Kinetics and Processivity of ATP Hydrolysis and DNA Unwinding by the RecBC Enzyme from *Escherichia coli*†

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ABSTRACT: The RecB and RecC subunits of the RecBCD enzyme from *Escherichia coli* were purified from cells containing plasmids overproducing these proteins [Boehmer, P. E., & Emmerson, P. T. (1991) *Gene 102*, 1–6]. RecB hydrolyzes ATP in the presence of either single- or double-stranded DNA. RecC stimulates ATP hydrolysis by RecB, particularly with double-stranded DNA. The steady-state kinetic parameters for ATP hydrolysis by RecBC with double-stranded DNA are k_{cat} = 1600 min^{-1}, K_m = 8.1 μM, and k_{cat}/K_m(ATP) = 1.97 × 10^8 M^{-1} min^{-1}. The RecBC enzyme acts processively, as measured by the effect of heparin on ATP hydrolysis stimulated by double-stranded DNA. About 2400 ATP molecules are hydrolyzed per enzyme bound to the end of a DNA molecule, using DNA substrates of 6250 or 21 400 base pairs. The enzyme is capable of unwinding a 6250 base pair double-stranded DNA molecule, in the presence of the single-stranded DNA binding protein of *Escherichia coli*. The steady-state kinetic parameters and the processivity are close to those found previously for the RecBCD-K177Q enzyme, with a lysine-to-glutamine mutation in the consensus ATP binding sequence in the RecD subunit, and are reduced compared to the RecBCD holoenzyme [Korangy, F., & Julin, D. A. (1992) *J. Biol. Chem. 267*, 1733–1740]. The most salient difference between RecBC and RecBCD-K177Q is the nuclease activity. RecBCD-K177Q produces a significant amount of acid-soluble DNA fragments from double-stranded DNA, while RecBC does not, even though the DNA does become unwound.

The RecBCD enzyme from *Escherichia coli* is one of several multifunctional, multisubunit enzymes whose substrate is DNA. The activities of the RecBCD enzyme include DNA binding, DNA-stimulated ATP hydrolysis, unwinding of double-stranded DNA, and nuclease [reviewed in Smith (1990)]. Information on the roles of the individual RecB, RecC, and RecD subunits in catalysis by this enzyme has come from a variety of experimental approaches, including study of the individual subunits (Hickson et al., 1985; Boehmer & Emmerson, 1992; Masterson et al., 1992), combinations of subunits (Lieberman & Oishi, 1974; Palas & Kushner, 1990; Masterson et al., 1992), and random and site-directed mutants (Amundsen et al., 1990; Korangy & Julin, 1992a,b,c; Hsieh & Julin, 1992), photochemical cross-linking (Julin & Lehman, 1987; Ganesan & Smith, 1993), and amino acid sequence comparisons (Hodgman, 1988; Gorbalenya, 1988). The individual subunits have not been found to have enzymatic activity, with the exception of the RecB protein, a DNA-dependent ATPase and weak helicase (Hickson et al., 1985; Boehmer & Emmerson, 1992). No catalytic activities have been reported to date for the isolated RecC and RecD subunits (Lieberman & Oishi, 1974; Hickson et al., 1984; Masterson et al., 1992), but RecD has been found to bind ATP (Julin & Lehman, 1987) and it has amino acid sequence homology to other helicases (Hodgman, 1988; Gorbalenya, 1988). Results from electron microscopy (Taylor & Smith, 1980), the kinetics of ATP hydrolysis and DNA unwinding (Roman & Kowalczykowski, 1989a,b), and DNA–protein cross-linking (Ganesan & Smith, 1993) have led to proposals that both the RecB and RecD subunits function as ATPases in the holoenzyme and that they are directly involved in DNA unwinding (Roman & Kowalczykowski, 1989b; Ganesan & Smith, 1993). A mutation in the consensus ATP binding sequence of RecD gives an active enzyme, but one which is slower and less processive than the wild-type enzyme in the reactions with double-stranded DNA (Korangy & Julin, 1992b,c). This finding supports a role for RecD in the unwinding reaction (Korangy & Julin, 1992b). Similar mutations in the corresponding sequence of RecB lead to loss of nuclease and ATPase activity with double-stranded but not with single-stranded DNA (Kushner et al., 1992; Hsieh & Julin, 1992).

The RecBC enzyme has been prepared previously by purification from cells lacking the *recD* gene (Palas & Kushner, 1990), by partial dissociation of the holoenzyme (Lieberman & Oishi, 1974; Amundsen et al., 1986), and most recently by association of the purified RecB and RecC subunits (Boehmer & Emmerson, 1991; Masterson et al., 1992). The purified RecBC enzyme has been reported to be a DNA-dependent ATPase and a DNA helicase (Palas & Kushner, 1990; Masterson et al., 1992), although helicase activity due to RecBC has not been observed in crude extracts of *recD* mutant cells (Amundsen et al., 1990).

We have investigated further the catalytic properties of the RecBC enzyme, essentially a RecD-deletion mutant, and compared the results to those we and others obtained previously with the RecBCD-K177Q mutant enzyme and the wild-type RecBCD enzyme. The results of these experiments show that the RecBC enzyme is a processive DNA helicase, capable of unwinding a 6250 base pair DNA molecule in the presence of the single-stranded DNA binding protein (SSB protein).[1] Its processivity, determined by heparin-trapping experiments, is less than RecBCD, but close to that of the RecBCD-K177Q enzyme (Korangy & Julin, 1992c). The k_{cat} and K_m(ATP)

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[1] Abbreviations: bp, base pair(s); DTT, dithiothreitol; kbp, kilobase pair(s); SDS, sodium dodecyl sulfate; SSB protein, single-stranded DNA binding protein.

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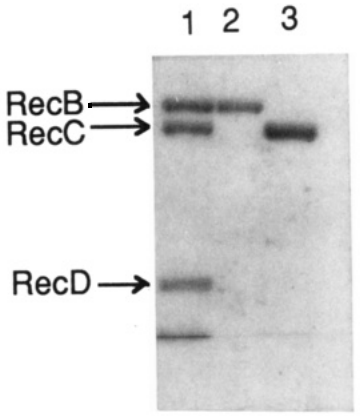

FIGURE 1: Purified RecB and RecC proteins. Samples of the purified subunits were analyzed by electrophoresis on a 5% polyacrylamide gel containing SDS. Lane 1, RecBCD enzyme (2 μg); lane 2, RecB protein (1.2 μg); lane 3, RecC protein (2 μg).

for double-stranded DNA-dependent ATP hydrolysis by RecBC are similar to those of the RecBCD-K177Q enzyme, and both are reduced compared to the RecBCD enzyme. The clearest difference between the RecBC and RecBCD-K177Q enzymes is in the nuclease reaction, since the former does not produce acid-soluble DNA fragments from double-stranded DNA. These quantitative measurements of the kinetics and processivity of the RecBC helicase and ATP hydrolysis activities provide insight into the functions of the RecB and RecD subunits.

EXPERIMENTAL PROCEDURES

Materials. ATP was purchased as a 100 mM solution from Pharmacia Corp. Heparin (sodium salt, 170 units/mg) was purchased from U.S. Biochemicals Corp. [*methyl*-^{3}H]-Thymidine and [γ-^{32}P]ATP were from Amersham. Restriction endonucleases were from U.S. Biochemicals Corp. or Fisher Scientific. The SSB protein from *E. coli* was purified as described (Korangy & Julin, 1992c).

The RecBCD and RecBCD-K177Q enzymes were purified from V186[pDJ05] or V186[pDJ05-DK177Q] cells as described (Korangy & Julin, 1992a). The individual RecB, RecC, and RecD proteins were purified from V186 cells (Δ*recBCD*; Chaudhury & Smith, 1984a) transformed with the plasmids pPB700 (*recB*$^+$), pPB500 (*recC*$^+$), or pPB100 (*recD*$^+$), and pNM52 (*lacI*^{q}). The plasmids were a generous gift from Prof. Peter Emmerson, University of Newcastle-upon-Tyne, England, and Dr. Paul E. Boehmer, Stanford University. Their construction and the subunit purification procedures are described in Boehmer and Emmerson (1991, 1992) and Masterson et al. (1992). The protein concentrations were determined from the absorbance at 280 nm using the following absorption coefficients: $\epsilon_{280} = 4 \times 10^5$ M^{-1} cm^{-1} (RecBCD enzyme; Roman & Kowalczykowski, 1989a); 164 150 M^{-1} cm^{-1} (RecB); and 199 400 M^{-1} cm^{-1} (RecC). The latter were calculated from the known number of aromatic amino acids in each subunit (Finch et al., 1986a,b) using absorption coefficients at 280 nm of 5700 and 1300 M^{-1} cm^{-1} for Trp and Tyr, respectively (Cantor & Schimmel, 1980). The yield of RecB was 4.4 mg from a 2-L culture, and that of RecC was 11.9 mg from a 2-L culture. These proteins were >95% pure (Figure 1). The RecD protein, which we have not studied in this work, contained a major contaminant with slightly lower mobility than RecB on a 5% SDS–polyacrylamide gel (not shown) and some insoluble material.

Tritium-labeled and unlabeled plasmid DNA substrates pPvSm19 (6.25 kbp) and pDJ01 (21.4 kbp) were prepared and linearized as described (Korangy & Julin, 1992b). Denatured substrates were prepared by immersing linearized DNA in a boiling water bath for 5 min and then immediately transferring the tube to ice water. The DNA concentrations were determined from the absorbance at 260 nm or by fluorescence in the presence of Hoechst 33258 using a TKO 100 fluorometer (Hoefer Scientific Instruments). The specific radioactivity of pPvSm19 [^{3}H]DNA was determined by counting small aliquots in mixtures containing the same concentration of trichloroacetic acid as would be present in quenched nuclease reaction aliquots.

Preparation of the RecBC Enzyme from Individual Subunits. The individual RecB and RecC proteins were mixed in 20 mM potassium phosphate, pH 7, 0.1 mM EDTA, 50 mM DTT, 100 mM sodium chloride, bovine serum albumin (1 mg/mL), and 10% (v/v) glycerol (Masterson et al., 1992). The mixtures were placed on ice for at least 30 min.

ATP Hydrolysis. ATP hydrolysis was measured using [γ-^{32}P]ATP and the thin-layer chromatography method described previously (Korangy & Julin, 1992b). The "standard conditions" for these and other experiments were 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.67 mM DTT. The concentrations of ATP stock solutions were determined from the absorbance at 259 nm using $\epsilon_{259} = 15.4$ mM^{-1} cm^{-1} [see p 449 of Maniatis et al. (1982)] (before [γ-^{32}P]ATP had been added).

The steady-state kinetic parameters for ATP hydrolysis by the RecBC enzyme were determined at 37 °C with pPvSm19 DNA digested with *Bgl*II or *Sac*I (100 μM nucleotide residues) and 0.73–1.46 nM RecBC enzyme prepared as above. The [γ-^{32}P]ATP concentration was varied from 2 to 200 μM. The initial reaction rates were determined by linear least-squares fits of the ADP concentration vs time, and steady-state kinetic parameters were determined from Eadie–Hofstee plots.

Heparin-trapping experiments were done as before (Korangy & Julin, 1992c) with 10–12 mg/mL heparin added either before the RecBC enzyme or along with the [γ-^{32}P]ATP substrate after preincubation of the enzyme and DNA.

Nitrocellulose Filter Binding. Filter binding experiments were done by the double-filter method devised by Lohman (Wong et al., 1992), using a Hoefer FH 124 single-filter holder connected to a water aspirator. DNA–protein complexes were trapped on a nitrocellulose filter (Millipore, HAWP), and the unbound DNA was trapped on a DE81 filter (Whatman) placed directly under the nitrocellulose filter. The nitrocellulose filters were soaked in 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$, and the DE81 filters in 2 M NaCl and then deionized water, before use.

The binding reactions contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.67 mM DTT, and pPvSm19 [^{3}H]DNA (10 μM nucleotides; 1.6 nM DNA ends). The plasmid DNA was linearized by cleavage with *Sac*I, *Bgl*II, or *Sma*I. The RecBC enzyme was added to the mixture, and then a sample was removed after 1 min and pipetted onto the nitrocellulose filter. The filter was immediately washed 3 times under vacuum with ice-cold 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$. Both filters were removed and dried under an infrared lamp, and the bound radioactivity was determined by liquid scintillation counting. A mixture containing no enzyme was also filtered to determine the background of DNA binding to the nitrocellulose. The fraction of DNA bound to the enzyme

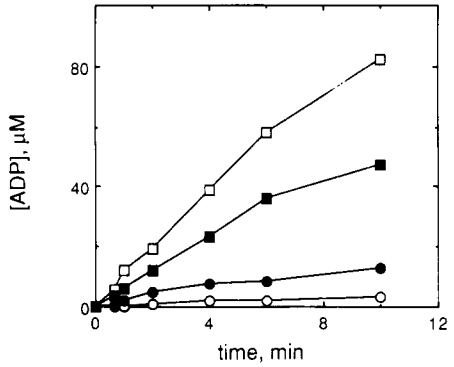

FIGURE 2: ATP hydrolysis by the RecB, RecC, and RecBC enzymes. Reaction mixtures (standard conditions) contained 200 μM [γ-^{32}P]-ATP, pPvSm19 DNA cleaved with *Sac*I (100 μM nucleotides), and 3 nM RecB or RecBC enzyme. Reactions with single-stranded DNA contained *Sac*I-cut DNA denatured by heating for 5 min in boiling water. (○) RecB protein and double-stranded DNA; (●) RecB protein and single-stranded DNA; (□) RecBC enzyme and double-stranded DNA; (■) RecBC enzyme and single-stranded DNA. The RecBC enzyme was prepared by mixing RecB (73 nM) and RecC (476 nM) in the buffer described under Experimental Procedures and incubating for 30 min on ice.

(f) was determined from

$$f = [cpm_{NC}/(cpm_{NC} + cpm_{DE})] - [cpm_{NC}/(cpm_{NC} + cpm_{DE})]_{BG}$$

where cpm_{NC} and cpm_{DE} are the counts bound to the nitrocellulose and DE81 filters, respectively, for a given sample, and $[cpm_{NC}/(cpm_{NC} + CPM_{DE})]_{BG}$ is the ratio in the absence of enzyme.

Nuclease and DNA Unwinding Measurements. Production of trichloroacetic acid-soluble DNA fragments and S1 nuclease coupled reactions were done essentially as described (Korangy & Julin, 1992b). Agarose gels were prepared in 1× TBE buffer (Maniatis et al., 1982), run, treated with EN^{3}HANCE, and visualized by autofluorography as described (Korangy & Julin, 1992c).

RESULTS

ATP Hydrolysis by RecB and RecBC. The purified RecB protein has ATPase activity (Figure 2), as reported before (Hickson et al., 1985; Boehmer & Emmerson, 1992; Masterson et al., 1992). Single-stranded DNA stimulates this activity more effectively than does double-stranded DNA [Figure 2 and Boehmer and Emmerson (1992) and Masterson et al. (1992)]. The RecC protein by itself had no ATPase activity with either single- or double-stranded DNA, at 9.5 nM RecC protein (data not shown; Masterson et al., 1992). However, addition of RecC to RecB stimulates ATP hydrolysis, presumably catalyzed by RecB. The activity of the two subunits together is greater with double-stranded DNA than with single-stranded DNA, the opposite of what we observe with RecB alone (Figure 2). The ATPase activity with single-stranded DNA increased by about 4-fold when RecC was added to RecB in this experiment, while that with double-stranded DNA increased by about 27-fold. This qualitative change in the ATPase activity of RecB shows that the two proteins associate to form the RecBC enzyme. ATPase activity has been reported previously for the RecBC enzyme (Palas & Kushner, 1990; Masterson et al., 1992), although with greater stimulation by single-stranded than double-stranded DNA.

The ATPase activity of the combination of RecB and RecC did not depend on the concentrations of the proteins in the reconstitution mixture nor on the time of incubation (Table I). It is therefore likely that all of the RecB protein can associate with RecC to make RecBC under these conditions. We included about a 6-fold excess of RecC over RecB and incubated for 30–60 min on ice in all subsequent experiments. The RecBC enzyme concentration was then assumed equal to the RecB protein concentration, since RecC was in excess.

Table I: ATP Hydrolysis Activity in Mixtures of RecB and RecC[a]

[RecB][b] (nM)	[RecC][b] (nM)	ATP hydrolysis rate (μM/min) after an incubation time (h) 0.5	1	4	4.5
73	73	1.99	2.11	1.97	1.77
73	146	2.34	2.11	2.20	2.05
73	438	2.14	1.84	2.20	1.89
73	876	1.90	1.95	1.92	1.90
290	580	1.85	2.12	1.86	2.06

[a] ATP hydrolysis was measured in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.67 mM DTT with double-stranded, *Sac*I-cut, pPvSm19 DNA (100 μM nucleotides; 16 nM ends) and 200 μM [γ-^{32}P]ATP.[b] The RecB and RecC proteins were mixed at the indicated concentrations in the buffer solution described under Experimental Procedures. The mixtures were placed on ice, and aliquots were removed and diluted into the assay mixture after the indicated incubation time. The final RecB concentration in each assay mixture was 1 nM.

Table II: Steady-State Kinetic Parameters for ATP Hydrolysis by the RecBC and RecBCD Enzymes[a]

enzyme	k_{cat} (min^{-1})	K_m(ATP) (μM)	k_{cat}/K_m(ATP) (M^{-1} min^{-1})	N[b]
RecBC[c]	1600 (±300)	8.1 (±1.2)	1.97 (±0.47) × 10^8	3
RecBC[d]	640 (±106)	7.4 (±3.5)	0.86 (±0.43) × 10^8	2
RecBCD[d]	35000	64	5.6 × 10^8	1
RecBCD[e]	36000	44	8.4 × 10^8	
RecBCD[f]	46200	85	5.4 × 10^8	
RecBCD-K177Q[e]	4300	9.2	4.7 × 10^8	

[a] ATP hydrolysis was measured as described under Experimental Procedures. Reaction mixtures contained [γ-^{32}P]ATP (2–200 μM), double-stranded DNA (100 μM nucleotide residues), and 0.73–1.46 nM RecBC or 0.085 nM RecBCD enzyme. [b] Number of determinations. [c] Measured with *Sac*I-cut pPvSm19 DNA. [d] Measured with *Bgl*II-cut pPvSm19 DNA. [e] Korangy & Julin (1992b). [f] Roman & Kowalczykowski (1989b).

The steady-state kinetic parameters for ATP hydrolysis by RecBC with double-stranded DNA are listed in Table II. We found in other experiments that the initial rate of ATP hydrolysis by RecBC was independent of the DNA concentration from 60 to 200 μM DNA (*Sac*I-cut pPvSm19), at 200 μM ATP and 0.73 nM RecBC (data not shown). The k_{cat} values for RecBC in Table II were calculated assuming that the RecB protein is 100% active (RecC is present in excess). The heparin-trapping results (Figure 4, below) suggest that the RecBC enzyme is at least 33% active enzyme, on the basis of the RecB and RecC protein concentrations determined by UV absorption measurements. Therefore, the true k_{cat} could be 3-fold larger than shown in Table II (4800 and 1920 min^{-1} with the *Sac*I-cut and *Bgl*II-cut DNA, respectively), if the RecBC enzyme is only one-third active enzyme. With this correction, the k_{cat} value for RecBC, with *Sac*I-cut DNA, is the same as that determined previously for the RecBCD-K177Q mutant enzyme with native calf thymus DNA (Table II; Korangy & Julin, 1992b). Both are reduced about 8–10-fold compared to RecBCD. The magnitudes of k_{cat}/K_m(ATP) [the apparent rate constant for ATP hydrolysis when the ATP concentration is below the K_m(ATP)] are virtually the same for all three enzymes. This observation implies that, at low ATP concentrations, the RecB subunit hydrolyzes ATP independently of RecD, as concluded previously (Korangy &

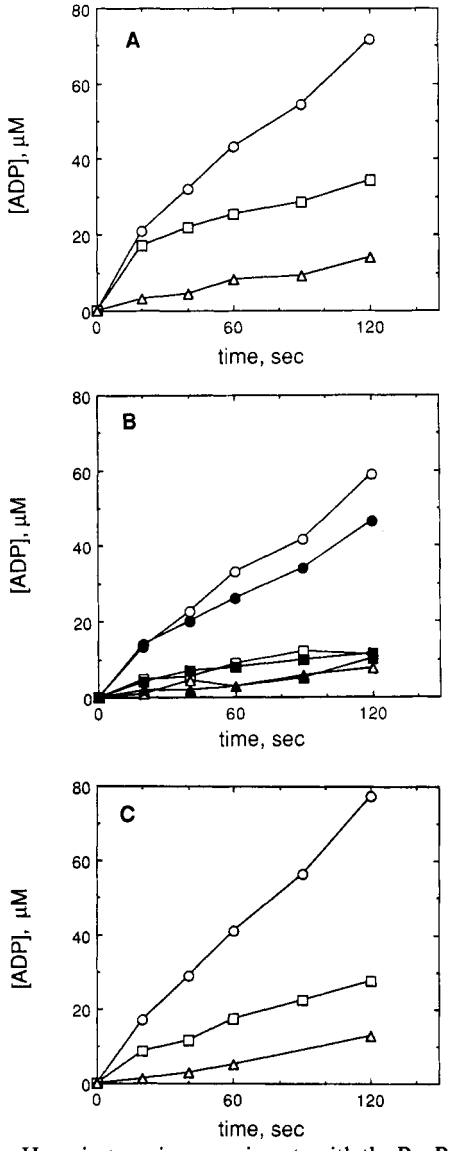

FIGURE 3: Heparin-trapping experiments with the RecBC enzyme. Reaction mixtures (standard conditions) contained pPvSm19 DNA (100 μM nucleotides; 16 nM ends) and RecBC enzyme (23 nM). (A) The DNA was cleaved with *Sac*I to leave a four-nucleotide 3′-single-stranded terminus. (○) 200 μM [γ-^{32}P]ATP added to start the reaction; (□) 200 μM [γ-^{32}P]ATP and heparin (12 mg/mL) added simultaneously to start the reaction; (△) heparin (12 mg/mL) present initially, before the RecBC enzyme was added. (B) The DNA was cleaved with *Sma*I to leave blunt ends (open symbols) or with *Bgl*II to leave a four-nucleotide 5′-single-stranded terminus (closed symbols). (○, ●) 200 μM [γ-^{32}P]ATP added to start the reaction; (□, ■) 200 μM [γ-^{32}P]ATP and heparin (12 mg/mL) added to start the reaction; (△, ▲) heparin (12 mg/mL) present initially, before the RecBC enzyme was added. (C) The DNA was pDJ01 plasmid DNA (100 μM nucleotides; 4.7 nM ends) cleaved with *Kpn*I, with 21 nM RecBC enzyme. Symbols are as defined in (A).

Julin, 1992b). Note that the determination of K_m for an enzyme-catalyzed reaction does not depend on knowledge of the percentage of active enzyme.

ATP Hydrolysis in the Presence of Heparin. We used ATP hydrolysis in the presence of heparin to measure the processivity of the RecBC enzyme, as we did previously for the wild-type and RecBCD-K177Q enzymes (Korangy & Julin, 1992c). There is a burst of ATP hydrolysis by RecBC in the presence of heparin, if the enzyme is allowed to bind to the DNA before ATP and heparin are added (Figure 3A). Heparin added before the RecBC enzyme greatly inhibits ATP hydrolysis. Similar results were obtained with the RecBCD and RecBCD-K177Q enzymes (Korangy & Julin, 1992c). The amount of ADP produced in the burst by RecBC, determined by extrapolation to the vertical axis (time = 0), was 15 μM, in the experiment shown in Figure 3A. The burst was somewhat smaller with DNA substrates having blunt ends or a 5′-single-stranded end (Figure 3B). The burst with *Sma*I-cut pPvSm19 DNA was 3.5 μM ADP, and that with *Bgl*II-cut DNA was 4 μM ADP, for the experiment shown in Figure 3B. The bursts for each type of DNA were identical whether the RecC subunit was present in an amount equal to RecB or in 6-fold excess (data not shown). This observation shows that the small bursts are not due to the excess RecC protein binding to the DNA ends and preventing the RecBC enzyme from binding.

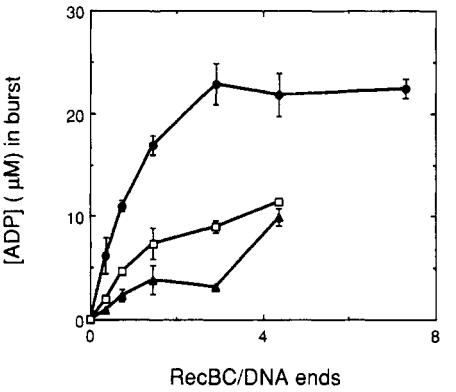

FIGURE 4: Burst of ADP production as a function of RecBC enzyme concentration. Heparin-trapping experiments were carried out as in Figure 3, with pPvSm19 DNA and RecBC enzyme present at the indicated enzyme:DNA ends ratio. The amount of ADP produced in the initial burst was calculated from the linear portion of the time course with heparin added along with ATP to initiate the reaction. This time course was fit by linear least squares, and the intercept on the *y*-axis was determined. (●) pPvSm19 DNA cleaved with *Sac*I, 8 nM ends; (▲) pPvSm19 DNA cleaved with *Sma*I, 9.6 nM ends; (□) pPvSm19 DNA cleaved with *Bgl*II, 9.6 nM ends.

The burst of ADP production by RecBC was small even with a much larger DNA substrate (pDJ01, 21.4 kbp, cleaved with *Kpn*I to produce a four-nucleotide 3′-overhang) rather than pPvSm19 (Figure 3C). The burst in this particular experiment was 6.6 μM ADP produced.

The amount of ADP produced in the initial burst in the heparin trap experiments reached a maximum value when about three molecules of RecBC were added per DNA end, with *Sac*I-cut pPvSm19 (Figure 4). This finding suggests that, as mentioned above, at least 33% of the RecBC protein determined from the A_{280} measurements is active enzyme, assuming that the enzyme acts as a heterodimer. The burst with the *Sma*I- or *Bgl*II-cut DNA was smaller than that with the *Sac*I-cut DNA even with about a 4-fold excess of RecBC over the DNA ends (Figure 4).

The amount of ADP produced per DNA end, under conditions where each end is occupied initially by an enzyme molecule, is a measure of the processivity of the ATP hydrolysis reaction and the DNA unwinding to which it is coupled (Korangy & Julin, 1992c). The average amount of ADP produced per DNA end with the *Sac*I-cut DNA and RecBC in at least 3-fold excess over the ends was 2400 (±600) ADP/end (13 determinations). In three experiments with *Kpn*I-cut pDJ01 (30–100 μM nucleotides; 1.34–4.7 nM ends), 200 μM ATP, and 4–5-fold excess of RecBC, the amount of ADP produced per DNA end was also 2400 (±400).

Previous studies have shown that the RecBCD enzyme is highly processive, able to unwind about 30 000 base pairs on average before it dissociates from the DNA (Roman et al., 1992). The burst of ATP hydrolysis by RecBCD in heparin-

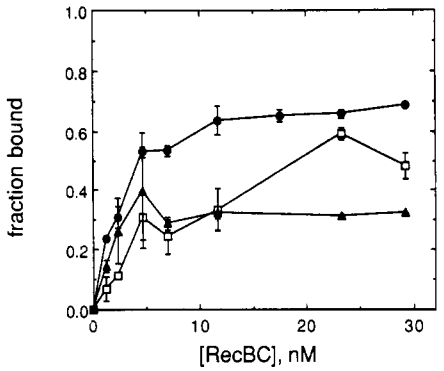

FIGURE 5: DNA binding by the RecBC enzyme. Binding was measured by the nitrocellulose filter binding assay as described under Experimental Procedures. Reaction mixtures contained [^{3}H]pPvSm19 DNA (1.6 nM ends) and RecBC enzyme at the indicated concentration. (●) pPvSm19 DNA cleaved with *Sac*I; (▲) pPvSm19 DNA cleaved with *Sma*I; (□) pPvSm19 DNA cleaved with *Bgl*II.

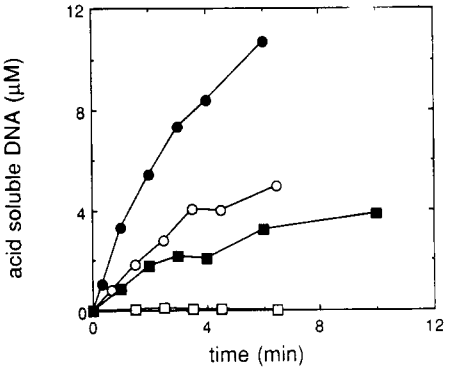

FIGURE 6: Nuclease and helicase reactions catalyzed by RecBC and RecBCD-K177Q enzymes. Reaction mixtures (standard conditions) contained 500 μM ATP, *Sac*I-cut pPvSm19 [^{3}H]DNA (30 μM nucleotides), and 7.5 μM single-stranded DNA binding protein. Open symbols: aliquots removed from the reaction mixtures were mixed with trichloroacetic acid, and the acid-soluble fragments were determined as described under Experimental Procedures. Closed symbols: aliquots removed from the reaction mixtures were treated with S1 nuclease (1.9 units) for 30 min at 37 °C and then treated with trichloroacetic acid. (○, ●) 0.22 nM RecBCD-K177Q enzyme; (□, ■) 0.16 nM RecBC enzyme.

trapping experiments (Korangy & Julin, 1992c) was about 26 000 ADP/end with the largest DNA substrate used (pDJ01, 21.4 kbp), consistent with its high processivity. The RecBCD-K177Q mutant enzyme is less processive than RecBCD, and similar to RecBC (2400 ATP hydrolyzed per DNA end; Korangy & Julin, 1992c). We have not tested directly the effect of the structure of the DNA ends on the processivity of the RecBCD or RecBCD-K177Q enzymes, except that we saw previously (Korangy & Julin, 1992c) that the burst size with RecBCD correlated well with the size of the DNA and did not seem to depend on the structure of the ends.

Binding of Double-Stranded DNA by RecBC. The dependence of the burst of ATP hydrolysis on the type of DNA end, in Figure 4, is surprising. The processivity of an enzyme is not expected to depend on the DNA end since the processive enzyme leaves the end as it acts on the DNA. One possible explanation for the small bursts with the blunt and 5′-single-stranded ends could be if the RecBC enzyme does not bind well to those ends. We examined binding of RecBC to linear double-stranded DNA by nitrocellulose filter binding. The DNA ends were either a four-nucleotide 3′-overhang, a blunt end, or a four-nucleotide 5′-overhang. We detect binding to all three DNAs in the presence of 10 mM $MgCl_2$ and no ATP (Figure 5). In no case is 100% of the DNA bound to the filter, even with large excesses of RecBC enzyme. We detect no significant binding to circular tritium-labeled plasmid DNA (at least 10-fold lower than to linear DNA; data not shown). The binding is consistently greatest with the DNA having the 3′-single-stranded terminus, and the binding appears to be weaker with the blunt ends and 5′-single-stranded overhangs. The lower extent of binding with the nonpreferred ends (particularly the blunt ends) when the enzyme is in excess could result from dissociation of the enzyme–DNA complexes on the filters. The small bursts with the nonpreferred ends may thus arise at least in part from a lower binding affinity for these ends.

DNA Unwinding by the RecBC Enzyme. The rate and processivity of ATP hydrolysis by RecBC suggest that this enzyme should also unwind DNA. Figure 6 shows DNA unwinding and DNA solubilization by the RecBC and RecBCD-K177Q enzymes. Unwound DNA is trapped by the SSB protein in this experiment. Samples removed from the reaction mixture either are added to trichloroacetic acid, to determine acid-soluble DNA fragments, or are treated with SDS, S1 nuclease, and then trichloroacetic acid, to determine the total amount of unwound DNA. The results show that the RecBC and RecBCD-K177Q enzymes unwind double-stranded DNA. Thus, ATP hydrolysis by RecBC is coupled to DNA unwinding in these experiments. However, while the RecBCD-K177Q enzyme solubilizes a substantial amount of the DNA on its own (Figure 6), no acid-soluble DNA fragments are produced by RecBC, in agreement with previous work (Palas & Kushner, 1990; Masterson et al., 1992). A mixture of the RecB, RecC, and RecD proteins did give ATP-dependent nuclease activity on double-stranded DNA (data not shown; Boehmer & Emmerson, 1991; Masterson et al., 1992).

We found previously that the RecBCD-K177Q mutant enzyme has both nuclease and helicase activities on double-stranded DNA and that they are about 5–7-fold reduced compared to the wild-type enzyme (Korangy & Julin, 1992b). The reaction rates determined from the initial slopes of the time courses in Figure 6 are 11 000 mol bp (mol of RecBCD-K177Q)$^{-1}$ min^{-1} and 3100 mol bp (mol of RecBC)$^{-1}$ min^{-1} under these conditions (using the uncorrected RecBC concentrations). If, as suggested above, the RecBC enzyme is 33% active, then its unwinding activity is comparable to the RecBCD-K177Q enzyme and about 5–7-fold less active than RecBCD. More recent measurements[2] of the unwinding rates indicate that RecBC unwinds DNA about 8-fold more slowly than RecBCD, using uncorrected concentrations. We also find[2] that SSB protein stimulates the ATPase activity of RecBC with double-stranded DNA by about 2.5-fold. This stimulation accounts for the rapid DNA unwinding in experiments containing SSB (Figure 6) compared to the ATP hydrolysis rates determined in the absence of SSB (Table II).

Full-length single-stranded pPvSm19 DNA produced by the RecBC enzyme can be seen on an agarose gel (Figure 7). Full-length single strands are visible in the RecBC-catalyzed reaction by 2 min (Figure 7A). The RecBCD-K177Q enzyme produces some full-length single strands in samples not treated with S1 nuclease, and a smear of partially unwound and degraded molecules, whether or not the samples are treated further with S1 nuclease, in agreement with previous results (Korangy & Julin, 1992c). There may be some degradation of the DNA by RecBC, indicated by the smearing at later time points. This observation agrees with previous reports

[2] F. Korangy and D. A. Julin, unpublished results.

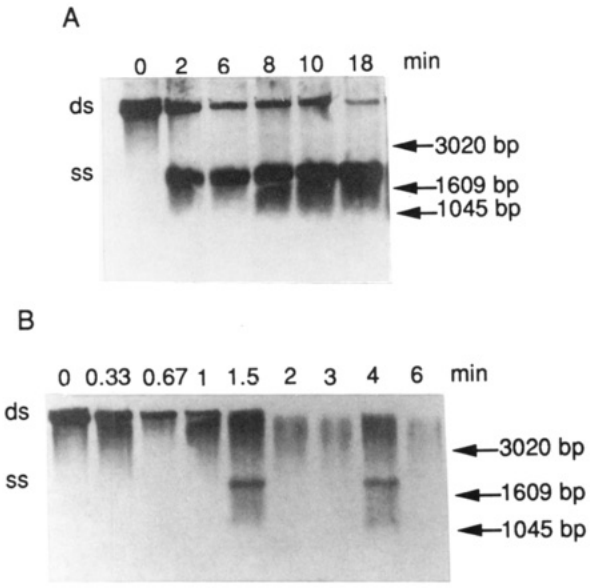

FIGURE 7: Agarose gel electrophoresis of RecBC and RecBCD-K177Q reaction mixtures. Reaction mixtures (standard conditions) contained 200 μM ATP, 40 μM *Sac*I-cut pPvSm19 [^{3}H]DNA, 8 μM single-stranded DNA binding protein, and 1.2 nM RecBC (A) or 1.6 nM RecBCD-K177Q enzyme (B). Aliquots removed from the reactions at the indicated times were run on a 0.7% agarose gel and treated with EN^{3}HANCE as described under Experimental Procedures. Markers run in adjacent lanes (not shown) were denatured linear pPvSm19 [^{3}H]DNA ("ss") and restriction fragments of the pPvSm19 [^{3}H]DNA. (A) Aliquots removed from the RecBC reaction mixtures were added to (final concentrations) 80 mM EDTA, 0.3% (w/v) SDS, and loading dye [8.5% (v/v) glycerol and 0.004% (w/v) bromophenol blue]. (B) Aliquots removed from the RecBCD-K177Q enzyme reaction were added to 50 mM sodium acetate, pH 5.2, 50 mM NaCl, 6 mM $ZnSO_4$, and 0.3% SDS. S1 nuclease (1.9 units) was added, and the samples were placed at 37 °C for 30 min. Loading dye was then added, and the mixtures were run on the gel. Aliquots removed at 1.5 and 4 min were treated in the same way except that no S1 nuclease was added.

that the RecBC enzyme has some nuclease activity but that it does not produce acid-soluble fragments (Palas & Kushner, 1990; Masterson et al., 1992). Neither the RecB nor the RecC subunit alone has nuclease activity (data not shown).

DISCUSSION

Catalytic Properties of RecBC. We have prepared and studied the RecBC enzyme in order to learn about the function of the RecD subunit and the mechanism of the RecBCD holoenzyme itself. We have compared the results with the RecBC enzyme to earlier results with the RecBCD and RecBCD-K177Q mutant enzymes.

The fact that the RecBC enzyme is capable of unwinding DNA shows that there is no absolute requirement for RecD in the helicase reaction. The RecD protein does bind ATP (Julin & Lehman, 1987) and has amino acid sequence similarity to other DNA helicases (Hodgman, 1988; Gorbalenya et al., 1988). The RecBCD-K177Q enzyme, which has a lysine-to-glutamine mutation in the consensus ATP binding motif in the RecD subunit, retains all of the catalytic activities of the wild-type enzyme for which we have tested, including double-stranded DNA-dependent ATP hydrolysis, ATP-dependent nuclease on either single- or double-stranded DNA, and DNA unwinding (Korangy & Julin, 1992b,c). The rates and processivity of the RecBCD-K177Q enzyme acting on double-stranded DNA are reduced compared to the wild-type enzyme (Korangy & Julin, 1992b,c), suggesting that RecD could be involved in translocation along the DNA and in DNA unwinding catalyzed by RecBCD. However, we do not know whether the reductions in catalytic activity in the RecBCD-K177Q enzyme result solely from the effect of the mutation on ATP binding to RecD, or whether there have been effects on other putative functions of the RecD subunit.

The RecBC enzyme, which totally lacks the RecD subunit, addresses this question. It seemed possible that an enzyme form (i.e., RecBC) missing a subunit likely to be involved in ATP hydrolysis and/or unwinding DNA might be much slower than the RecBCD enzyme or have low processivity. The rate and processivity of ATP hydrolysis by RecBC are indeed reduced compared to the RecBCD enzyme, but by a factor of only about 10-fold. RecBC can act processively, and it is able to unwind DNA substrates several thousand base pairs in length. Moreover, the RecBC enzyme is very similar to RecBCD-K177Q in these properties, implying that the lysine-to-glutamine mutation in the RecBCD-K177Q enzyme has essentially completely eliminated the ATP-dependent functions of RecD.

Processivity of ATP Hydrolysis and DNA Unwinding by RecBC. The use of the heparin trap experiment to measure processivity requires the following: (1) The enzyme binds the DNA in the absence of ATP. This is clearly true for RecBC on the basis of the results shown in Figure 5. (2) Some or all of the bound enzymes begin to hydrolyze ATP when ATP and heparin are added. This is shown by Figure 3. (3) Enzyme not bound to the DNA is inhibited by the heparin. The inhibition is not total, since heparin is likely to be a reversible inhibitor which binds to the enzyme competitively with DNA. (4) Heparin does not inhibit the enzyme when it is bound to the DNA. The fact that ATP is hydrolyzed in the presence of heparin if the enzyme binds the DNA first shows that this condition is met.

The processivity of the reaction catalyzed by RecBC is given by the amount of ATP hydrolyzed per DNA end under conditions where all ends are occupied initially by a RecBC enzyme molecule (Bryant et al., 1983; Korangy & Julin, 1992c). The processivity of RecBC is quite close to that we obtained with the RecBCD-K177Q enzyme, and both are less than the wild-type enzyme (Korangy & Julin, 1992c; Roman et al., 1992). It is significant that the burst size per end, and therefore the processivity, is the same with the large DNA substrate (pDJ01, with 3′-single-stranded termini) as it is with pPvSm19. A small burst could result if a substantial fraction of the bound enzymes did dissociate, without hydrolyzing any ATP, when ATP and heparin were added. The remaining enzymes could act with *high* processivity, but the burst would nonetheless be small because there would be significantly fewer than one enzyme acting on each DNA molecule. However, the burst would increase with a larger DNA compared to the smaller one if this were true (assuming that the same fraction of bound enzymes dissociated from each DNA). We find that the burst is about the same with both the pPvSm19 and pDJ01 substrates (Figure 3A,C). Thus, we feel that we are seeing the true processivity of the RecBC enzyme, at least with DNA molecules having a 3′-single-stranded end.

We would expect that the processivity of the RecBC enzyme should be independent of the structure of the ends, since the enzyme leaves the ends when it starts unwinding. The lower apparent processivity of RecBC with the nonpreferred ends (blunt or 5′-single-stranded end) could result in part from the lower apparent affinity of RecBC for these ends, as indicated by the filter binding measurements (Figure 5). The burst in the heparin-trapping experiment with those ends increases somewhat as the RecBC enzyme concentration is increased

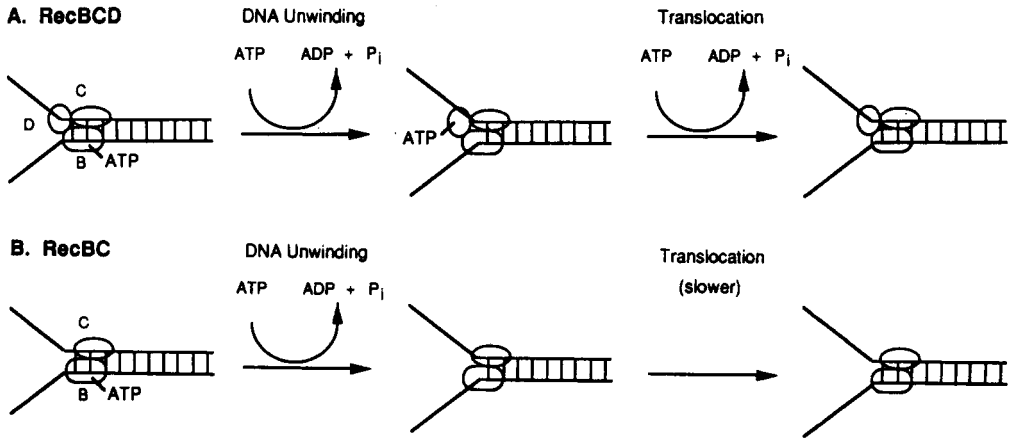

FIGURE 8: Proposed mechanism for RecBCD (A) and RecBC (B). (A) RecBCD. ATP hydrolysis by RecB leads to unwinding of a DNA base pair in the first step. ATP hydrolysis by RecD causes the enzyme to move forward on the DNA so that RecB can unwind the next base pair. (B) RecBC. ATP hydrolysis coupled to DNA unwinding by RecB is followed by slower, *ATP-independent* movement in the absence of RecD.

(Figure 4), as would be expected if more of the ends become occupied at a higher enzyme:DNA ends ratio. It is also possible that some of the RecBC enzymes bound initially to the nonpreferred ends dissociate from these ends and are trapped by the heparin after ATP is added and before they are able to carry out substantial ATP hydrolysis [see point (2) above].

The preference of RecBC for 3′-single-stranded termini is consistent with recent results of photo-cross-linking experiments carried out with the RecBCD enzyme (Ganesan & Smith, 1993). These investigators found that the RecB subunit could be cross-linked to the 3′-end of a duplex DNA molecule. This finding, along with our results, suggests that the RecB subunit of RecBC initiates DNA unwinding by binding to the 3′-strand of a duplex DNA molecule.

ATP Hydrolysis Kinetics. A lower k_{cat} and K_m for ATP in the double-stranded DNA-dependent ATP hydrolysis reaction for both the RecBCD-K177Q enzyme and RecBC compared to the RecBCD enzyme could result from (at least) two mechanisms. The k_{cat} and K_m for the RecBCD enzyme could reflect hydrolysis by two active sites (i.e., RecB and RecD) acting independently. Recent results obtained with the RecB-K29Q-CD mutant enzyme (a lysine-to-glutamine mutation in the consensus ATP binding site of RecB; Hsieh & Julin, 1992) provide evidence that RecD does hydrolyze ATP in the presence of single-stranded DNA, although the isolated RecD subunit has so far not been found to be an ATPase (Lieberman & Oishi, 1974; Masterson et al., 1992). The steady-state kinetics of ATP hydrolysis by RecBCD are consistent with a single active site in the holoenzyme (Roman & Kowalczykowski, 1989b; Korangy & Julin, 1992b), although two ATPase sites in the holoenzyme could be difficult to detect in steady-state kinetic measurements, particularly if their intrinsic kinetic parameters were similar. If the observed k_{cat} for RecBC is the same as that for RecB in the RecBCD holoenzyme, then the reduction in k_{cat} for RecBC compared to RecBCD would have to arise from the loss of ATP hydrolysis by the missing subunit, RecD. This implies that, if the independent sites mechanism is true, RecD must hydrolyze ATP at a rate about 10-fold greater than RecB, to account for the at least 10-fold reduction in the k_{cat} for ATP hydrolysis by RecBC compared to RecBCD.

A second possible mechanism is one in which the RecBCD-catalyzed reaction on double-stranded DNA requires the sequential action of RecB and the RecD. The RecB and RecD subunits could alternate in catalysis of ATP hydrolysis, in a mechanism in which one subunit normally does not act until the other has. Two tightly coupled, alternating sites would appear as one in a steady-state kinetics experiment. A possible mechanism is shown in Figure 8. We propose that ATP hydrolysis by the RecB subunit in RecBCD (Figure 8A) or RecBC (Figure 8B) is directly coupled to DNA unwinding. The RecD subunit is not required for DNA unwinding, since RecBC is a helicase, and so ATP hydrolysis by RecD in the RecBCD enzyme must have some other function. One possibility is that the RecD subunit, stimulated by single-stranded DNA produced by RecB, hydrolyzes ATP to propel the enzyme forward so that RecB can unwind the next base pair. This translocation step must occur in the absence of RecD, since RecBC does unwind DNA. The slow, ATP-independent translocation step in the absence of RecD could be the rate-determining step for the RecBC-catalyzed reaction, but not for RecBCD, leading to the observed reduction in the k_{cat} for RecBC (and RecBCD-K177Q) compared to RecBCD. The overall reaction rate for RecBC and RecBCD-K177Q would also become independent of the ATP concentration at a lower ATP concentration than does that for RecBCD. In other words, the K_m(ATP) would be lower for RecBC and RecBCD-K177Q than for the RecBCD enzyme, as is also observed. This is the reasoning offered previously to explain the difference in the K_m(ATP) between the RecBCD and RecBCD-K177Q enzymes (Korangy & Julin, 1992b).

The reduced processivity caused by the loss of RecD function (either due to the ATP binding site mutation in RecBCD-K177Q or due to its complete absence in RecBC) could follow from a reduction in the rate of translocation. The mechanism shown in Figure 8A also requires that the RecBCD enzyme hydrolyze at least two ATP molecules per base pair unwound, as has been observed (Roman & Kowalczykowski, 1989b). Recent experiments with RecBC[2] show that this enzyme hydrolyzes less than two ATP per base pair unwound, as would be expected for the mechanism in Figure 8B. A mechanism similar to that in Figure 8A was proposed for the rep helicase based on its efficiency of DNA unwinding (Yarranton & Gefter, 1979). Further work is clearly required to test the validity of the mechanism shown in Figure 8A.

Nuclease Activity. The most notable difference between RecBC and RecBCD-K177Q is in the nuclease activity. Although the two enzymes are both helicases on double-stranded DNA, the RecBCD-K177Q enzyme degrades much of the unwound DNA to acid-soluble fragments. This finding suggests that the RecD-K177Q subunit retains some functions of the RecD protein. It may be that the ATP binding site

mutation has essentially knocked out the ATP-dependent functions of the subunit, so the ATP hydrolysis rates and processivity are similar for the RecBC and RecBCD-K177Q enzymes. The RecD subunit may have a second functional site, unaffected by the mutation, which participates in the nuclease activity. Since the RecBC enzyme completely lacks the RecD subunit and therefore also this site, it has essentially no nuclease activity. It could be that the nuclease active site resides in RecD and is shared between RecD and another subunit or that RecD binds the DNA and holds it near the nuclease active site on another subunit. The low level of nuclease activity in RecBC [Figure 7 and Palas and Kushner (1990) and Masterson et al. (1992)] argues against the first possibility.

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