unwinding of the DNA fragment catalyzed by helicase II. These data are consistent with previous results obtained by others (27) and with the NTP preference observed when the DNA-dependent ATPase reaction catalyzed by helicase II was analyzed. Both ATP and dATP are hydrolyzed by the DNA-dependent NTPase activity of helicase II to essentially the same extent and with nearly identical K_m values. GTP, dGTP, and CTP are the only other NTPs hydrolyzed to any detectable extent. It is also interesting to note that the apparent K_m values for ATP and dATP are similar whether helicase II is assayed as a DNA-dependent NTPase or as a helicase.

The Amount of Duplex DNA Unwound is Directly Proportional with Helicase II Concentration—Previous reports have suggested that helicase II interacts stoichiometrically with a

duplex DNA substrate (27–29). Studies with the bacteriophage T4 gene 41 protein (31) and the phage T7 gene 4 protein (30) have shown that the fraction of partial duplex substrate unwound decreases as the length of the partial duplex substrate increases. To determine the effect of the length of duplex DNA available for unwinding on the helicase reaction catalyzed by helicase II, we have systematically increased the length of duplex DNA in the helicase substrate and analyzed the helicase reaction. The DNA substrates used in this series of experiments are shown in Fig. 4. Each helicase substrate is a circular partial duplex molecule constructed as described under "Experimental Procedures." One substrate has 71 bp of duplex DNA, one substrate has 119 bp of duplex DNA, and one substrate has 343 bp of duplex DNA. The duplex region occurs at a different site on the single-stranded M13mp7 DNA molecule for each helicase substrate.

The results of experiments utilizing each of the three partial duplex DNA molecules and increasing concentrations of helicase II are presented in Fig. 5*A*. The unwinding reaction is proportional with helicase II concentration to approximately 2 μg/ml for all three helicase substrates. At this enzyme concentration slightly more than 80% of the 71-bp substrate is unwound by helicase II. Incubation with increasing concentrations of helicase II or for longer periods of time does not increase the amount of 71-nucleotide DNA fragment unwound. The reasons we are unable to achieve a greater degree of unwinding remains unknown. However, it may be partially due to reannealing of the displaced fragment during the course of the reaction. In addition, it is known that some of the radioactivity incorporated into the DNA substrate is in the long single-stranded DNA molecule and therefore not in the restriction fragment to be displaced. This is probably due to breaks introduced into the M13 phage DNA during isolation which allow DNA synthesis to occur from a 3′-OH at a hairpin on the single-stranded phage DNA molecule. If we assume that all helicase II molecules in solution are active, then at a concentration of 2 μg/ml of helicase II there are approximately 80 molecules of helicase II per DNA substrate molecule. As we show later, one reason for an excess of helicase II molecules compared with DNA substrate molecules is the fact that helicase II interacts with the duplex DNA in a protein concentration dependent reaction.

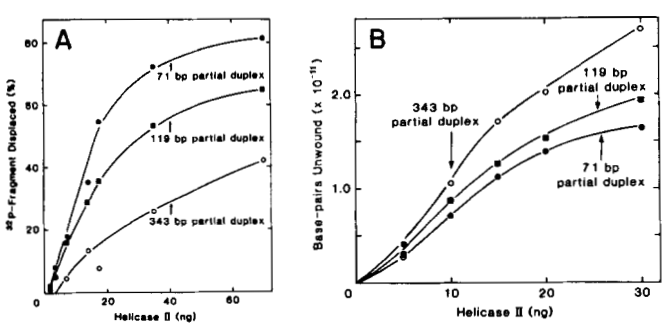

FIG. 5. **The unwinding reaction is directly proportional with helicase II concentration.** Helicase assays were as described under "Experimental Procedures" using the indicated concentration of helicase II and the indicated circular partial duplex DNA substrate. *Panel A*, the displacement reaction was quantitated as described under "Experimental Procedures" for each concentration of helicase II and each DNA substrate. The data points presented represent the average of three or more experiments. ●, 71-bp partial duplex; ■, 119-bp partial duplex; ○, 343-bp partial duplex. *Panel B*, the total number of base pairs unwound on each DNA substrate and for each concentration of helicase II was calculated from the data presented in *panel A* assuming a DNA concentration of 2 μM in each reaction. ●, 71-bp partial duplex; ■, 119-bp partial duplex; ○, 343-bp partial duplex.

Comparing the fraction of DNA fragment displaced on the 71, 119, and 343-bp DNA substrates reveals that as the length of the duplex DNA increases the fraction of the ^{32}P fragment that is unwound by a specific concentration of helicase II decreases. At a helicase II concentration of 1.75 μg/ml approximately, 75% of the ^{32}P-labeled DNA fragment is displaced from the 71-bp partial duplex while only 50% of the labeled DNA fragment is displaced from the 119-bp partial duplex and less than 30% of the labeled DNA fragment is displaced from the 343-bp partial duplex. Similar results were obtained at other concentrations of helicase II (Fig. 5*A*).

The helicase assay measures the displacement of a complete DNA fragment; partial displacement events are not observed using this assay. The fraction of 343-nucleotide DNA fragments unwound by a specific concentration of helicase II is lower than the fraction of 119-nucleotide DNA fragments or 71-nucleotide DNA fragments unwound by the same concentration of helicase II. However, at helicase II concentrations less than 0.5 μg/ml, the total number of *base pairs* of duplex DNA unwound on each partial duplex DNA substrate is essentially the same (Fig. 5*B*). This is due to the greater length of duplex DNA present in the 119 and the 343-bp partial duplex DNA substrates as compared to the 71-bp partial duplex DNA substrate. In addition, the total number of base pairs of duplex DNA unwound by helicase II is directly proportional with helicase II concentration to 0.5 μg/ml and does not appear to depend on the length of duplex DNA available for unwinding (*i.e.* on the DNA substrate used in the reaction). At helicase II concentrations greater than 0.5 μg/ml, the total number of base pairs of 71-bp partial duplex unwound begins to level off compared to the 343-bp partial duplex DNA substrate since this substrate is nearly 50% unwound (Fig. 5*B*). In the case of the 343-bp partial duplex DNA substrate, the total number of base pairs of duplex DNA unwound continues to increase linearly to a helicase II concentration of approximately 1.5 μg/ml. At this concentration of helicase II, less than 35% of the 343-bp partial duplex substrate has been unwound. Therefore, it is not surprising that the total number of base pairs unwound increases linearly to a concentration of 1.5 μg/ml on the 343-bp partial duplex while remaining linear to just 0.75 μg/ml using the 71-bp partial duplex. The total number of base pairs of 119-bp partial duplex unwound by helicase II is nearly linear up to 1.0 μg/ml which is intermediate between what is observed with the 71-bp partial duplex and the 343-bp partial duplex substrates (Fig. 5*B*). These data suggest that the amount of duplex DNA unwound by helicase II depends primarily on the amount of enzyme present in the reaction mixture and less on the length of duplex DNA being unwound. This is consistent with a mechanism of interaction between helicase II and duplex DNA that requires helicase II concentrations that are directly proportional with the duplex DNA concentration.

Using the same three partial duplex DNA substrates, the kinetics of the unwinding reaction catalyzed by helicase II have also been investigated (Fig. 6, *A* and *B*). In this series of experiments, each helicase substrate has been incubated with a specific concentration of helicase II for increasing lengths of time, and the fraction of ^{32}P fragment displaced has been quantitated. The unwinding reaction is essentially linear with time for about 10 min using each DNA substrate and reaches a maximum (different for each substrate) at about 25 min regardless of the length of duplex DNA fragment present (Fig. 6*A*). The fraction of ^{32}P-DNA fragment unwound in a specific period of time decreases as the length of the partial duplex increases. A relatively low concentration of helicase II unwinds greater than 50% of the 71-bp partial duplex in a 20-

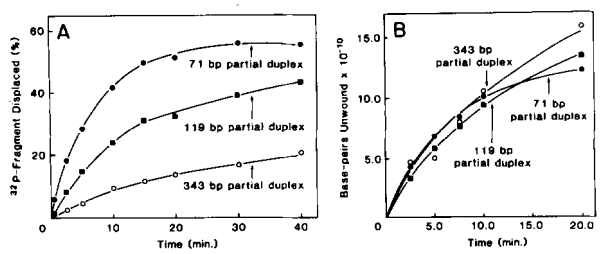

FIG. 6. **The rate of the unwinding reaction is independent of the length of the duplex to be unwound.** Helicase reactions were as described under "Experimental Procedures" using 0.85 μg/ml helicase II and the indicated circular partial duplex DNA substrates. *Panel A*, the displacement reaction was quantitated as described under "Experimental Procedures" for each time point and each helicase substrate. The data points presented represent the average of three or more experiments. ●, 71-bp partial duplex; ■, 119-bp partial duplex; ○, 343-bp partial duplex. *Panel B*, the total number of base pairs unwound at each time point on each DNA substrate was calculated from the data in *panel A* assuming a DNA concentration of 2 μM in each reaction. ●, 71-bp partial duplex; ■, 119-bp partial duplex; ○, 343-bp partial duplex.

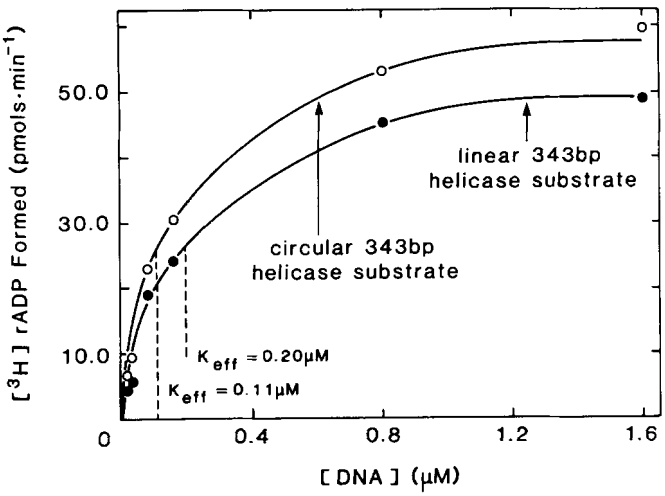

FIG. 7. **Helicase II dissociates frequently from a circular partial duplex DNA substrate.** ATPase reactions were as described under "Experimental Procedures" using 0.3 units of helicase II. The DNA effector was either a circular (○) or a linear (●) 343-bp partial duplex helicase substrate. Linear partial duplex molecules were obtained by incubating circular partial duplex DNA molecules with *Bam*HI restriction endonuclease. The *Bam*HI recognition site occurs in the single-stranded region of the circular DNA molecule and not in the duplex region of the helicase substrate. K_{eff} values were determined from a Lineweaver Burke plot of initial reaction rate *versus* DNA effector concentration. Lines were determined by linear regression analysis.

min reaction but unwinds only about 30% of the 119-bp partial duplex and less than 20% of the 343-bp partial duplex in the same period of time.

When the total number of base pairs of duplex DNA unwound in each reaction is calculated for the individual DNA substrates it is essentially the same at early times in the reaction irrespective of the length of the partial duplex (Fig. 6*B*). This suggests that the rate of unwinding catalyzed by helicase II is not influenced by the total length of duplex DNA to be unwound in the range from 71 to 343 bp.

Helicase II Dissociates Frequently from Partial Duplex DNA Substrates—The helicase substrates used in the experiments described above are all circular DNA molecules. Helicase II binds single-stranded DNA, apparently randomly, and then processively translocates in a 3′ to 5′ direction along single-stranded DNA (32), presumably utilizing the energy provided by ATP hydrolysis to fuel translocation. Therefore, helicase II approaches the 5′-end of the fragment to be displaced first no matter where it binds initially. To determine whether the unwinding reaction catalyzed by helicase II is processive or distributive with regard to translocation of helicase II through the duplex region of the DNA substrate, we have utilized the kinetic approach for analyzing translocation described above. K_{eff} values have been determined using both circular and linear *partial duplex* helicase substrates in a DNA-dependent ATPase reaction.

A comparison of the initial rate of the helicase II ATPase reaction *versus* the partial duplex DNA substrate concentration using either a linear or a circular helicase substrate is shown in Fig. 7. The linear partial duplex helicase substrate was obtained by converting a circular helicase substrate to a linear form by digesting with *Bam*HI. This restriction enzyme will cleave M13mp7 DNA at its recognition site in the hairpin formed by the polylinker region in M13mp7 DNA. The helicase substrate used as a DNA effector of the ATPase reaction in these experiments contains a duplex region of 343 bp. It is important to note that the linear helicase substrate has a long 3′ single-stranded region to which helicase II can bind and initiate 3′ to 5′ translocation toward duplex DNA (see Fig. 4).

The ATPase reaction initial rates *versus* DNA effector concentration curves are nearly the same for the linear and circular partial duplex DNA molecules. This is in contrast to what is observed using linear or circular *single-stranded* DNA effectors of the helicase II ATPase reaction (see Fig. 1). The K_{eff} value measured for the circular helicase substrate is 0.11 μM and K_{eff} value measured for the linear helicase substrate is 0.20 μM. The V_{max} value for the ATPase reaction using each DNA molecule is nearly the same (less than 10% difference). The K_{eff} values measured using linear and circular helicase substrates as DNA effectors can be compared to K_{eff} values measured using *single-stranded* linear and circular DNA molecules as DNA effectors of the helicase II ATPase reaction (Table II). The K_{eff} value determined for the linear helicase substrate is nearly the same as that determined for the linear single-stranded DNA effector. However, the K_{eff} value determined for the circular helicase substrate is 7-fold higher than that determined for the circular single-stranded DNA effector. Similar results have been obtained using linear and circular 119-bp helicase substrates as effectors of the DNA-dependent ATPase activity of helicase II.

These data suggest that helicase II dissociates from a circular DNA molecule with a region of duplex DNA more frequently than it dissociates from a single-stranded circular DNA molecule. In fact, the probability of helicase II dissociating from the partial duplex DNA substrate is essentially the same on both circular and linear DNA molecules. This is in contrast to what is observed using circular and linear single-stranded DNA effectors. In this case, the probability of dissociation from a linear molecule is much higher than the probability of dissociation from a circular DNA molecule. Thus, we conclude that helicase II dissociates more frequently from a circular partial duplex DNA molecule than from a circular single-stranded DNA molecule probably due to the presence of the duplex region of DNA. This result is consistent with results obtained previously by others (27) using a challenge technique to determine whether or not helicase II remains bound to the duplex substrate being unwound. These experiments do not distinguish between dissociation of helicase II after the duplex is unwound and dissociation of the enzyme molecules during the unwinding reaction.

E. coli SSB Stimulates the Helicase Reaction—Using the

343-bp partial duplex DNA substrate (see Fig. 4) the effect of a single-stranded DNA binding protein, *E. coli* SSB, on the unwinding reaction catalyzed by helicase II has been investigated (Fig. 8). The previous results of others (27) suggested that *E. coli* SSB inhibited the unwinding reaction catalyzed by helicase II. However, in those studies a single concentration of *E. coli* SSB was used to determine the effect of the single-stranded DNA-binding protein on the helicase reaction.

In the experiments shown in Fig. 8, several concentrations of SSB have been added to helicase reaction mixtures. In each case SSB was added just prior to the addition of helicase II. The addition of increasing concentrations of SSB stimulates the helicase reaction catalyzed by helicase II approximately 2-fold. Maximum stimulation of the helicase reaction occurs when *E. coli* SSB is at a concentration that is approximately one-third of that expected to coat the single-stranded DNA present in the reaction mixture. Concentrations of SSB above this inhibit the helicase reaction completely. This is expected as *E. coli* SSB inhibits the translocation of helicase II along single-stranded DNA. When the helicase II concentration is reduced by half essentially the same 2-fold stimulation of the unwinding reaction is observed and at the same *E. coli* SSB concentration (Fig. 8). This indicates that the effect of *E. coli* SSB on the helicase II reaction is most likely due to an interaction between SSB and the DNA substrate. Moreover, when the single-stranded DNA binding protein from bacteriophage T4, the gene 32 protein, is substituted for *E. coli* SSB the result is identical to the result shown in Fig. 8 (data not shown). Thus, there is apparently no specific interaction between helicase II and *E. coli* SSB.

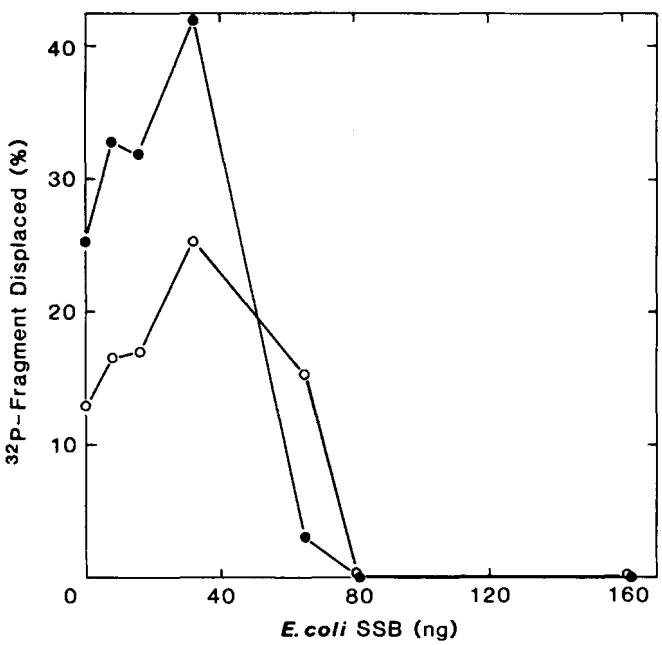

FIG. 8. **E. coli SSB stimulates the helicase reaction catalyzed by helicase II.** Helicase reactions using the 343-bp partial duplex helicase substrate were as described under "Experimental Procedures" using either 0.85 μg/ml helicase II (○) or 1.75 μg/ml helicase II (●) and the indicated concentrations of *E. coli* SSB. *E. coli* SSB was added to the reaction mixture just prior to the addition of helicase II. The helicase reaction was terminated as described under "Experimental Procedures" and proteinase K was added to a concentration of 200 μg/ml. Incubation was continued at 37 °C for 30 min to completely digest the SSB in the reaction mixture. Control experiments were performed to show that this additional step had no effect on the unwinding reaction catalyzed by helicase II. The addition of approximately 130 ng of *E. coli* SSB is expected to coat the single-stranded DNA present in the reaction mixture.

DISCUSSION

The recent discovery of the gene encoding helicase II, *uvrD* (8–11), has led to a greater understanding of the role of helicase II in *E. coli*. Cells lacking functional helicase II show an increased sensitivity to ultraviolet light (16), an increased spontaneous mutation frequency (17), a deficiency in recombination (18), and fail to carry out methyl-directed mismatch repair (22). These genetic analyses implicate helicase II in a variety of processes in the cell. In this report we have extended the biochemical studies of helicase II by characterizing the helicase and DNA-dependent ATPase reactions catalyzed by this enzyme. The *in vitro* assay developed to measure helicase activity detects the unwinding of a radioactively labeled DNA fragment from a single-stranded circular DNA molecule. An advantage of this assay is the DNA molecule chosen as a helicase substrate. The method used for construction of this DNA molecule allows for almost infinite variation in the secondary structure of the final helicase substrate. Molecules with long duplex regions or single-stranded tails can be easily constructed. In addition, both linear and circular partial duplex helicase substrates can be conveniently made. In this study we have used partial duplex DNA substrates with 71 bp of duplex DNA, 119 bp of duplex DNA, and 343 bp of duplex DNA to probe the biochemical mechanism of the helicase II unwinding reaction.

Helicase II catalyzes the unwinding of a 343-bp DNA fragment in the absence of any additional proteins. There is no absolute requirement for a single-stranded tail on the fragment to be displaced as has been observed for the *E. coli* dnaB protein (14) and bacteriophage T7 gene 4 protein (30). Displacement of each of the three different length DNA fragments used in this study is proportional with enzyme concentration. The helicase reaction catalyzed by helicase II is also linear with time for somewhat less than 10 min at 37°C.

To date, all helicases have been shown to be DNA-dependent NTPases (3). The degree of specificity for NTP in the hydrolysis reaction determined for helicase II is similar to that exhibited by other prokaryotic helicases (12,14,40,43,46). However, the apparent K_m value of approximately 100 μM determined for helicase II is lower than observed for all other helicases except rep protein (46). In addition, the helicase reaction depends on ATP hydrolysis. Simple interaction between helicase II and a poorly hydrolyzed ATP analog is not sufficient to promote an unwinding reaction. Only ATP and dATP are able to serve effectively as hydrolysis substrates in the helicase reaction. The apparent K_m value for these two NTPs is essentially identical at approximately 200 μM. This is consistent with what was observed when helicase II was analyzed as a DNA-dependent ATPase. Thus, we conclude that the ATP hydrolysis reaction catalyzed by helicase II reflects the helicase reaction or at least some aspect of it such as the unidirectional translocation of the enzyme along single-stranded DNA.

The experiments presented in Fig. 1 and Table II suggest (but do not prove) that helicase II translocates processively along single-stranded DNA using the energy of ATP hydrolysis to fuel translocation. This fact, coupled with the knowledge that the helicase reaction requires ATP hydrolysis, suggests a role for ATP hydrolysis in allowing helicase II to reach duplex DNA. These experiments do not allow a determination of whether or not ATP hydrolysis is required for the enzyme to move through duplex DNA. Presumably ATP hydrolysis is required for the unwinding event. Direct proof of this will require helicase substrates that contain substantial amounts of duplex DNA in relation to the amount of single-stranded DNA present.

Helicase II is capable of displacing (unwinding) DNA fragments 71, 119, and 343 nucleotides in length. However, the fraction of ^{32}P-DNA fragment that is displaced decreases as the length of the duplex region increases. Careful analysis of this data reveals that the same number of base pairs of duplex DNA are being unwound by a specific concentration of helicase II with each DNA substrate. Therefore, the number of base pairs of duplex DNA unwound is directly proportional with the concentration of helicase II in the reaction mixture. Based on this data a model for the interaction between helicase II and duplex DNA is presented in Fig. 9. Helicase II translocates in a 3′ to 5′ direction to the junction of single-stranded and double-stranded DNA (32). As the first enzyme molecule invades the duplex region, another molecule of helicase II moves in behind the first molecule and helps to maintain the unwound DNA in a single-stranded conformation. The complex of helicase II molecules and partially unwound DNA is then joined by another molecule of helicase II and so on until the duplex DNA is completely unwound. Such a model accommodates the requirement for amounts of helicase II proportional to the amount of duplex DNA to effect the complete unwinding of the double-stranded region. The data presented in Fig. 7 suggest that helicase II molecules are dissociated from the DNA substrate in the course of the unwinding reaction. Whether this occurs as the DNA is being unwound or after the unwinding reaction is complete is not known. However, the results do suggest that the likelihood of helicase II dissociating from the DNA substrate is greater during an unwinding reaction than it is when helicase II is migrating along a single-stranded DNA molecule.

Since no cooperativity between helicase II molecules is observed in the kinetics of the helicase reaction it is not clear why helicase II molecules should become concentrated at the region of duplex DNA. Under the reaction conditions used, there are between 8 and 80 molecules of helicase II per DNA substrate molecule. It is presumed that initial binding is random, and therefore the enzyme molecules should be evenly distributed, at least initially, along the DNA substrate. It is possible that movement of helicase II through duplex DNA is slow compared to translocation along single-stranded DNA, and therefore helicase II molecules begin to concentrate behind the first enzyme molecule to reach the duplex region. This may help to force enzyme molecules through duplex DNA. Alternatively, helicase II could bind preferentially at the single-stranded/double-stranded DNA junction and subsequently complex with duplex DNA to effect the unwinding reaction. Measurements of the rate of movement through duplex DNA and on single-stranded DNA will help to resolve this question.

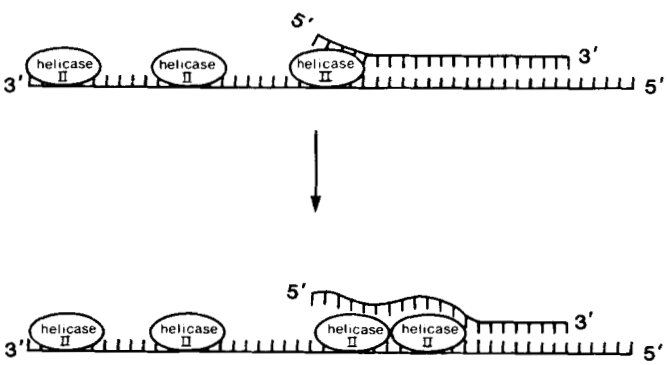

FIG. 9. **Model for the helicase reaction catalyzed by helicase II.** Helicase II translocates processively in a 3′ to 5′ direction along single-stranded DNA. Presumably the energy provided by ATP hydrolysis is used to fuel translocation of the enzyme to the junction of duplex and single-stranded DNA. For reasons that are currently unknown, helicase II molecules become concentrated at the single-stranded DNA:duplex DNA junction. This allows unwinding of the duplex DNA using concentrations of helicase II that are directly proportional to the concentration of duplex DNA.

A concentration-dependent interaction between a helicase and duplex DNA is not expected for a helicase that unwinds DNA ahead of the advancing DNA polymerase at the replication fork. Such a replicative helicase would be expected to move through duplex DNA catalytically, perhaps with the aid of additional proteins. The available genetic evidence (16–24) suggests a role for helicase II in many processes that require unwinding of short lengths of duplex DNA. This is entirely consistent with a requirement for stoichiometric amounts of helicase II in the unwinding reaction. In fact, helicase II is induced as part of the SOS response when greater amounts of the protein may be required. Thus the concentration-dependent mechanism by which helicase II unwinds duplex DNA seems to be reasonable in light of its apparent role in unwinding short duplex regions of DNA in the cell.

Although helicase II is able to unwind 343 base pairs of duplex DNA in the absence of additional proteins, this reaction is stimulated approximately 2-fold by *E. coli* SSB. The stimulation of the helicase reaction is due to an interaction between SSB and the DNA substrate for the following reasons: (i) a reduction in the concentration of helicase II in the reaction mixture results in essentially the same stimulation by SSB at the same SSB concentration and (ii) a similar stimulation of the helicase reaction can be achieved using the gene 32 protein from bacteriophage T4. A possible explanation for the stimulation of the helicase reaction is that *E. coli* SSB or the phage T4 gene 32 protein may help to maintain single-stranded DNA in a denatured conformation and thereby prevent reannealing of the duplex DNA that has been unwound by helicase II. This might be reflected by a change in the helicase II reaction mechanism from concentration-dependent to catalytic or by an increase in the unwinding rate. These possibilities have not been tested. Alternatively, *E. coli* SSB may artificially increase the concentration of helicase II near the duplex region by competing for available binding sites on the remaining single-stranded DNA. Helicase II is unable to form a complex with DNA that is coated with SSB and furthermore will not displace bound SSB encountered in its path. The presence of SSB could cause a higher fraction of the helicase II molecules to bind at or near the duplex region of the DNA substrate. At present we are unable to distinguish between these possibilities.

Although *E. coli* SSB and phage T4 gene 32 protein stimulate the helicase reaction catalyzed by helicase II, both proteins inhibit the helicase II DNA-dependent ATPase reaction. It is interesting to note the differences in the inhibition curves obtained using these two DNA-binding proteins (Fig. 2). Using *E. coli* SSB the slope of the line defined by the initial portion of the inhibition curve is much greater than the slope of the corresponding line for the gene 32 protein inhibition curve. In fact, if the line between the first two points of the SSB inhibition curve is extrapolated to the *abscissa*, the intersection is at 0.06 μg of SSB per reaction mixture. This is one-third of the SSB required to coat the single-stranded DNA present in the reaction mixture. A similar extrapolation of the gene 32 protein inhibition curve yields an intercept of 0.5 μg gene 32 protein, approximately 2-fold more gene 32 protein than is required to coat the single-stranded DNA in the reaction. One explanation for these results derives from the different binding characteristics of the two single-stranded DNA binding proteins and the mechanism by which

helicase II interacts with single-stranded DNA. Phage T4 gene 32 protein binds cooperatively to single-stranded DNA; *E. coli* SSB does not bind cooperatively under the conditions used (47, 48). At SSB concentrations lower than that required to coat the single-stranded DNA, SSB protein molecules are expected to be relatively evenly disbursed over the DNA molecules present in the reaction mixture. Helicase II can translocate processively along single-stranded DNA and does not displace bound SSB molecules encountered in its path. Thus, the presence of even one molecule of SSB on a DNA molecule will impede the translocation of helicase II and presumably inhibit ATP hydrolysis. Gene 32 protein, on the other hand, binds cooperatively to single-stranded DNA and therefore will coat some DNA molecules while leaving others free of bound protein at a low concentration of gene 32 protein. In this case some fraction of the DNA molecules present in the reaction mixture will be free of bound protein. These DNA molecules should serve as effectors on which helicase II can bind and translocate with concomitant ATP hydrolysis.

The different inhibition curves obtained using either *E. coli* SSB or gene 32 protein may reflect just such a situation. The pronounced inhibition of helicase II ATP hydrolysis at low SSB concentrations may indicate that processive translocation of helicase II has been impeded by the binding of one or a few SSB molecules to each DNA molecule. It should be noted that such an effect would not be expected if the interaction between helicase II and single-stranded DNA were distributive. The less pronounced inhibition of ATP hydrolysis by the gene 32 protein would be expected since some DNA molecules contain many bound gene 32 protein molecules while other DNA molecules are free of bound gene 32 protein. The latter DNA molecules represent good effectors of the helicase II ATPase reaction. Thus the different inhibition curves obtained with the two DNA binding proteins may provide further evidence to support a processive translocation mechanism for helicase II.

The current view of the helicase reaction catalyzed by helicase II is presented in Fig. 9. The unwinding of duplex DNA requires amounts of helicase II directly proportional with the amount of duplex DNA unwound, and the rate of unwinding is independent of the length of duplex being unwound. Although the unidirectional translocation of helicase II along single-stranded DNA appears to be processive, the enzyme dissociates more frequently from DNA substrates containing regions of duplex DNA. Whether dissociation occurs within the region of duplex DNA or at the end of the displacement reaction is unknown. It is possible that some of the helicase II molecules are bound on the fragment being displaced and are dissociated from the circular DNA molecule when the fragment is displaced. The hydrolysis of ATP is required, at least for movement along single-stranded DNA, and most likely for movement through regions of double-stranded DNA. This reaction mechanism is suitable for an enzyme that is primarily responsible for unwinding short lengths of duplex DNA. Thus, this view of the helicase reaction catalyzed by helicase II is consistent with the role helicase II is currently thought to play in nucleic acid metabolism in the cell.

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