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Biochemical Characterization of the DNA Helicase Activity of the *Escherichia coli* RecQ Helicase*

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We demonstrate that RecQ helicase from *Escherichia coli* is a catalytic helicase whose activity depends on the concentration of ATP, free magnesium ion, and single-stranded DNA-binding (SSB) protein. Helicase activity is cooperative in ATP concentration, with an apparent $S_{0.5}$ value for ATP of 200 μM and a Hill coefficient of 3.3 ± 0.3. Therefore, RecQ helicase utilizes multiple, interacting ATP-binding sites to mediate double-stranded DNA (dsDNA) unwinding, implicating a multimer of at least three subunits as the active unwinding species. Unwinding activity is independent of dsDNA ends, indicating that RecQ helicase can unwind from both internal regions and ends of dsDNA. The K_M for dsDNA is 0.5–0.9 μM base pairs; the k_{cat} for DNA unwinding is 2.3–2.7 base pairs/s/monomer of RecQ helicase; and unexpectedly, helicase activity is optimal at a free magnesium ion concentration of 0.05 mM. Omitting *Escherichia coli* SSB protein lowers the rate and extent of dsDNA unwinding, suggesting that RecQ helicase associates with the single-stranded DNA (ssDNA) product. In agreement, the ssDNA-dependent ATPase activity is reduced in proportion to the SSB protein concentration; in its absence, ATPase activity saturates at six nucleotides/RecQ helicase monomer and yields a k_{cat} of 24 s^{-1}. Thus, we conclude that SSB protein stimulates RecQ helicase-mediated unwinding by both trapping the separated ssDNA strands after unwinding and preventing the formation of non-productive enzyme-ssDNA complexes.

DNA helicases are the motor proteins responsible for separating the individual strands of dsDNA[1] to provide ssDNA for key cellular processes such as DNA repair, recombination, and replication (for reviews see Refs. 1 and 2). An important group of DNA helicases is the RecQ helicase family (for review see Ref. 3); members of this group span a wide cross-section of organisms, including bacteria (4), yeast (5–7), fungi (8), fly (9), frog (10), and humans (11–14). The proteins in this family share extensive amino acid homology within seven characteristic helicase motifs (15) and display common biochemical attributes (16–19). In the yeast *Saccharomyces cerevisiae*, loss of Sgs1 helicase, a RecQ helicase homologue, leads to premature aging due to genetic instability at the rDNA locus (20, 21). In humans, there are five homologues of RecQ helicase, and mutations in two of these, the WRN and BLM helicases, are responsible for Werner and Bloom syndromes, respectively (12, 17). A third human homologue, RECQ4, has recently been implicated in a subset of cases of Rothmund-Thompson syndrome (14, 22). A common phenotype exhibited by these diseases is pronounced genomic instability. Thus, RecQ helicase-like proteins in eukaryotes appear to be important in maintaining genomic stability (for review see Ref. 23).

The role in DNA metabolism played by the majority of the RecQ helicase family members remains to be determined; however, the function of the RecQ helicase from *Escherichia coli*, the founding member of this family, is now becoming clear. The gene that encodes RecQ helicase, *recQ*, was originally identified in *E. coli* as a thymineless death-resistant mutant in *thyA* cells (24). The same *recQ* null mutation also reduced conjugal and plasmid recombination by up to 100-fold in a *recB(C)sbcCD* background (24). In addition, loss of *recQ* function in the same genetic background rendered *E. coli* cells highly sensitive to UV irradiation and other DNA-damaging agents (25, 26). These results were consistent with the product of this gene being needed for homologous recombination mediated by the RecF recombination pathway (27). Furthermore, *recQ* function is required to suppress illegitimate recombination; *E. coli* cells lacking an intact copy of the *recQ* gene display 30–300-fold higher levels of non-homologous recombination between λ Spi^- phage and the *E. coli* chromosome (28).

The recombination functions of RecQ helicase have been examined *in vitro* using purified components (29). RecQ helicase was found to unwind a dsDNA substrate to provide a suitable ssDNA substrate for binding by RecA protein. These findings were interpreted as a clear indication that RecQ helicase acts to initiate homologous recombination mediated by the RecF recombination pathway. RecQ helicase was also capable of disrupting homologous pairing intermediates formed by RecA protein, in addition to unwinding a broad spectrum of other DNA substrates. These results suggested that RecQ helicase may antagonize illegitimate recombination by unwinding aberrantly paired DNA molecules formed at regions of limited homology. Thus, RecQ helicase is a multifunctional protein in DNA recombination, which appears to be responsible for both promoting homologous recombination and discouraging illegitimate recombination.

An alternative means by which RecQ helicase may suppress illegitimate recombination events is through its functional interaction with *E. coli* topoisomerase III (Topo III). Together these two proteins display a strong DNA strand passage activ-

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[1] The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; scDNA, covalently-closed supercoiled DNA; bp, base pairs; nt, nucleotides; SSB, single-stranded DNA binding; PAGE; polyacrylamide gel electrophoresis; P-enolpyruvate, phosphoenolpyruvate; DTT, dithiothreitol; H33258, Höechst 33258; ATPγS, adenosine 5′-*O*-(thiotriphosphate); kbp, kilobase pair; Topo III, topoisomerase III.

ity *in vitro*, which is both absent from the individual proteins and requires ATP. Fully catenated dsDNA molecules are produced by these two proteins when RecQ helicase unwinds a covalently closed dsDNA substrate to provide a preferential substrate for Topo III. A similar activity is observed with RecQ helicase and yeast Topo III from *S. cerevisiae*. This is consistent with the finding that yeast Topo III and SGS1 helicase interact both genetically and physically (5). A physical interaction between the BLM helicase and human Topo III has also been reported (30). In both yeast and human cells, the cognate RecQ helicase homologues are needed to suppress recombination events (5, 12). Based on the similarities between the prokaryotic and eukaryotic systems, it stands to reason that in *E. coli* the combined activities of RecQ helicase and Topo III are also important for suppressing recombination events.

Earlier characterizations of RecQ helicase had assayed its helicase activity using agarose gel electrophoresis and employing DNA substrates that generally consisted of an ssDNA fragment (between 70 and 350 bp in length) annealed to either a full-length circular M13 ssDNA molecule to generate a partially dsDNA substrate or a homologous partner of the same length to make a fully dsDNA substrate (16, 31). In these experiments, RecQ helicase was found to translocate $3' \rightarrow 5'$ along the DNA strand to which it is bound. In addition, this helicase displayed the capacity to unwind both partial ssDNA and full-dsDNA substrates. Unwinding by RecQ helicase was also greatly stimulated by both *E. coli* SSB protein and phage T4 gene 32 protein. However, agarose gel electrophoresis assays have the limitation that unwinding is scored only when the DNA substrate is unwound (*i.e.* the complete dissociation of the dsDNA substrate) and, therefore, do not directly provide information concerning the initial rate of dsDNA unwinding by RecQ helicase. To characterize further the helicase activity of RecQ helicase, with respect to both its initial rate and the final extent of unwinding, we examined the activity of RecQ helicase on plasmid-length (>2 kbp) dsDNA substrates using a continuous fluorescent dye-displacement assay (32).

Here we describe experiments that examine the effects on RecQ helicase activity of buffer components, DNA concentration and composition, enzyme concentration, and SSB protein concentration. We find that RecQ helicase is capable of fully unwinding the plasmids pUC19 and pBSKM, which are 2.7 and 2.9 kbp in length, respectively. As expected, unwinding by RecQ helicase is dependent on a hydrolyzable nucleotide cofactor and is stimulated by *E. coli* SSB protein. Unexpectedly, the helicase activity of RecQ helicase is sensitive to the concentration of both ATP and free magnesium ion. We also provide the first evidence that RecQ helicase functions as a multimer: the helicase activity of RecQ helicase displays a cooperative dependence on ATP concentration, which yields an apparent $S_{0.5}$ value of 200 μM and a Hill coefficient of 3.3 ($\pm$0.3). A Hill coefficient of 3 or greater suggests that at least three interacting ATP-binding sites are utilized by the enzyme to mediate unwinding. RecQ helicase does not display a preference for initiating unwinding from dsDNA ends compared with internal regions of dsDNA: the kinetic parameters for DNA unwinding are similar when the helicase was provided with three separate DNA substrates that differed in the ratio of the total number of base pairs to dsDNA ends. Finally, we find that SSB protein is needed not only to trap the displaced ssDNA strands produced by RecQ helicase-mediated unwinding but is also important to prevent the formation of abortive RecQ helicase-ssDNA complexes.

EXPERIMENTAL PROCEDURES

Reagents—Chemicals were reagent grade, and all solutions were prepared using Barnstead NanoPure water. P-enolpyruvate and dATP were purchased from Sigma, and ATP was from Amersham Pharmacia Biotech. ATPγS was from Roche Molecular Biochemicals. Nucleotides were dissolved as concentrated stock solutions at pH 7.5, and the concentrations were determined spectrophotometrically using an extinction coefficient at 260 nm of 1.54×10^4 M^{-1} cm^{-1}. The fluorophore, Höechst 33258 (H33258) dye (Molecular Probes, Eugene, OR), was dissolved as a concentrated stock solution in water, and the concentration was determined spectrophotometrically using an extinction coefficient at 344 nm of 4.6×10^4 M^{-1} cm^{-1}.

Proteins—RecQ helicase was purified, and its concentration was determined as described previously (29). The specific activity of the RecQ helicase preparation used here was 2×10^5 ATPase units/mg as defined by Umezu *et al.* (16) and 1×10^6 helicase units/mg as defined by Roman and Kowalczykowski (33). SSB protein was purified from *E. coli* strain RLM727 as described (34), and its monomer concentration was determined spectrophotometrically using an extinction coefficient at 280 nm of 3.0×10^4 M^{-1} cm^{-1} (35). The restriction endonucleases *Hin*dIII and *Ban*I were from New England Biolabs. Pyruvate kinase and lactate dehydrogenase were from Sigma. Wheat germ topoisomerase I was from Promega.

DNA—pUC19 scDNA was purified using alkaline lysis followed by CsCl-ethidium bromide equilibrium centrifugation (36). Linear pUC19 dsDNA was produced by incubating pUC19 scDNA with either *Hin*dIII or *Ban*I for 1 h at 37 °C under the conditions recommended by the vendor. The DNA was recovered by ethanol precipitation following phenol extraction. pBSKM nicked dsDNA, produced by digestion of pBSKM scDNA with the M13 gene2 protein, was the kind gift of Dr. Eugene Zaitzev (University of California, Davis). The concentration of dsDNA was determined using an extinction coefficient at 260 nm of 6500 M (bp)$^{-1}$ cm^{-1}. Poly(dT) was purchased from Amersham Pharmacia Biotech, and its concentration was determined using an extinction coefficient at 260 nm of 8520 M (nts)$^{-1}$ cm^{-1}.

Helicase Assays—RecQ helicase-mediated unwinding was monitored using a continuous fluorescent dye-displacement assay (32). The standard reaction buffer consisted of 25 mM Tris acetate (pH 7.5), 300 nM H33258 dye, 0.1 mM DTT, and an ATP regeneration system composed of 1 mM P-enolpyruvate and 25 units ml^{-1} pyruvate kinase. Standard conditions were reactions (300 μl total volume) having 1 mM magnesium-acetate, 1 mM ATP, 1 μM SSB protein, 2.5 μM (bp) linear dsDNA, and 100 nM RecQ helicase. When the unwinding of relaxed pUC19 dsDNA substrate was assayed, 20 units (as defined by the vendor) of wheat germ topoisomerase I were present in the reaction. All reactions were carried out at 37 °C with constant stirring. Fluorescent measurements were carried out on an SLM 8100C spectrofluorometer (SLM-Aminco, Rochester, NY) with the photomultiplier tube voltage set to 800 V, a gain setting of 1, and the excitation and emission wavelengths set to 344 and 487 nm, respectively. The bandwidths for both excitation and emission were 8 nm. The total change in fluorescent signal corresponding to 100% unwinding (ΔF_T) was determined for each assay by subtracting the value for the free H33258 dye from the fluorescence obtained for the dye-dsDNA complex. This is possible since the fluorescence of the H33258 dye is not altered by ssDNA (32). The extent of unwinding (*i.e.* the percentage of total DNA unwound) was calculated by dividing the observed change in fluorescence at the completion of unwinding (ΔF_{obs}) by ΔF_T. The initial rate of unwinding in nM (bp) s^{-1} was calculated by multiplying the absolute value of the initial slope of the unwinding curve by the DNA concentration in nM (bp), and then dividing this product by ΔF_T. Where indicated, the concentration of free magnesium ion was determined using the CHELATOR software package (Dr. J. M. Schoenmakers, University of Nijmegen, The Netherlands).

Reactions that were analyzed by agarose gel electrophoresis were carried out as above, including the presence of 300 nM H33258, except that aliquots of the reaction were taken at the indicated times, and the reaction was stopped with 1% SDS and 50 mM EDTA. These samples were then loaded onto a 1% agarose gel and run in 1× TAE buffer at 1.5 V/cm for 12 h. Gels were stained with 0.5 μg/ml ethidium bromide in water for 1 h followed by extensive (>2 h) de-staining with water. The amount of DNA in each band was determined using ImageQuant software.

PAGE Bandshift Assays—Binding of RecQ helicase to the synthetic DNA substrates was analyzed using non-denaturing PAGE bandshift assays as described previously (29). The sequences of the oligonucleotide substrates were as follows: oligo 1, 5′-TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATTGC-3′; and oligo 2, 5′-GCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGA-3′. The ssDNA substrate was oligo 1, and the 48-bp dsDNA substrate was oligo 2 annealed to oligo 1 as described previously (29). Reactions contained RecQ helicase from 1 nM to 1.0 μM, and either the

ssDNA substrate or the dsDNA substrate at a concentration of 960 nM (nts) or 960 nM (bp), respectively. Complexes were formed by incubation at 37 °C for 4 min in binding buffer (25 mM Tris acetate (pH 7.5), 1.2 mM magnesium acetate, 28 mM NaCl, 0.1 mM DTT, 1 mM ATPγS, and 14% glycerol), and then they were immediately loaded onto a 6% polyacrylamide gel (30:1 acrylamide:bisacrylamide). Electrophoresis was carried out at 380 V for 1–1.5 h in a Tris glycine buffer (25 mM Tris, 190 mM glycine, and 0.1 mM EDTA). Gels were dried onto DEAE paper (Whatman) and visualized using a Storm PhosphorImager (Molecular Dynamics). The concentration of DNA in bands corresponding to free DNA substrate was determined using ImageQuant software. Apparent binding affinity (M^{-1}) was calculated as the reciprocal of the RecQ helicase concentration at which half the available DNA substrate was bound.

ATP Hydrolysis Assays—The hydrolysis of ATP by RecQ helicase was monitored spectrophotometrically as described previously (37). The standard reaction buffer consisted of 25 mM Tris acetate (pH 7.5), 1 mM magnesium acetate, 0.1 mM DTT, 1 mM P-enolpyruvate, 25 units ml^{-1} pyruvate kinase, 25 units ml^{-1} lactate dehydrogenase, 150 μM NADH, and 1 mM ATP. Reactions had the indicated concentration of RecQ helicase, and 1.0 μM (nt) heat-denatured *Hin*dIII-cut pUC19 linear DNA. SSB protein was present, where indicated, at a concentration of 1 μM. All reaction components, except for SSB protein and RecQ helicase, were incubated together at 37 °C for 2 min prior to initiation of the reaction by the addition of a mixture of SSB protein and RecQ helicase.

RESULTS

RecQ Helicase Unwinds Plasmid-length dsDNA Substrates—Unwinding by RecQ helicase was monitored using a continuous fluorescent dye-displacement assay (32), previously used to monitor the unwinding of oligonucleotide-derived substrates by RecQ helicase (29). This assay is based on the observation that the binding of certain fluorescent dyes, such as H33258 dye, to dsDNA results in a large increase in fluorescent signal relative to that for the dye in the presence of ssDNA (Fig. 1*A*). Helicase-mediated unwinding of this highly fluorescent dsDNA-H33258 dye complex results in a decrease in the fluorescent signal that is proportional to the amount of ssDNA produced (32). Therefore, the rate and extent of helicase-mediated unwinding can be determined from the change in fluorescence that accompanies unwinding of the dsDNA-H33258 dye complex.

The initial dsDNA substrate used here was the plasmid pUC19 digested with *Hin*dIII to yield a single 2.7-kbp fragment with nearly blunt dsDNA ends. The standard H33258 dye concentration of 300 nM was empirically determined by analyzing the effect of dye concentration on the unwinding activity of RecQ helicase (data not shown); this concentration of H33258 dye was chosen because the unwinding activity of RecQ helicase, as measured by DNA strand separation in agarose gel electrophoresis assays, was unaffected by the presence of the dye, and this amount of dye was sufficient to determine accurately the initial rate of unwinding (32). Typical time courses for RecQ helicase-mediated unwinding of this DNA substrate (in the presence of 1 μM SSB protein) appear in Fig. 1*B*. Addition of RecQ helicase to the linear pUC19 dsDNA-H33258 dye complex and subsequent unwinding results in a rapid decrease in the fluorescent signal. An incremental increase in the RecQ helicase concentration from 10 to 100 nM resulted in a higher rate of DNA unwinding (Table I). Note that the observed extent of unwinding calculated for 10 nM RecQ helicase is likely to underestimate the true extent of unwinding because the fluorescent signal at 10 nM RecQ helicase continued to decrease slowly beyond the end of the 10-min time course presented in Fig. 1*B*. A time-dependent inactivation of RecQ helicase was uniformly observed whenever RecQ helicase was present at low concentrations (≤10 nM). Nevertheless, these data demonstrate that RecQ helicase can rapidly unwind this 2.7-kbp linear dsDNA substrate.

To confirm that the loss of fluorescent signal observed in this assay was due to dsDNA unwinding by RecQ helicase and was not simply due to dsDNA binding, similar to that observed for

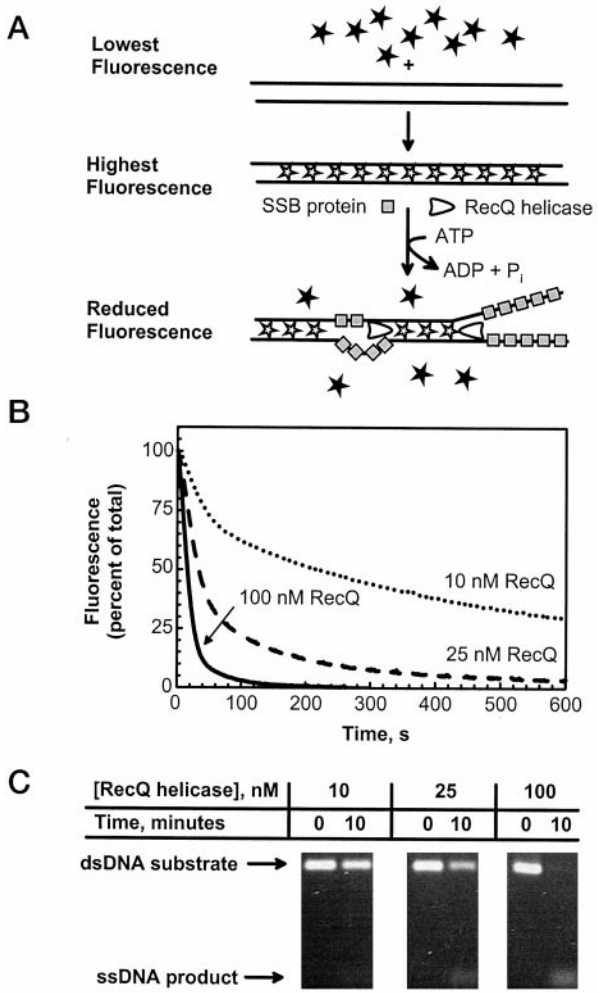

FIG. 1. **The fluorescent dye-displacement helicase assay applied to the helicase of RecQ helicase.** *A,* schematic of the fluorescent dye-displacement helicase assay. *Dark stars* are free H33258 dye with low fluorescence. The *open stars* represent H33258 dye with high fluorescence by virtue of its interaction with dsDNA. *B,* time courses of unwinding of 2.5 μM (bp) *Hin*dIII-cut pUC19 linear dsDNA under standard conditions with the following concentration of RecQ helicase: 10 (*dotted line*), 25 (*dashed line*), and 100 nM (*solid line*). *C,* unwinding of *Hin*dIII-cut pUC19 linear dsDNA monitored by agarose gel electrophoresis under identical conditions, at the indicated RecQ helicase concentrations. Samples were taken at the indicated times, separated on a 1% agarose gel, and the DNA visualized by staining with ethidium bromide.

RecA protein (38), the effect of nucleotide cofactor on dye displacement was determined. Previously, studies have shown that RecQ helicase requires ATP to mediate unwinding (16). In addition, RecQ helicase must hydrolyze ATP to promote unwinding; ATPγS, a non-hydrolyzable analogue of ATP, does not support RecQ helicase-mediated unwinding. In agreement, we find that RecQ helicase displays no detectable helicase activity either in the absence of ATP or when ATPγS is provided as nucleotide cofactor (Table I). These results clearly demonstrate that the helicase-dependent drop in fluorescence observed in the fluorescent dye-displacement assay is due to unwinding of the dsDNA substrate by RecQ helicase.

The lack of helicase activity in the presence of ATPγS does not stem from an inability of RecQ helicase to bind this cofactor, since the addition of 1 mM ATP to a helicase assay initiated with 1 mM ATPγS does not allow RecQ helicase to unwind the dsDNA (Table I). Accordingly, 1 mM ATPγS immediately inhibited dsDNA unwinding when added to an ongoing standard reaction having 1 mM ATP present (data not shown). A compa-

TABLE I
Unwinding of pUC19 linear dsDNA by RecQ helicase

Rate and extent of unwinding were determined under standard conditions, with the indicated changes, using the fluorescent dye-displacement helicase assay (see "Experimental Procedures"). In all cases, the dsDNA substrate was *Hin*dIII-cut pUC19 linear dsDNA at a concentration of 2.5 μM (bp).

Variable	Initial rate	Extent[a]
	nM (bp) s^{-1} (±5%)	*% (±5%)*
[RecQ] (nM)		
10	16	85[b] (25)
25	43	95 (50)
100	73	100 (100)
[SSB] (μM monomers)		
0	33	29
1	75	100
Nucleotide cofactor		
None	NA[c]	NA
ATP	78	100
dATP	38	76
ATPγS	NA	NA
ATPγS/ATP[d]	NA	NA

[a] The numbers in parentheses are the extents of unwinding determined by agarose gel electrophoresis (see text).
[b] This value represents an apparent extent of unwinding, since RecQ helicase progressively lost activity throughout the course of the reaction due to nonspecific inactivation of the enzyme.
[c] No activity detected.
[d] 1 mM ATP was added to an ongoing standard reaction that previously contained only 1 mM ATPγS as nucleotide cofactor.

rable inhibitory effect of ATPγS on the unwinding activity of RecQ helicase was also observed previously (29). Thus, RecQ helicase is fully capable of binding to ATPγS, but this nonhydrolyzable cofactor does not support dsDNA unwinding; instead, it is an inhibitor of RecQ helicase. Helicase activity was also supported by 1 mM dATP, but both the initial rate and extent of unwinding were 50% that observed with ATP (Table I). Thus, ATP appears to be a superior cofactor compared with dATP. It remains to be determined, however, whether the differences in helicase activity that are elicited by these two cofactors are due to differences in the binding affinity or the intrinsic turnover rate for each cofactor.

Strand separation by RecQ helicase was confirmed in separate reactions by agarose gel electrophoresis (Fig. 1*C*). As expected, electrophoresis demonstrated that 100% of the *Hin*dIII-cut pUC19 linear dsDNA was unwound in 10 min by RecQ helicase at a concentration of 100 nM (Fig. 1*C* and Table I). Unwinding was also apparent in the gel assay at RecQ helicase concentrations of 10 and 25 nM, except that the observed extents of unwinding were found to be lower at 25 and 50%, respectively (Fig. 1*C* and Table I). The discrepancy between the extents of unwinding measured by the dye-displacement assay and by the agarose gel method is likely due to differences intrinsic to the assays. The gel assay is only an approximate measurement of unwinding at the DNA molecule level because removal of proteins prior to agarose gel electrophoresis may allow DNA molecules that are only partially unwound to reanneal, producing the original substrate. Thus, many DNA molecules that are partially unwound will go undetected with this technique. Because of these limitations, the gel assay is more likely to under-estimate the actual extent of unwinding, especially under conditions where unwinding is incomplete. On the other hand, the fluorescent dye assay is highly sensitive because it measures unwinding at the base pair level. With this assay, the change in fluorescence brought on by unwinding is directly proportional to the number of base pairs unwound by the helicase (32). For this reason, this assay provides a more accurate measurement of the extent and rate of dsDNA unwinding. In addition, the dye-displacement method monitors the unwinding reaction in real time, so it is not subject to artifacts introduced by deproteinizing of the DNA prior to analysis.

In the absence of *E. coli* SSB protein, the extent of unwinding for RecQ helicase on short dsDNA fragments was found to be significantly reduced (31). To confirm these findings for the longer dsDNA substrates and to determine the effect of SSB protein on the initial rate of unwinding, RecQ helicase-mediated unwinding was examined in the absence of SSB protein (Table I). As expected, the extent of unwinding was significantly reduced when SSB protein was omitted. In addition, the rate of unwinding without SSB protein was approximately 40% that with 1 μM SSB protein present. Thus, SSB protein is a stimulatory factor that increases both the initial rate and extent of unwinding by RecQ helicase. The mechanism by which SSB protein stimulates RecQ helicase is explored further below.

The Helicase Activity of RecQ Helicase Is Uncharacteristically Sensitive to the Concentrations of ATP and Free Magnesium Ion—To determine the optimal conditions for DNA unwinding by RecQ helicase, the initial rate and extent of unwinding were measured over a range of magnesium ion concentrations (Fig. 2). Curiously, the helicase activity of RecQ helicase displays an optimum in the magnesium ion concentration profile. In the presence of 1 mM ATP, the initial rate of RecQ helicase-mediated unwinding displayed a sharp magnesium ion concentration optimum, with maximal activity occurring at 0.8 mM magnesium (Fig. 2*A, filled circles*); the extent of unwinding displayed a similar optimum but with a broader peak extending from 0.5–1.0 mM magnesium (*open circles*). It is important to note that the extent of unwinding obtained at each magnesium acetate concentration represents the final maximal degree of dsDNA unwinding, since the fluorescent time traces for each determination reached a stable plateau value (data not shown). Clearly, helicase activity (*i.e.* both initial rate and extent) is reduced when the magnesium ion concentration is low. The decrease in activity beyond the maximum implies that excess magnesium ion concentration is also inhibitory.

Since ATP chelates magnesium ion, it was not clear whether the reduced activity at the lower magnesium ion concentrations was due to a low total concentration or a low free-magnesium ion concentration. To discriminate between these possibilities, the magnesium ion concentration-dependence was re-examined at a higher ATP concentration (3 mM rather than 1 mM). At 3 mM ATP, the unwinding activity of RecQ helicase also displays a pronounced magnesium ion optimum (Fig. 2*A*), with 2.2 mM magnesium representing the peak of activity (*filled squares*). The extent of unwinding displays an equivalent sensitivity to the magnesium ion concentration, with the maximal extent of unwinding reaching 100% at magnesium ion concentrations ranging from 2 to 2.6 mM (*open squares*). Interestingly, the rate profile obtained at 3 mM ATP is similar to that obtained at 1 mM ATP when the data are replotted *versus* the *ratio* of magnesium ion and ATP concentrations; under both conditions, the maximal initial rate of unwinding corresponds to conditions where the Mg^{2+}:ATP ratio is slightly less than 1.0 (Fig. 2*B*). These data clearly demonstrate that RecQ helicase is sensitive to the ratio of the magnesium ion to ATP concentration, with maximal activity exhibited at approximately equimolar concentrations of magnesium ion and ATP. Thus, the concentration of free magnesium ion appears to affect the activity of RecQ helicase.

To confirm that the unwinding activity of RecQ helicase is influenced by free magnesium ion, the concentration of free magnesium ion for each data point in Fig. 2*B* was calculated using the CHELATOR software package. The initial rates of unwinding at each ATP condition (*i.e.* either 1 mM or 3 mM ATP) were the

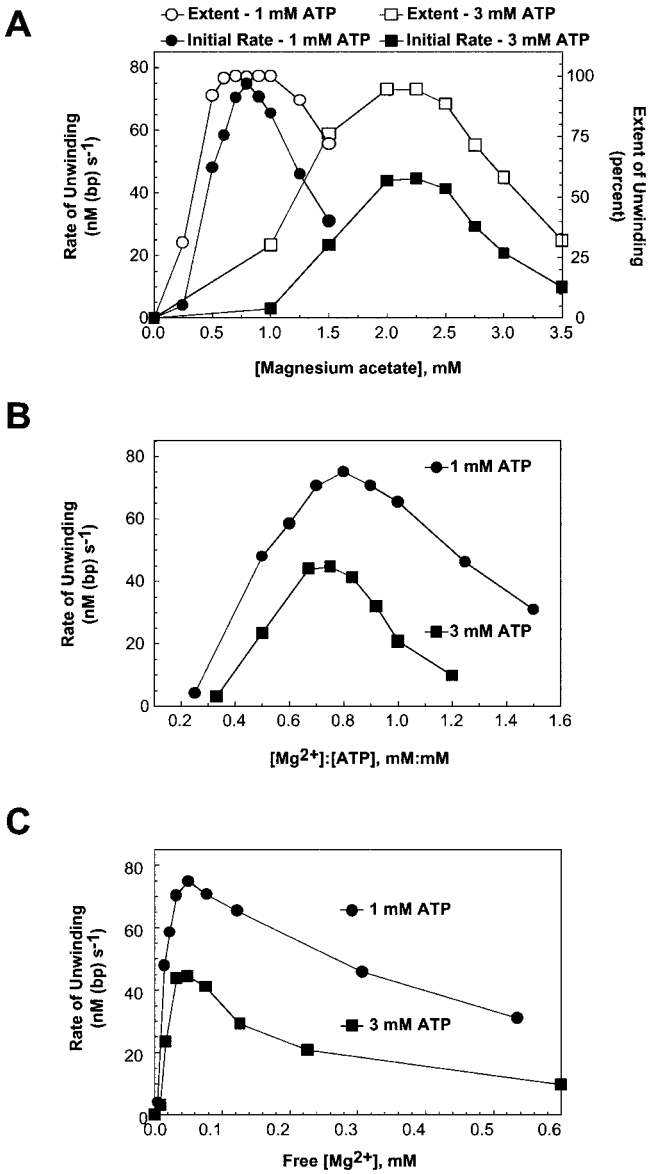

FIG. 2. **The helicase activity of RecQ helicase displays a sharp dependence on the free magnesium ion concentration.** *A,* initial rate (*closed symbols*) and final extent (*open symbols*) of unwinding as a function of magnesium acetate concentration at 1 mM ATP (*circles*) and 3 mM ATP (*squares*). *B,* initial rate of unwinding at either 1 (*circles*) or 3 mM (*squares*) ATP as a function of the Mg^{2+}:ATP ratio. *C,* the initial rate of unwinding by RecQ helicase at 1 and 3 mM ATP as a function of the free magnesium ion concentration. Reactions were carried out under standard conditions, with the indicated changes. The data are the mean of at least two determinations. Free magnesium ion concentration was calculated as indicated under "Experimental Procedures."

replotted as a function of free magnesium ion concentration (Fig. 2*C*). At both ATP concentrations, the helicase activity increased with increasing free magnesium ion concentration up to its peak at a concentration of approximately 0.05 mM. Beyond this optimum, the rate steadily declined with increasing free magnesium ion concentration. A comparable effect of free magnesium ion concentration on the rate of unwinding was observed when the magnesium acetate concentration was held to 1 mM and the ATP concentration varied from 0.25 to 3.0 mM (data not shown). Thus, the helicase activity of RecQ helicase is quite sensitive to the concentration of free magnesium ion. Interestingly, the initial rates of unwinding at 3 mM ATP reach only 60% of those observed at 1 mM ATP (Fig. 2*C*, compare *squares* to *circles*), even when the equivalent free magnesium ion concentrations are compared.

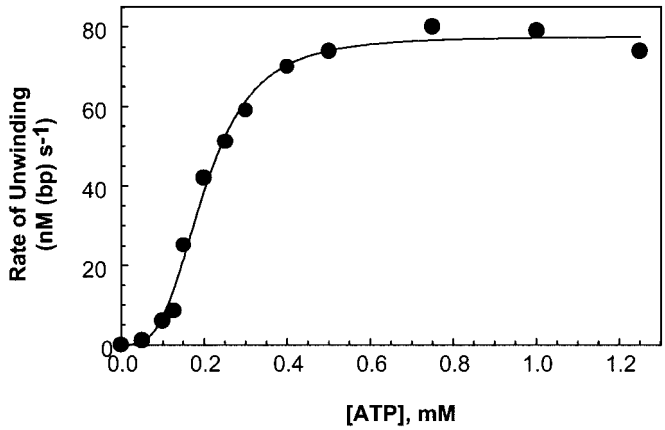

FIG. 3. **The unwinding activity of RecQ helicase displays a sigmoid dependence on the ATP concentration.** The helicase activity of RecQ helicase as a function of ATP concentration at a constant Mg^{2+}:ATP ratio of 0.8. The data are the mean of two determinations. The line corresponds to a fit of the data to the Hill equation ($Y = (V_{max}(X^n))/(K_M{}^n + X^n)$), where Y is the observed rate of unwinding; X is the ATP concentration, and n is the Hill coefficient, using Graphpad Prism 3.0. The R^2 value of fit was 0.993. The slope, or Hill coefficient, of the resultant line was 3.3. The derived value for $S_{0.5}$ (K_M) was 200 μM. Reactions were carried out under standard conditions, with the indicated changes.

Therefore, RecQ helicase is also sensitive to the ATP concentration, and this influence is separate from the effect of free magnesium ion.

RecQ Helicase May Function as a Multimer, Composed Minimally of Three Interacting Monomers—To obtain an estimate of the affinity that RecQ helicase has for ATP, the initial rates of RecQ helicase-mediated unwinding were determined over a range of ATP concentrations. Since RecQ helicase is inhibited by free magnesium ion, the Mg^{2+}:ATP ratio was maintained at 0.8 for these experiments. The initial rates of unwinding obtained from this analysis were then plotted as a function of ATP concentration (Fig. 3). The resultant curve is sigmoid, which is consistent with RecQ helicase utilizing multiple ATP-binding sites to mediate dsDNA unwinding (39). To determine both the apparent $S_{0.5}$ for ATP and the minimum number of ATP-binding sites involved in the unwinding reaction, the data were fit to the Hill equation (Fig. 3). The apparent $S_{0.5}$ for ATP derived from this analysis was 200 μM ATP. In addition, the slope of the resultant line, or Hill coefficient, provides an estimate of the minimum number of interacting ATP-binding sites involved in the unwinding reaction (39). In this case the slope is 3.3 $\pm$ 0.3, which is consistent with RecQ helicase utilizing at least three interacting ATP-binding sites in the course DNA unwinding. Since each RecQ helicase monomer contains a single ATP binding/hydrolysis site (4), these data suggest that the minimal functional oligomer of RecQ helicase is composed of at least three monomers.

Effect of Enzyme Concentration on RecQ Helicase Unwinding Activity—To determine the optimal amount of RecQ helicase required to unwind dsDNA, its concentration was varied in the presence of 0.5 μM (bp) pUC19 linear dsDNA (Fig. 4*A, closed squares*). The DNA concentration is presented in units of μM (bp) because, as we will show later, RecQ helicase is not limited to DNA ends as sites from which to initiate unwinding. The initial rate of unwinding saturated at a rate of 24 nM (bp)/s, and at a RecQ helicase concentration of 19 nM, which yields an apparent stoichiometry of approximately 25 bp/RecQ helicase monomer (Table II). Similar stoichiometries were also obtained at DNA concentrations of 2.5 and 5.0 μM (bp) (Table II). Based on these data, the apparent dsDNA binding stoichiometry for RecQ helicase is, on average, 30 bp/RecQ helicase monomer,

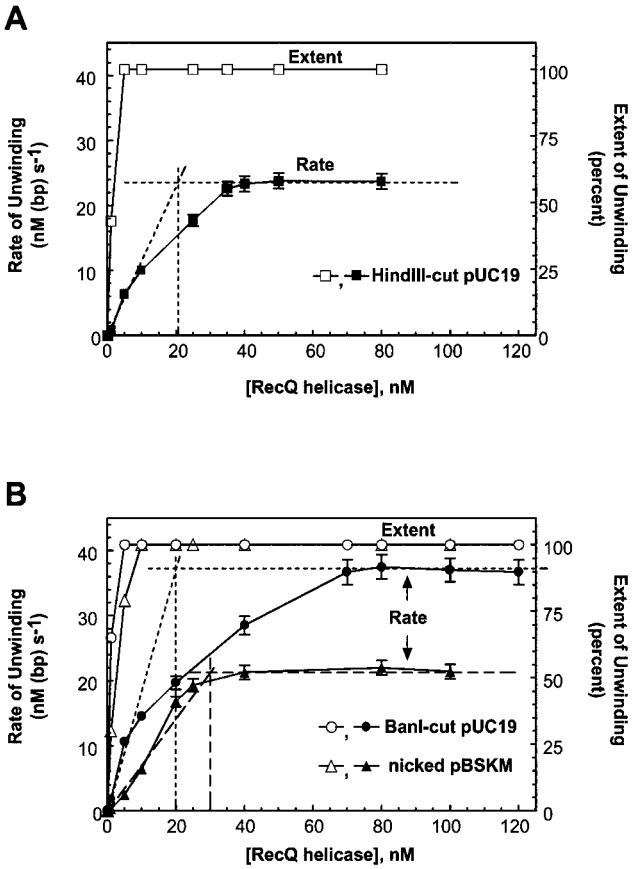

FIG. 4. **The DNA unwinding activity of RecQ helicase as a function of enzyme concentration.** The initial rate (*closed symbols*) and extent (*open symbols*) of unwinding were determined at the indicated RecQ helicase concentrations. *A,* the dsDNA was *Hin*dIII-cut pUC19 dsDNA (*squares*), which is a 2.7-kbp linear DNA molecule. *B,* the dsDNA substrates were either *Ban*I-cut pUC19 linear dsDNA (*circles*) or nicked pBSKM circular dsDNA (*triangles*). Digestion of pUC9 DNA with *Ban*I produces four dsDNA fragments of 142, 173, 1097, and 1274 bp. Nicked pBSKM circular dsDNA contains a single site-specific nick and is 2.9 kbp in length. All reactions were carried out under standard conditions, except that the DNA substrates were present at a concentration of 0.5 μM bp. In both *A* and *B*, the data are the mean of two determinations, and the *error bars* represent the minimum and maximum values. Saturating RecQ helicase and the apparent dsDNA binding stoichiometry were calculated from these data as indicated in Table II. The *dashed* and *dotted lines* indicate the tangents drawn to the curves in each case to determine the saturating concentration of RecQ helicase.

and this parameter is independent of the DNA concentration. At almost all of the RecQ helicase concentrations tested, the extent of unwinding was 100% (Fig. 4*A*). However, at 1 nM RecQ helicase, the apparent extent of unwinding was approximately 45%, due to nonspecific loss of enzyme activity at this low concentration.

Effect of DNA Concentration on the Unwinding Activity of RecQ Helicase—The concentration of the pUC19 linear dsDNA substrate was varied to establish values for both the apparent K_M for dsDNA and the apparent k_{cat} (*i.e.* turnover number) for the DNA helicase activity of RecQ helicase (Fig. 5*A*). The apparent K_M and k_{cat} values were calculated by fitting the data to the Michaelis-Menten equation, as indicated in Table III. The apparent K_M value for this linear dsDNA substrate was 767 nM (bp), and the apparent k_{cat} was 2.3 s^{-1} (Table III). Interestingly, compared with the values obtained at 20 nM RecQ helicase, similar experiments conducted at 100 nM RecQ helicase yielded an apparent K_M value of 2030 ± 300 nM (bp), a V_{max} value of 148 ± 5 nM (bp) s^{-1}, and an apparent k_{cat} value of 1.5 ± 0.1 s^{-1} (data not shown); thus, the apparent k_{cat} at this higher

TABLE II
Apparent dsDNA binding stoichiometry for the helicase activity of RecQ helicase

All reactions were carried out under standard conditions.

dsDNA substrate[a]	Saturating [RecQ][b]	Apparent stoichiometry[c]
μM (bp)	*nM*	*bp/RecQ monomer*
[*Hin*dIII-cut pUC19]		
0.5	19 ± 2	25 ± 3
2.5	68 ± 7	37 ± 4
5.0	177 ± 18	28 ± 3
[*Ban*I-cut pUC19]		
0.5	20 ± 2	25 ± 2
2.5	85 ± 8	29 ± 3
[Nicked pBSKM]		
0.5	30 ± 5	17 ± 3

[a] See legend of Fig. 4 for description of DNA substrates.
[b] Determined from a plot of initial rate of unwinding *versus* RecQ helicase concentration as the mean RecQ helicase concentration at the intersection of lines drawn tangent to the initial slope and to the final plateau value for the initial rate of unwinding (see Fig. 4). The error is the deviation from the mean.
[c] The ratio of the DNA concentration to the RecQ helicase concentration.

concentration was lower by 35%. These data suggested that RecQ helicase was a more active helicase at the lower concentration. As we will show below, this behavior is a manifestation of the competition between RecQ helicase and SSB protein for the ssDNA products of unwinding (see below). The extent of unwinding was 100% at all the dsDNA concentrations of ≤2.5 μM (bp); however, above this concentration, the apparent extent of unwinding decreased with increasing dsDNA concentration. The reduction in the extent of unwinding above 2.5 μM (bp) DNA is due to nonspecific binding of the protein to the walls of the quartz cuvette. In this case, the increased time required to complete unwinding of the DNA at the higher substrate concentrations (approximately 20 min) presumably exacerbated protein inactivation.

RecQ Helicase Is Capable of Initiating Unwinding from Both the Ends and the Internal Regions of dsDNA—An interesting characteristic of RecQ helicase is that, unlike many DNA helicases, it lacks a rigid specificity for a particular DNA substrate (29). Moreover, RecQ helicase is capable of unwinding covalently closed dsDNA molecules (40). Even though RecQ helicase does not necessarily require a dsDNA end to initiate unwinding, we were interested in determining the relative importance of dsDNA ends as sites for initiation of unwinding by RecQ helicase. A simple means of assessing the importance of dsDNA ends was to obtain the apparent dsDNA binding stoichiometry for DNA substrates that differ in the ratio dsDNA ends to the total number of base pairs. If dsDNA ends are a preferred initiation site for RecQ helicase, then the amount of protein needed to saturate the DNA should be related to the number of dsDNA ends. For example, the helicase activity of RecBCD enzyme, which is limited to initiating unwinding from dsDNA ends (41), saturates at a functional protein concentration that is equivalent to the concentration of dsDNA ends (33).

The additional DNA substrates used for these experiments are as follows: 1) pUC19 dsDNA digested with the restriction enzyme *Ban*I (*Ban*I-cut pUC19 dsDNA) to produce four different linear DNA fragments (see legend of Fig. 4) and, therefore, has 4-fold more dsDNA ends per total number of base pairs relative to the standard *Hin*dIII-cut pUC19 linear dsDNA substrate; and 2) the 2.9-kbp plasmid pBSKM with a single site-specific nick ("nicked pBSKM" dsDNA), which is a nicked, circular molecule that lacks dsDNA ends. The initial rate of unwinding was determined for these two DNA substrates at

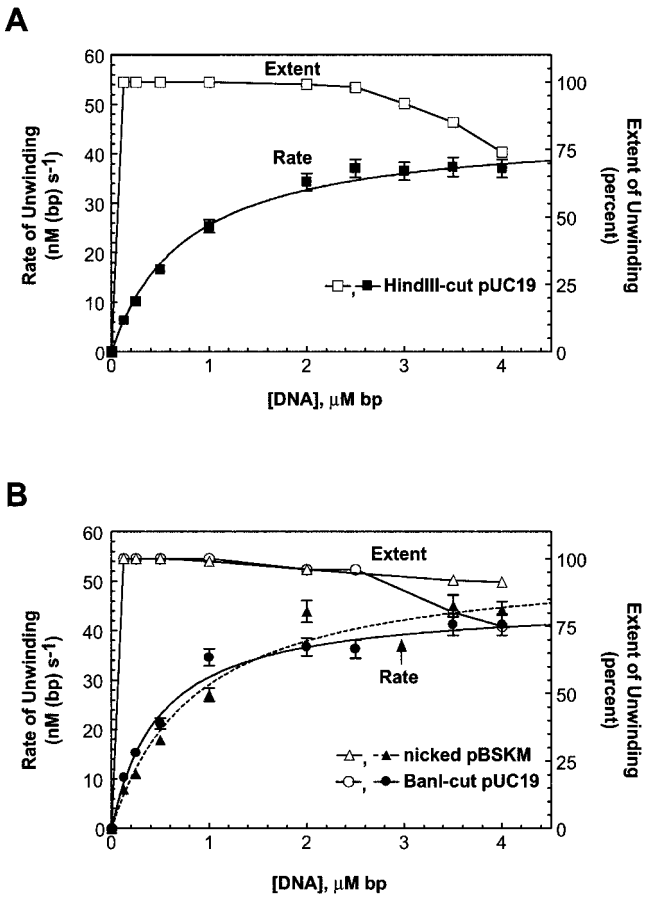

FIG. 5. **The dsDNA unwinding activity of RecQ helicase as a function of DNA concentration.** The initial rate (*closed symbols*) and extent (*open symbols*) of unwinding were determined at the indicated dsDNA concentrations. In all cases, the reactions were carried out under standard conditions, except the RecQ helicase concentration was 20 nM. *A,* the dsDNA substrate was *Hin*dIII-cut pUC19 linear dsDNA (*squares*). *B,* the dsDNA substrates were either *Ban*I-cut pUC19 linear dsDNA (*circles, solid line*) or nicked pBSKM circular dsDNA (*triangles, dashed line*). See legend of Fig. 4 for a description of the DNA substrates. In both *A* and *B*, the data are the mean of two determinations, and the *error bars* represent the minimum and maximum values. The *curves* for the initial rate data were generated by a least squares fit of the data to the Michaelis-Menten equation (see Table III), and the R^2 values for each fit were >0.97.

TABLE III

Kinetic parameters for the DNA helicase activity of RecQ helicase

All reactions were carried out under standard conditions, with 20 nM RecQ helicase.

dsDNA substrate[a]	Apparent K_M, DNA[b]	V_{max}[b]	Apparent k_{cat}[c]
	nM (bp)	*nM (bp) s^{-1}*	*s^{-1}*
*Hin*dIII-cut pUC19	767 ± 62	45.2 ± 1.0	2.3 ± 0.1
*Ban*I-cut pUC19	465 ± 42	45.4 ± 1.0	2.3 ± 0.1
Nicked pBSKM	869 ± 107	54.4 ± 2.0	2.7 ± 0.2

[a] See legend of Fig. 4 for description of DNA substrates.

[b] Determined from a least squares fit of the data in Fig. 5 to the Michaelis-Menten equation ($v = (V_{max}(s))/(K_M + s)$; where v is the observed rate of unwinding; s is the concentration of RecQ helicase; V_{max} is the maximal rate of unwinding at a saturating concentration of DNA, and the K_M is the DNA concentration at one-half the maximal rate of unwinding, using Graphpad Prism 3.0. The error is the deviation from the mean.

[c] The ratio of V_{max} and the RecQ helicase concentration.

the indicated RecQ helicase concentrations (Fig. 4*B*), and the apparent dsDNA binding stoichiometry for each was calculated as above for *Hin*dIII-cut pUC19 dsDNA (Table II).

For the *Ban*I-cut pUC19 dsDNA substrate, the initial rates of unwinding at all RecQ helicase concentrations were approximately 40% greater than with *Hin*dIII-cut dsDNA (Fig. 4*B, closed circles*). The magnitude of this difference, however, was significantly less than the 4-fold increase expected if RecQ helicase acted strictly from dsDNA ends. More importantly, the apparent dsDNA binding stoichiometry obtained at 0.5 μM (bp) *Ban*I-cut pUC19 DNA was identical to that observed for *Hin*dIII-cut pUC19 linear dsDNA (*i.e.* 25 bp/RecQ helicase monomer; Table II). In addition, a comparable apparent dsDNA binding stoichiometry was observed when RecQ helicase was titrated against a 5-fold higher concentration of this DNA substrate (Table II); thus, this apparent dsDNA binding stoichiometry, like that for *Hin*dIII-cut pUC19 linear dsDNA, was independent of DNA concentration. Therefore, a 4-fold increase in dsDNA ends does not significantly alter the apparent dsDNA binding stoichiometry of RecQ helicase. As for the *Hin*dIII-cut pUC19 linear dsDNA substrate, 100% of the *Ban*I-cut pUC19 dsDNA substrate was unwound at every RecQ helicase concentration, with the exception of the lowest concentration tested.

RecQ helicase unwound the nicked pBSKM dsDNA nearly as well as the two linear dsDNA substrates (Fig. 4*B, closed triangles*). With the nicked DNA, the initial rates of unwinding were reduced, on average, by 15%, relative to initial rates for *Hin*dIII-cut pUC19 dsDNA. The dsDNA binding stoichiometry for the nicked DNA substrate was 17 bp/RecQ helicase monomer, which is nearly half of the value obtained for the linear DNA substrates. The nick was not necessary for unwinding by RecQ helicase; a dsDNA binding stoichiometry of 22 bp/RecQ helicase was obtained for relaxed pUC19 dsDNA (*i.e.* a covalently-closed DNA substrate) at a concentration of 2.5 μM (bp), when the initial rates of RecQ helicases-mediated unwinding were determined in the presence of wheat germ topoisomerase I (data not shown). The topoisomerase was present to remove supercoils introduced into the DNA by RecQ helicase-promoted unwinding. These results are in agreement with our previous observations that RecQ helicase unwinds covalently closed dsDNA (40); therefore, this helicase does not require a dsDNA end to initiate unwinding. Furthermore, and more importantly, the data from both the nicked and linear dsDNA substrates indicate that RecQ helicase is almost equally capable of initiating unwinding from an internal region of dsDNA as it is from a dsDNA end. In contrast, RecBCD enzyme is unable to initiate unwinding from the internal regions of a dsDNA substrate (41).

RecQ Helicase Displays Similar Affinities for the Ends and Internal Sites of dsDNA—To determine quantitatively the relative affinity of RecQ helicase for dsDNA ends *versus* the internal regions of dsDNA, the apparent K_M value for both the *Ban*I-cut pUC19 and the pBSKM dsDNA was determined by varying the DNA concentration. The data for both of these DNA substrates appear in Fig. 5*B*, and the apparent K_M values calculated from each data set are presented in Table III. The apparent K_M value of 465 nM (bp) for the *Ban*I-cut pUC19 DNA was only 1.6-fold lower than that determined for *Hin*dIII-cut pUC19 DNA, even though the former substrate possessed 4-fold more dsDNA ends. Additionally, the apparent K_M value for the substrate without dsDNA ends, nicked pBSKM DNA, was 869 nM (bp), which was only slightly higher than that for *Hin*dIII-cut pUC19 DNA. If the apparent K_M is assumed to be a rough estimate of the relative dsDNA binding affinity of RecQ helicase, then these data indicate that RecQ helicase does not have a substantially higher affinity for dsDNA ends relative to its affinity for the internal base pairs of dsDNA, at least with regard to its DNA unwinding activity. By comparison, the affinity of RecBCD enzyme for dsDNA ends is more than 1×10^6-fold greater than its affinity for internal dsDNA sites (33).

Further evidence of this unusual property of RecQ helicase is

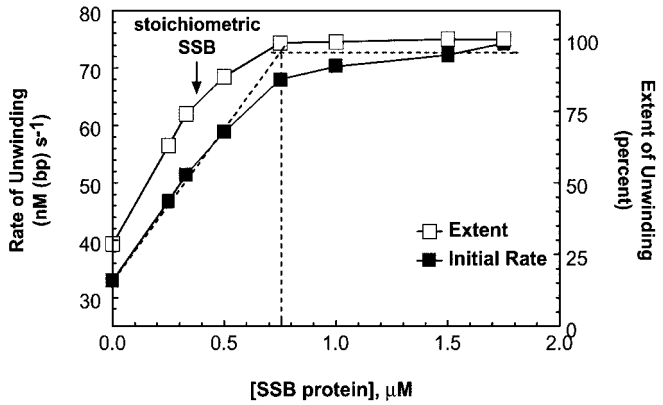

FIG. 6. **SSB protein stimulates the DNA unwinding activity of RecQ helicase.** The initial rate (*closed squares*) and extent (*open squares*) of unwinding as a function of the SSB protein concentration. The concentration of SSB protein needed to saturate the DNA in its fully unwound state is 0.37 μM. Reactions were carried out under standard conditions. The data are the mean of two determinations. The *dashed lines* indicate the tangents drawn to the curves to determine the SSB protein concentration where stimulation was saturated.

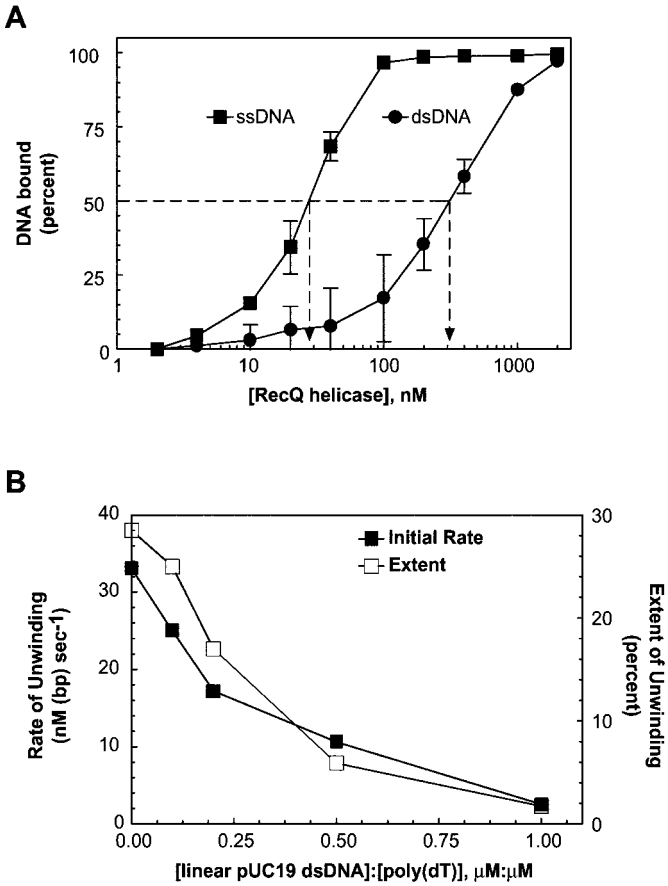

FIG. 7. **RecQ helicase preferentially binds to ssDNA over dsDNA, resulting in inhibition of helicase activity.** *A,* the binding of RecQ helicase to either a 48-nt ssDNA substrate (*squares*) or a 48-bp dsDNA substrate (*circles*), determined by PAGE bandshift assays. The apparent DNA binding affinity for each DNA substrate was calculated as the protein concentration at which binding was 50% (indicated by the *dashed lines*). The apparent DNA binding affinity values for ssDNA and dsDNA were 3.3×10^7 and 3.2×10^6 M^{-1}, respectively. *B,* the effect of single-stranded poly(dT) on the initial rate (*closed squares*) and extent of unwinding (*open squares*) by RecQ helicase. The ratio of the dsDNA and ssDNA concentrations is based on the concentrations of each DNA in nucleotides. In both *A* and *B*, the data are the mean of two determinations, and the *error bars* in *A* represent the minimum and maximum values.

provided by the apparent k_{cat} values calculated from the data in Fig. 5*B* (Table III). The apparent k_{cat} of 2.3 s^{-1} for the *Ban*I-cut pUC19 DNA substrate was the same as the k_{cat} value for *Hin*dIII-cut pUC19 dsDNA. The nicked pBSKM DNA substrate yielded a k_{cat} value of 2.7 s^{-1}, which was essentially that observed for the linear dsDNA substrates. Clearly, the affinity that RecQ helicase has for the internal regions of dsDNA substrates is comparable to that which it displays for dsDNA ends.

Greater Than Stoichiometric Concentrations of SSB Protein Are Needed to Fully Stimulate Unwinding by RecQ Helicase—Previous work demonstrates that SSB protein stimulated the extent of RecQ helicase-promoted unwinding by trapping the individual strands of the ssDNA product to prevent their reannealing (31). Under the assay conditions used here, SSB protein had a similar stimulatory effect on the helicase activity of RecQ helicase (Table I). To determine the concentration of SSB protein needed for maximal stimulation, both the initial rate and the final extent of unwinding were determined at concentrations of SSB protein that ranged from sub-saturating to excess with respect to the total concentration of DNA in nucleotides (*i.e.* potential ssDNA produced). Under the conditions used here, the DNA binding stoichiometry for SSB protein is 13.5 nt/SSB protein monomer (data not shown); consequently, 0.37 μM SSB protein would be sufficient to saturate completely the 2.5 μM of DNA in its fully unwound state.

At every protein concentration tested, SSB protein positively influenced both unwinding parameters for RecQ helicase (Fig. 6). In the absence of SSB protein, 100 nM RecQ helicase unwound approximately 30% of the substrate at an initial rate of 33 nM (bp)/s; subsequent addition of SSB protein to this reaction allowed complete unwinding by RecQ helicase (data not shown). On the other hand, RecQ helicase unwound 100% of the DNA at a 2-fold higher rate when 1 μM SSB protein was present (*i.e.* standard conditions). Increasing the SSB protein concentration up to 0.75 μM SSB protein led to a proportional increase in both the initial rate and the extent of unwinding by RecQ helicase. Above 0.75 μM SSB protein, a plateau level was achieved at which further increases in SSB protein concentration had little effect on unwinding. Thus, twice the saturating amount of SSB protein was required to achieve maximal unwinding by RecQ helicase. This phenomenon is likely to be a reflection of the competition between RecQ helicase and SSB protein for ssDNA-binding sites (see below).

SSB Protein Stimulates RecQ Helicase-promoted Unwinding by Preventing the Formation of Non-productive Complexes of RecQ Helicase and ssDNA—In addition to its capacity to bind to dsDNA, RecQ helicase can also bind ssDNA (16). In fact, using PAGE bandshift assays, we established that RecQ helicase (in the absence of SSB protein) displays a 10-fold higher affinity for a single-stranded oligonucleotide over a fully duplex DNA of the same length (Fig. 7*A*). The apparent DNA binding affinities for a 48-nucleotide ssDNA substrate and a 48-bp dsDNA substrate are 3.3×10^7 and 3.2×10^6 M^{-1}, respectively (Fig. 7*A*).

As might be expected, the ssDNA-dependent ATP hydrolysis activity of RecQ helicase is as much as 10-fold greater than its dsDNA-dependent activity (16); this behavior is similar to that of other DNA helicases such as helicase II (42), helicase IV (43), and Rep helicase (44). Furthermore, the unwinding activity of RecQ helicase is inhibited by ssDNA: the initial rate of unwinding for 100 nM RecQ helicase (in the absence of SSB protein) was reduced by 90% when the enzyme was provided with a equimolar mixture of *Hin*dIII-cut pUC19 linear dsDNA (2.5 μM (bp)) and poly(dT) (5.0 μM (nt)) (Fig. 7*B*). This reduction is in close agreement with the 10-fold difference in apparent binding affinity that RecQ helicase displays for ssDNA relative to dsDNA. Even when present at one-fifth the dsDNA concentra-

tion, poly(dT) inhibited the rate of unwinding by approximately 2-fold. In addition, the extent of unwinding displayed a comparable sensitivity to the presence of poly(dT) competitor (Fig. 7*B*). Thus, ssDNA is inhibitory to the helicase activity of RecQ helicase, presumably because RecQ helicase is sequestered by the ssDNA.

Since RecQ helicase shares the capacity to bind ssDNA with SSB protein, it is possible that these two proteins compete for binding to the ssDNA produced by unwinding. To determine whether these two proteins compete for ssDNA-binding sites, the effect of SSB protein on the ssDNA binding activity of RecQ helicase was examined. In these experiments, the binding of RecQ helicase to *Hin*dIII-cut pUC19 linear ssDNA (produced by heat denaturation) was monitored indirectly by assaying the ssDNA-dependent ATP hydrolysis activity of RecQ helicase (see "Experimental Procedures"). Fig. 8*A* shows a time course of ATP hydrolysis mediated by 10 nM RecQ helicase incubated with 1.0 μM (nt) ssDNA either in the absence or the presence of 1 μM SSB protein. In these experiments, the final steady-state rate of ATP hydrolysis is reduced by 8-fold from 262 to 34 nM ATP/s when SSB protein is present. Thus, approximately 8-fold less RecQ helicase binds to the ssDNA substrate in the presence of SSB protein. Furthermore, SSB protein (1 μM) inhibits ssDNA-dependent ATP hydrolysis 3-fold, with a half-time of approximately 20 s, upon addition of the protein to complexes of RecQ helicase (20 nM) and *Hin*dIII-cut pUC19 linear ssDNA (1 μM (nt)) (data not shown). Hence, SSB protein also competes with RecQ helicase that is already bound to ssDNA.

To determine the concentration of SSB protein needed for maximal inhibition of RecQ helicase, the ssDNA-dependent ATP hydrolysis activity of RecQ helicase was surveyed over a range of SSB protein concentrations (Fig. 8*B*). At each concentration of SSB protein, ATP hydrolysis activity was reduced, with the largest reduction being approximately 8-fold. In these experiments, saturation of the ssDNA should have occurred at approximately 75 nM SSB protein, based on a DNA binding stoichiometry of 13.5 nt/SSB monomer (see above) However, full inhibition of the ATPase activity occurred at more than 120 nM SSB protein or a >1.5-fold excess of SSB protein. Thus, the full inhibitory effect of SSB protein required greater than saturating concentrations of SSB protein, relative to the total concentration of DNA nucleotides. These data are in agreement with previous studies showing that the ATP hydrolysis activity of RecQ helicase is fully inhibited only in the presence of excess SSB protein (31).

To further characterize ssDNA binding by RecQ helicase and the effect of SSB protein on this activity, the apparent ssDNA binding stoichiometry of RecQ helicase was determined with and without 1 μM SSB protein (Fig. 8*C*). In the absence of SSB protein, the ATP hydrolysis activity of RecQ helicase saturated at an enzyme concentration of 165 nM and at a maximal rate of 4000 nM ATP/s (Fig. 8*C*) or an apparent k_{cat} value for ATP hydrolysis of 24.2 s^{-1}. The apparent ssDNA binding stoichiometry calculated from these data was 6 nucleotides/RecQ helicase monomer. Identical results were obtained when the DNA substrate was M13mp7 circular ssDNA.[2]

In similar reactions having SSB protein, 1.7-fold more enzyme, or 280 nM RecQ helicase, was needed to saturate the linear ssDNA substrate (Fig. 8*C*). As a result, the apparent ssDNA binding stoichiometry of RecQ helicase in the presence of SSB protein was reduced to 3.5 nucleotides/RecQ helicase monomer. In addition to affecting the observed ssDNA binding stoichiometry of RecQ helicase, SSB protein also inhibited the maximal rate of ATP hydrolysis achieved by RecQ helicase;

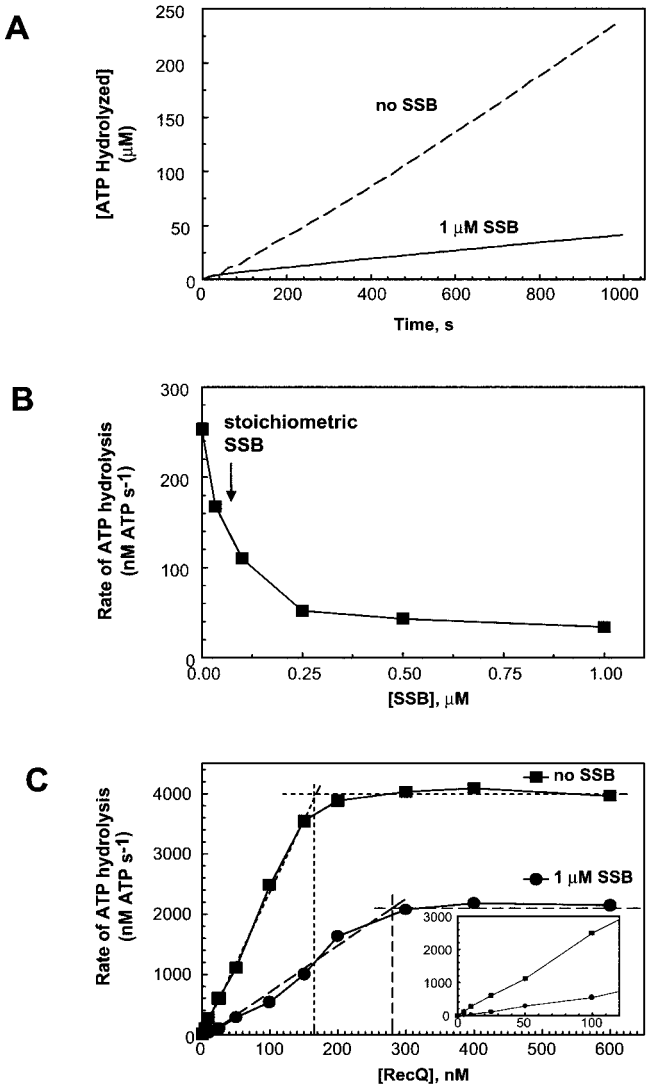

FIG. 8. **SSB protein limits the ability of RecQ helicase to bind to ssDNA.** *A,* time course of ATP hydrolysis by 10 nM RecQ helicase in the presence (*solid line*) or absence (*dashed line*) of 1 μM SSB protein. The final steady-state rates of ATP hydrolysis with and without SSB protein were 34 and 262 nM ATP/s, respectively. *B,* the ATP hydrolysis activity of 10 nM RecQ helicase as a function of SSB protein. The *arrow* indicates the SSB protein concentration (*i.e.* 75 nM) that is saturating for the 1.0 μM (nt) concentration of ssDNA present in the reaction. *C,* ssDNA-dependent ATP hydrolysis activity of RecQ helicase as a function of enzyme concentration in the presence (*circles*) or absence (*squares*) of 1 μM SSB protein, which is a 13.5-fold excess of SSB protein relative to the total DNA concentration. The *inset* shows the data for RecQ helicase concentrations between 0 and 100 nM. The DNA substrate was *Hin*dIII-cut pUC19 linear ssDNA, and its concentration was 1.0 μM (nt). The *dashed* and *dotted lines* indicate the tangents drawn to the *curves* to calculate the saturating concentrations of RecQ helicase.

saturating concentrations of RecQ helicase yielded a rate of 2200 nM ATP/s, which is approximately 60% of the value observed in the absence of SSB protein.

These results also reveal that RecQ helicase competes less effectively with SSB protein as enzyme concentration decreases. For example, at 100 nM RecQ helicase, ATP hydrolysis (*i.e.* the amount of enzyme bound to the ssDNA) was reduced by 4-fold when SSB protein was present (Fig. 8*C, inset*); at 50 nM RecQ helicase SSB protein reduces ATP hydrolysis by 5-fold, whereas at 5 nM RecQ helicase the reduction is the greatest at 12-fold. These data indicate that as the RecQ helicase concentration is reduced, SSB protein more effectively blocks the binding of RecQ helicase to ssDNA.

To test whether SSB protein stimulates the helicase activity

[2] F. G. Harmon and S. C. Kowalczykowski, unpublished observations.

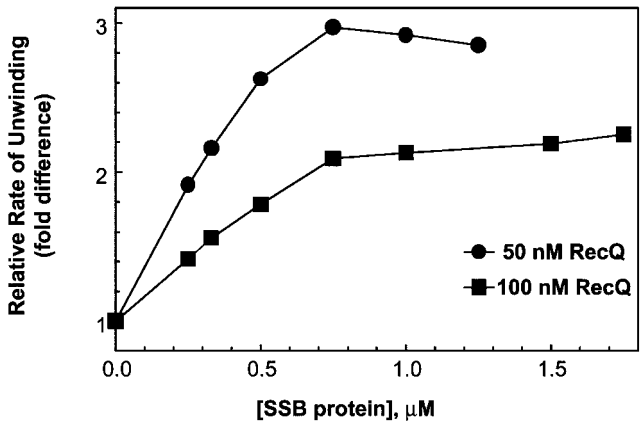

FIG. 9. **SSB protein stimulates the helicase activity of RecQ helicase by blocking the formation of inactive RecQ helicase-ssDNA complexes.** Relative stimulation of the initial rate of unwinding by SSB protein under standard conditions with either 50 (*circles*) or 100 nM (*squares*) RecQ helicase. The SSB protein concentration is expressed in terms of monomers. The data are the means of two determinations.

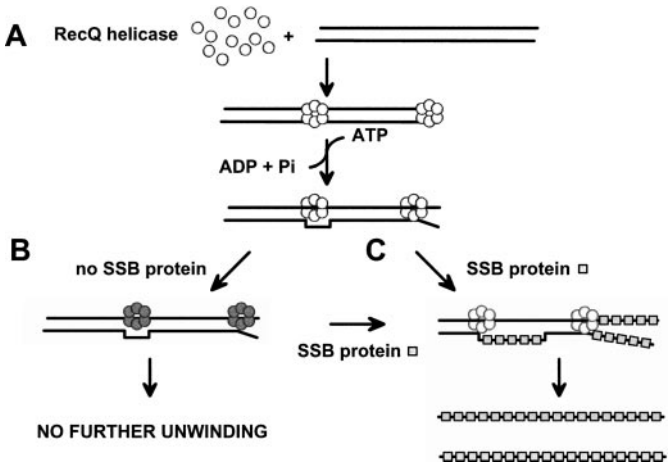

FIG. 10. **Model for dsDNA unwinding by RecQ helicase and for stimulation of this activity by SSB protein.** *A,* RecQ helicase binds to and, in the presence of ATP, begins unwinding a dsDNA substrate to produce ssDNA. RecQ helicase can initiate unwinding both at the ends and within internal regions of the dsDNA. *B,* in the absence of SSB protein, unwinding activity is limited because the helicase forms non-productive complexes with its ssDNA product. The presence of SSB protein alleviates this inhibition, since SSB protein competes with RecQ helicase for ssDNA-binding sites ($B \rightarrow C$). *C,* SSB protein stimulates unwinding by RecQ helicase both by maintaining the ssDNA in its unwound state and by displacing RecQ helicase from the ssDNA product. RecQ helicase monomers that are capable of participating in DNA unwinding are depicted by *open circles.* Although RecQ helicase is depicted as a hexamer, the actual makeup of the unwinding complex remains to be determined. *Shaded circles* represent non-productive RecQ helicase-ssDNA complexes. SSB protein is depicted as *shaded squares.*

of RecQ helicase by blocking formation of inactive RecQ helicase-ssDNA complexes, the effect of SSB protein on RecQ helicase-promoted unwinding was determined at two different concentrations of RecQ helicase, 50 and 100 nM. The prediction was that the relative stimulation of unwinding by SSB protein would be greater at 50 nM RecQ helicase than at 100 nM enzyme, because RecQ helicase at the lower concentration will compete less effectively with SSB protein for binding to the ssDNA product of unwinding. As anticipated, stimulation by SSB protein at the lower RecQ helicase concentration was 1.5-fold greater than at the higher enzyme concentration (Fig. 9). Thus, the stimulatory effects of SSB protein on RecQ helicase-mediated unwinding are inversely proportional to the helicase concentration. These data, taken together with those demonstrating that RecQ helicase and SSB protein compete for ssDNA-binding sites, are consistent with a model in which SSB protein stimulates the helicase activity of RecQ helicase by acting to antagonize the ssDNA binding of the helicase. In this view, SSB protein increases the effective concentration of RecQ helicase in solution by blocking sequestration of the enzyme by ssDNA.

DISCUSSION

We have extensively characterized the helicase activity of RecQ helicase using plasmid-length dsDNA substrates (pUC19 and pBSKM, which are 2.7 and 2.9 kbp in length, respectively). RecQ helicase fully unwinds these substrates in a reaction that is dependent on a hydrolyzable nucleotide cofactor and is stimulated by *E. coli* SSB protein. The helicase activity of RecQ helicase is sensitive to the ratio of magnesium ion concentration to ATP concentration because the enzyme is inhibited by excess free magnesium ion. In addition, the dependence of helicase activity on ATP concentration was sigmoidal with an apparent $S_{0.5}$ value of 200 μM and a Hill coefficient of 3.3 ± 0.3. Thus, RecQ helicase utilizes three or more interacting ATP-binding sites to mediate unwinding; this is the first evidence that RecQ helicase functions as an oligomer. The apparent dsDNA binding stoichiometry for RecQ helicase is 30 bp/RecQ helicase monomer, and the ssDNA binding stoichiometry is 6 nucleotides/RecQ helicase monomer. Significantly, RecQ helicase does not display a preference for initiating unwinding from dsDNA ends compared with internal regions of dsDNA. As reported previously by Nakayama and co-workers (31), SSB protein greatly stimulates the helicase activity of RecQ helicase. This stimulation of unwinding by SSB protein is a consequence of both trapping the displaced ssDNA strands and preventing the formation of non-productive RecQ helicase-ssDNA complexes.

In Fig. 10, we present a model that depicts the essential features of RecQ helicase-mediated dsDNA unwinding. RecQ helicase is illustrated as a helicase that can act catalytically but that is subject to inhibition by its ssDNA product due to formation of non-productive RecQ helicase-ssDNA complexes. SSB protein stimulates the helicase activity of RecQ helicase both by maintaining the unwound DNA strands in their ssDNA state and by abrogating the inhibition imposed by ssDNA. When provided with a dsDNA substrate, free RecQ helicase binds with almost equal affinity to either the dsDNA ends or the internal regions of the substrate (Fig. 10*A*). The active species formed by RecQ helicase upon binding to the DNA substrate is expected to be oligomeric, since multiple ATP-binding sites are utilized by the protein to achieve strand separation. The unwinding complex is depicted as a hexameric ring, since the BLM helicase, a human homologue of RecQ helicase, has been shown to adopt this structure (45). However, the higher order structure of the RecQ helicase unwinding complex remains to be determined. After gaining access to the substrate, RecQ helicase proceeds to unwind the dsDNA in the $3' \rightarrow 5'$ direction. When SSB protein is absent (Fig. 10*B*), RecQ helicase catalyzes only limited unwinding because RecQ helicase is sequestered by its ssDNA product. The inactive RecQ helicase-ssDNA complexes are preferentially formed because RecQ helicase has a significantly higher affinity for ssDNA than it does for dsDNA.

In the presence of SSB protein (Fig. 10*C*), RecQ helicase displays maximal helicase activity. The data presented here confirm that SSB protein is an important stimulatory factor for the unwinding activity of RecQ helicase. In its strand-trapping role, SSB protein coats the individual DNA strands, thereby preventing their reannealing. In this capacity, SSB protein is

expected to affect mainly the extent of unwinding. SSB protein also stimulates RecQ helicase by competing with it for ssDNA binding. As described above, RecQ helicase can become associated with its ssDNA product (Fig. 10*B*), blocking further unwinding of the dsDNA substrate. SSB protein alleviates this inhibition by sequestering the ssDNA away from RecQ helicase, thereby allowing RecQ helicase to continue unwinding the dsDNA substrate (Fig. 10, *B–C*). This is likely to be the means by which SSB protein stimulates the steady-state rate of unwinding. In general, SSB protein plays the role of a trapping protein to stimulate helicase-mediated unwinding by binding to the separated ssDNA strands after unwinding to prevent their reannealing (33, 31). In addition to this traditional role for SSB protein in unwinding, our data highlight a second role for SSB protein in DNA helicase function, namely SSB protein blocks the formation of helicase-ssDNA complexes that are inhibitory to the unwinding activity of the enzyme; this is the case for RecQ helicase, as we show here, and for the RecBCD helicase (46).

As expected, the helicase activity of RecQ helicase requires a hydrolyzable nucleotide cofactor; ATPγS, the essentially nonhydrolyzable ATP analogue, is bound by the enzyme, but it does not support dsDNA unwinding. Both the initial rate and extent of unwinding are greatest when ATP is the nucleotide cofactor, but the enzyme also displays limited activity when provided with dATP. Thus, ATP is a better cofactor for the helicase activity of RecQ helicase; however, the enzymatic basis for this preference was not determined. Interestingly, RecQ helicase, provided with ssDNA, hydrolyzes dATP at the same rate as it does ATP (16). Therefore, the ssDNA-dependent ATP hydrolysis activity of the enzyme appears not to be part of the rate-limiting step of DNA unwinding but likely reflects a post-unwinding step.

We investigated the dependence of RecQ helicase activity on the concentration of both magnesium ion and ATP, and we found that, regardless of total ATP concentration, optimal activity occurred at a ratio of total magnesium ion to ATP of 0.7–0.8. Since ATP chelates magnesium ion, the free magnesium ion concentration was calculated for each condition. In each case, the peak of RecQ helicase activity corresponded to a free magnesium concentration of 0.05 mM. In addition to its sensitivity to free magnesium ions, RecQ helicase is also affected by the absolute concentration of ATP; the optimal initial rate of unwinding at 3 mM ATP is 2-fold lower than the optimal initial rate obtained at 1 mM ATP. These results demonstrate that RecQ helicase is inhibited by both excess free magnesium ion and ATP. One possible explanation for this sensitivity is that RecQ helicase requires Mg^{2+}-ATP to be bound to the protein as a complex; and for an unknown reason, association of either of these cofactors in their free form inhibits enzyme function.

The dependence of unwinding activity on the concentration of the Mg^{2+}-ATP complex revealed that RecQ helicase may function as an oligomer, since its activity is cooperative with ATP concentration. The apparent $S_{0.5}$ value for ATP calculated from these data 200 μM, which is well below the intracellular concentration of ATP (approximately 3 mM (47)). In addition, this $S_{0.5}$ value for RecQ helicase is similar to the apparent K_M values for ATP reported for other *E. coli* helicases including 110 μM for helicase II (42), 147 μM for RuvB protein (48), 50–100 μM for DnaB (49), and 85–130 μM for RecBCD enzyme (33, 50). Furthermore, a fit of the data to the Hill equation yielded a slope, or Hill coefficient, of 3.3. As the Hill coefficient describes the minimum number of interacting active sites utilized by an enzyme (39), RecQ helicase utilizes three or more ATP-binding sites in the course of unwinding a dsDNA substrate. Since each RecQ helicase monomer possesses a single ATP-binding site (4), the functional unit of RecQ helicase is minimally a trimer. However, this analysis does not rule out the possibility that the unwinding complex is a higher order multimer. Interestingly, recent work (45) on the BLM helicase demonstrates that this helicase exclusively forms hexameric rings. A similar cooperative dependence on ATP concentration is observed for the ATP hydrolysis activity of *E. coli* Rep helicase (51). The dTTP hydrolysis activity of bacteriophage T7 gene 4 helicase is also cooperative with nucleotide concentration (52), and this helicase is active as a hexamer (53). Interestingly, gel filtration chromatography of free RecQ helicase (*i.e.* in the absence of DNA) in the presence of ATP and magnesium acetate indicated that this protein exists almost exclusively as a monomer in solution.[2] Consequently, the oligomeric unwinding species formed by RecQ helicase must assemble upon binding to the DNA substrate.

The apparent dsDNA binding stoichiometry for RecQ helicase obtained here (*i.e.* 30 bp/RecQ helicase) indicates that a relatively high concentration of RecQ helicase is needed to unwind a linear dsDNA substrate. A trivial explanation for this requirement is that the RecQ helicase preparation contains a large proportion of inactive enzyme. This possibility is unlikely, however, given that similar experiments with several different preparations of RecQ helicase yielded comparable DNA binding stoichiometries.[2,3] Another possible explanation for this observation is that RecQ helicase is a helicase that acts stoichiometrically. In the strictest case of a stoichiometric helicase, each enzyme monomer catalyzes a single round of unwinding. However, the data presented here are consistent with each RecQ helicase monomer being capable of mediating multiple rounds of unwinding. For example, a direct comparison of the values obtained for the apparent dsDNA binding and the ssDNA binding stoichiometries indicates that the helicase activity of RecQ helicase saturates at 8.5-fold less enzyme than does its ssDNA-dependent ATP hydrolysis activity. Therefore, unlike a classical stoichiometric helicase, far less RecQ helicase is required to saturate a dsDNA substrate than would be needed to bind stoichiometrically to either one or both strands of the ssDNA product. In addition, a titration of RecQ helicase against a constant concentration of dsDNA substrate revealed that under conditions where the enzyme concentration was far less than saturating (relative to the apparent dsDNA binding stoichiometry), RecQ helicase fully unwound each of the dsDNA molecules. In fact, RecQ helicase at a concentration that is 20-fold less than saturation unwound nearly half of the dsDNA molecules. RecQ helicase behaved similarly in the DNA titration experiments; at all the DNA concentrations tested, the protein unwound at least 75% of the dsDNA substrate. Thus, more dsDNA is unwound by RecQ helicase than is expected based on the apparent DNA binding stoichiometry, demonstrating that each RecQ helicase monomer participates in multiple DNA unwinding events. Taken together, these data clearly indicate that RecQ helicase is a catalytic DNA helicase. Our experiments, however, do not provide a measure of the average processivity of a RecQ helicase unwinding complex. Thus, it is in unclear whether RecQ helicase achieves complete unwinding of a single dsDNA molecule following a single association event with the substrate or after multiple, successive association and dissociation events.

The requirement for high concentrations of RecQ helicase in DNA unwinding is readily explained by two of the observations made here. 1) RecQ helicase initiates unwinding from the in-

[3] A. K. Eggleston, N. A. Rahim, and S. C. Kowalczykowski, unpublished observations.

ternal regions of a dsDNA substrate with nearly the same facility as it does from dsDNA ends. 2) The rate of RecQ helicase-mediated unwinding is relatively slow. In agreement with our previous work (40), we have shown here that each base pair in a dsDNA substrate is a potential site of binding for RecQ helicase. The strongest support for this is provided by the similar apparent K_M values obtained for the nicked and the two linear dsDNA substrates. If these K_M values are interpreted as the relative affinity of RecQ helicase for each of the substrates, then it is clear that this helicase does not possess a significantly greater affinity for dsDNA ends relative to the internal portions of dsDNA. Similarly, the apparent k_{cat} values for all three of the DNA substrates are comparable. Since the unwinding activity of RecQ helicase is not significantly influenced by the number of dsDNA ends present in a DNA substrate, this protein can initiate unwinding from almost any internal position along a dsDNA molecule. These findings are in contrast to what has been described for the majority of DNA helicases, which generally require a DNA end from which to initiate unwinding (for review see Ref. 2). For example, RecBCD enzyme can initiate unwinding only from the ends of a dsDNA molecule (41). This property of RecBCD enzyme arises from the fact that, unlike RecQ helicase, the affinity of RecBCD enzyme for dsDNA ends is vastly higher than its affinity for the internal sites of a dsDNA molecule (33). A direct physical consequence of this unique feature of RecQ helicase is that the amount of enzyme needed to saturate a dsDNA substrate is achieved at a stoichiometry of protein that is on the order of the total concentration of DNA base pairs.

The second contributor to the observed dsDNA binding stoichiometry is that RecQ helicase is a relatively "slow" helicase; the apparent k_{cat} for dsDNA unwinding activity is on the order of 2.5 s^{-1}. By comparison, the apparent k_{cat} values determined for other helicases from *E. coli* are significantly higher; the k_{cat} for RecBCD enzyme is 250–1000 s^{-1} (33), that of DnaB helicase is 30–40 s^{-1} (54), and the turnover number for Rep helicase is 23 s^{-1} (55). A predicted outcome of a slower rate of unwinding is that each DNA substrate molecule remains intact for a longer time. With this longer temporal window for binding, each DNA molecule is likely to support the binding of more RecQ helicase unwinding complexes. This is especially the case with RecQ helicase, since this protein is not limited to binding to dsDNA ends. The expected result is that saturation of the dsDNA substrate occurs at an almost stoichiometric ratio of enzyme to DNA base pairs, as seen here. Thus, the unusual apparent dsDNA binding stoichiometry for a catalytic helicase is a direct result of the biochemical properties of RecQ helicase.

Our finding that RecQ helicase unwinds dsDNA from both dsDNA ends and the internal portions of a DNA substrate is consistent with the genetic evidence indicating that the majority of the crossovers occurring at internal regions of dsDNA are mediated by the RecF recombination pathway (56–58). It is now clear that RecQ helicase is responsible for initiating homologous recombination mediated by the RecF pathway (see Introduction). As we have shown here and elsewhere (40), RecQ helicase is fully capable of acting on the internal portions of a dsDNA substrate. Thus, RecQ helicase has the proper biochemical flexibility to participate in the initiation of the internal crossover events mediated by the RecF pathway.

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