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Articles

Interactions of the RecBCD Enzyme from *Escherichia coli* and Its Subunits with DNA, Elucidated from the Kinetics of ATP and DNA Hydrolysis with Oligothymidine Substrates†

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ABSTRACT: Oligothymidines eight nucleotides or longer stimulate ATP hydrolysis by the RecBC and RecBCD enzymes, and they are substrates for the ATP-stimulated nuclease activity of RecBCD. The steady-state kinetics of ATP hydrolysis by the RecBC enzyme are consistent with a single ATPase and DNA binding site. Results with RecBCD and RecBCD-K177Q [an enzyme with a Lys-to-Gln mutation in the ATP binding motif of the RecD subunit [Korangy, F., & Julin, D. A. (1992) *J. Biol. Chem. 267*, 1727−1732]] indicate that ATP hydrolysis by the RecB subunit is stimulated by pd(T)$_{12}$ binding to a high-affinity site, while the RecD subunit hydrolyzes ATP stimulated by pd(T)$_{12}$ binding to a low-affinity site. The site which stimulates RecB has about 50-fold greater affinity for DNA in either RecBCD or RecBCD-K177Q than does the corresponding site in RecBC. The rates of ATP hydrolysis observed for the RecBCD enzyme at low concentrations of pd(T)$_{12}$ are best explained by a mechanism where the enzyme binds to the DNA and catalyzes multiple rounds of ATP hydrolysis before dissociating. Larger DNA molecules [pd(T)$_{25-30}$ and poly(dT)] are bound more tightly by RecBCD, are hydrolyzed more rapidly, and are much more effective in stimulating ATP hydrolysis than is pd(T)$_{12}$. The results at low ATP concentrations where the nuclease activity is minimal (5 μM) suggest that ATP hydrolysis is stimulated by the DNA ends, but there is no evidence that the RecBCD enzyme moves along these DNA molecules in an ATP-dependent manner under these conditions.

The RecBCD enzyme from *Escherichia coli* is one of many enzymes which use the energy from ATP hydrolysis to catalyze other reactions on DNA, including DNA unwinding, damage recognition, branch migration, and topological changes (Maxwell & Gellert, 1986; Alberts & Miake-Lye, 1992; Sancar & Hearst, 1993; Lohman & Bjornson, 1996). These enzymes have vital roles in all aspects of cellular DNA metabolism. The RecBCD enzyme consists of three protein subunits encoded by the *recB*, *recC*, and *recD* genes, and it catalyzes the following reactions: single- or double-stranded DNA-dependent ATP hydrolysis, ATP-dependent exonuclease on single- or double-stranded DNA, ATP-stimulated endonuclease on single-stranded DNA, and ATP-dependent helicase [reviewed in Kowalczykowski et al. (1994), Myers and Stahl (1994), and Smith et al. (1995)]. The catalytic activities exhibited by RecBCD on double-stranded DNA depend on the reaction conditions, particularly on the relative concentrations of ATP and divalent metal ion (Mg^{2+} or Ca^{2+}). The enzyme degrades double-stranded DNA during processive movement along the DNA at high Mg^{2+} ion

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concentrations relative to the ATP (Goldmark & Linn, 1972; MacKay & Linn, 1974; Muskavitch & Linn, 1982). Under other conditions (high ATP, low Mg^{2+}, or when Ca^{2+} is present), the enzyme unwinds the DNA with little concomitant degradation and is thus a DNA helicase (Rosamond et al., 1979; Taylor & Smith, 1980; Muskavitch & Linn, 1982). The nuclease activity, which is thought to serve a protective function against foreign DNA in the cell (Myers & Stahl, 1994), is suppressed when the enzyme encounters a correctly oriented χ sequence in double-stranded DNA (Taylor & Smith, 1992; Dabert et al., 1992; Dixon & Kowalczykowski, 1993; Kuzminov et al., 1994). The enzyme continues to unwind the DNA past the χ site, and the single-stranded DNA thus produced serves in the initiation of genetic recombination (Dixon & Kowalczykowski, 1991, 1995; Smith, 1994). Recent experiments suggest that the inhibitory effect of χ sequences is by inactivating or causing dissociation of the RecD subunit (Stahl et al., 1990; Dixon et al., 1994; Myers et al., 1995; Köppen et al., 1995).

We expect RecBCD to share some mechanistic aspects with other helicases, but the nuclease activity adds another level of complexity. RecBCD is capable of very rapid and highly processive movement on double-stranded DNA, starting from a blunt duplex end (Roman & Kowalczykowski, 1989a; Roman et al., 1992). However, it has been found to be unable to initiate DNA unwinding from single-stranded termini of large duplex molecules (Taylor & Smith, 1985). A number of other helicases require single-stranded DNA as an initiation site to which the enzyme binds and then unwinds adjacent double-stranded DNA (Lohman & Bjornson, 1996). There is evidence for some helicases, from examining the efficacy of single-stranded DNA molecules of varying length in stimulating ATP hydrolysis (Liu & Alberts, 1981; Matson & Richardson, 1983; Young et al., 1994b), that ATP hydrolysis is coupled to processive movement along a single-stranded DNA molecule.

We have been studying the functions of the two putative ATPase subunits, RecB and RecD, in the catalytic activity of this enzyme. The purified RecB protein is a DNA-dependent ATPase and a helicase at high protein concentrations (Boehmer & Emmerson, 1992; Villani et al., 1993). The RecBC enzyme is an ATP-dependent, processive helicase (Korangy & Julin, 1993, 1994), with little if any nuclease activity on double-stranded DNA (Palas & Kushner, 1990; Masterson et al., 1992; Korangy & Julin, 1993). It is thus clear that ATP hydrolysis by RecB enables RecBC, and presumably also RecBCD, to unwind DNA. Several lines of evidence indicate that the RecD subunit also binds and hydrolyzes ATP. These include amino acid sequence analysis (Finch et al., 1986a,b), photoaffinity labeling (Julin & Lehman, 1987), and comparative studies of the effects of mutations in the ATP binding motifs of RecB (Hsieh & Julin, 1992) and RecD (Korangy & Julin, 1994). Experiments with the mutant enzymes led us to conclude that RecD hydrolyzes ATP with single-stranded DNA and that the ATP hydrolysis can support nuclease activity on single-stranded DNA (Hsieh & Julin, 1992). We also concluded that the RecD subunit hydrolyzes ATP during the reaction with double-stranded DNA (Korangy & Julin, 1994), although it is not essential for DNA unwinding. We and others have proposed mechanisms for catalysis by the RecBCD enzyme in which both the RecB and RecD subunits are DNA-dependent ATPases (Korangy & Julin, 1994; Roman & Kowalczykowski, 1989b; Ganesan & Smith, 1993).

The steady-state kinetics of ATP hydrolysis with double-stranded DNA are consistent with there being only one ATPase catalytic site (Roman & Kowalczykowski, 1989b; Korangy & Julin, 1992b). However, this reaction is very complicated, as it involves ATP hydrolysis, processive DNA unwinding, and, depending on the conditions, nuclease activity. We have attempted to simplify the RecBCD-catalyzed reactions by using small single-stranded DNA oligomers as cosubstrates for ATP hydrolysis and as substrates in the nuclease reaction. It is possible that small DNA oligomers would individually fill DNA binding sites and thus stimulate ATP hydrolysis by individual subunits of the enzyme. Analysis of the steady-state kinetics of ATP hydrolysis is a way to examine the substrate specificity of the enzyme, to provide additional evidence for two ATP hydrolysis active sites, and to investigate whether the enzyme couples ATP hydrolysis to movement along the DNA. Oligothymidines were chosen because they form neither intra- nor intermolecular structures (Saenger, 1983).

We report here results for the steady-state kinetics of ATP hydrolysis by RecBCD, RecBC, and the RecBCD-K177Q mutant enzyme (Korangy & Julin, 1992a), using small oligothymidines as the DNA cosubstrate. The results indicate that ATP hydrolysis by RecBCD is stimulated by two DNA binding sites, while only one site is evident from the kinetics of the RecBCD-K177Q mutant enzyme, and RecBC. ATP hydrolysis by the RecB subunit is stimulated by DNA binding to a high-affinity site, and RecD hydrolyzes ATP stimulated by a low-affinity DNA binding site.

MATERIALS AND METHODS

Materials

ATP (100 mM solution), $pd(T)_n$,[1] with $n = 4$, 6, 8, 12, or 16, and $pd(T)_{25-30}$ were from Pharmacia or Midland Certified Reagent Co., Midland, TX. An oligo(dT) ladder (4−22 nt) was purchased from Gibco-BRL. Poly(dT) was purchased from U.S. Biochemical Corp. Poly(ethylenimine)−cellulose TLC plates were from Sigma or Baker. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont NEN Corp. Hexokinase (yeast, type VI) was from Sigma.

Oligothymidine concentrations were determined from their absorbance at 260 nm. Absorbance coefficients, calculated as in Cantor et al. (1970), were 33 000 [M $pd(T)_4]^{-1}$ cm^{-1}; 49 000 [M $pd(T)_6]^{-1}$ cm^{-1}; 65 400 [M $pd(T)_8]^{-1}$ cm^{-1}; 97 800 [M $pd(T)_{12}]^{-1}$ cm^{-1}; 130 200 [M $pd(T)_{16}]^{-1}$ cm^{-1}; 223 300 [M $pd(T)_{25-30}]^{-1}$ cm^{-1}; and 8100 (M nucleotide residues)$^{-1}$ cm^{-1} for poly(dT). All DNA concentrations are given in terms of oligomers, unless stated otherwise. The size distributions of $pd(T)_{25-30}$ and poly(dT) were determined by electrophoretic analysis of 5′-^{32}P end-labeled DNA, prepared by the polynucleotide kinase exchange reaction (Maniatis et al., 1982). Labeled $pd(T)_{25-30}$ was analyzed on a 20% polyacrylamide gel and the distribution of radioactivity was quantitated using a PhosphorImager (Mo-

[1] Abbreviations: $pd(T)_n$, $n = 4$, 6, 8, 12, 16, or 25−30, 5′-phosphorylated oligothymidines of length 4, 6, 8, 12, 16, or 25−30 nucleotide residues; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; nt, nucleotide residue; TLC, thin-layer chromatography.

lecular Dynamics Corp.). Six labeled bands of approximately equal intensity were observed. An average size of 27.5 nt was used in converting from nucleotide concentration to oligomer concentration. The labeled poly(dT) was analyzed by electrophoresis on a denaturing 1.2% agarose gel with labeled, heat-denatured restriction fragments as markers. About 97% of the radioactivity migrated as a single band centered slightly below a 1647-nt marker band, in agreement with the average size (1200 nt) given by the manufacturer. We used 1200 nt as the average size when estimating the concentrations of poly(dT) molecules.

The concentration of ATP solutions was determined from absorbance measurements at 259 nm (ϵ_{259} = 15.4 mM^{-1} cm^{-1}).

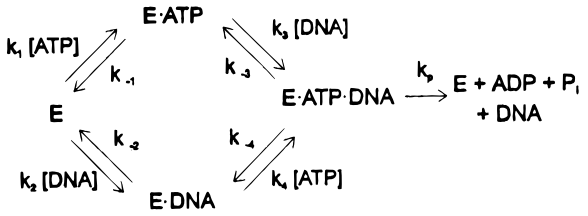
The RecBCD enzyme was purified as described (Korangy & Julin, 1992a). The specific activity of the preparation was 92 000 units/mg using the assay conditions and unit definition given previously (Korangy & Julin, 1992a). The RecBCD-K177Q enzyme (Korangy & Julin, 1992a) was a gift from Dr. Firouzeh Korangy of this laboratory, and it had a specific activity of 20 000 units/mg in the same assay. Both specific activities are about 50% that obtained in our previous work (Korangy & Julin, 1992a). We also measured the nuclease activity of the wild-type enzyme at various ratios of enzyme to DNA ends (Korangy & Julin, 1992b). About 2 enzyme molecules/DNA end were required. These results suggest that about 50% of the purified enzyme is active. All enzyme concentrations are therefore expressed as the concentration of active molecules (i.e., half the concentration based on the absorbance measurements).

The RecBC enzyme was a gift from Hua-Wei Chen of this laboratory and was prepared as follows: the purified RecB and RecC proteins were mixed as described (Masterson et al., 1992) and the RecBC complex was isolated by chromatography on heparin−agarose as for the RecB protein alone (Boehmer & Emmerson, 1991). Analysis by SDS−PAGE showed that equal amounts of RecB and RecC subunits are present. The RecBC enzyme migrates as a single band on a nondenaturing polyacrylamide gel, with mobility intermediate between that of the isolated RecB and RecC proteins. These results, together with the fact that the RecC protein alone does not bind to heparin−agarose (Hickson et al., 1984), show that the two subunits are associated to form the RecBC enzyme. The RecBC concentration was calculated from the A_{280} using ϵ_{280} = 369 500 M^{-1} cm^{-1}. The ATPase activity of the enzyme was maximal with about 2 RecBC molecules/DNA end, indicating that this enzyme is about 50% active as well.

Methods

ATP Hydrolysis. ATP hydrolysis was measured using the TLC assay described previously (Korangy & Julin, 1992b). Standard reaction mixtures contained (final concentrations) 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.67 mM DTT, and [γ-^{32}P]ATP and pd(T)$_n$ as indicated. Stock solutions of enzyme were prepared by diluting small aliquots of concentrated stock into 25 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM DTT, 0.14 mg/mL BSA, and 10% glycerol. Reaction mixtures (20 μL total volume) were placed in a water bath set at 37 °C for about 1 min and then the reaction was initiated by adding the enzyme. Aliquots were removed and analyzed by TLC as described (Korangy & Julin, 1992b).

Scheme 1



The data for most steady-state kinetics experiments were obtained using the PhosphorImager. The spots corresponding to the [γ-^{32}P]ATP and [^{32}P]P_i in the resulting image were integrated using the volume integration utility in the ImageQuant 3.3 program. The concentration of ATP hydrolyzed at each time point was calculated from

$$[ATP]_{hydro} = [ATP]_0 V_{P_i}/(V_{P_i} + V_{ATP}) \qquad (1)$$

where $[ATP]_0$ is the initial ATP concentration and V_{P_i} and V_{ATP} are the integrated volumes of the [^{32}P]P_i and [γ-^{32}P]-ATP spots for each time point. In a few experiments, the radioactivity was determined by liquid scintillation counting.

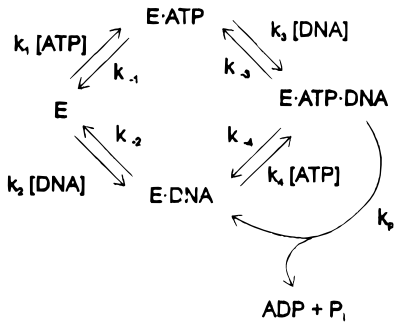
The initial reaction rates were determined from the slope of time courses for low extents of reaction (usually less than about 10% of the ATP hydrolyzed). Because this assay measures the percent change in the ATP concentration, rather than the absolute concentration of the product, it is most accurate at low ATP concentrations, where the extent of reaction is relatively high. Rates are more difficult to determine at high ATP, since the percent change in substrate is lower when the substrate concentration is near or above the saturating level. We were reluctant to carry out long time courses, as that allowed more opportunity for nucleolytic degradation of the DNA substrate. Initial rate data (v_i *vs* substrate concentration) were fit to equations (see below) using the nonlinear least-squares curve-fitting program in SigmaPlot (Jandel Scientific).

ATP Hydrolysis Data Interpretation. The ATP hydrolysis data were interpreted in terms of a mechanism where both ATP and DNA must be bound by the enzyme before ATP can be hydrolyzed (Scheme 1). We have assumed that the substrates can bind in any order, and we have made no assumption as to the relative rates of the catalytic step (rate constant k_p) and the substrate binding and dissociation steps. The general equation for the dependence of the initial rate of ATP hydrolysis (v_i) on the varied concentration of one substrate (S), at a fixed concentration of the second substrate, is given by Segel (1975, pp 646 and 657) and by Ferdinand (1966):

$$\frac{v_i}{E_T} = \frac{iS^2 + jS}{k + lS^2 + mS} \qquad (2)$$

where E_T is the enzyme concentration and i, j, k, l, and m are functions of the individual rate constants (Scheme 1) and of the fixed substrate concentration (Segel, 1975). As discussed by Segel and by Ferdinand, the mechanism in Scheme 1 can give rise to a variety of hyperbolic and nonhyperbolic v_i *vs* S dependencies, depending on the substrate concentrations and on the relative magnitudes of the rate constants. Nonhyperbolic curves can result when the catalytic step is fast and there is a kinetic preference for binding one substrate before the other (Segel, 1975).

Scheme 2



The data in most experiments appeared hyperbolic and were fit well to the Michaelis−Menten equation:

$$v_i = \frac{V_{max}^{app} S}{K_m^{app} + S} \quad (3)$$

The meaning of the apparent parameters in eq 3 depends on the specific mechanism under consideration. Thus, when the DNA concentration is varied at a fixed, saturating ATP concentration, the rate equation for Scheme 1 can simplify to a hyperbolic form:

$$\frac{v_i}{E_T} = \frac{k_p[\text{DNA}]}{(k_{-3} + k_p)/k_3 + [\text{DNA}]} \quad (4)$$

and $K_m^{app} = (k_{-3} + k_p)/k_3$ and $V_{max}^{app} = k_p E_T$.

The DNA is treated in Scheme 1 as a substrate which must dissociate from the enzyme and reassociate before each ATP hydrolysis turnover. Scheme 2 is a variation of Scheme 1, in which the enzyme does not necessarily dissociate from the DNA for every ATP hydrolysis reaction. In this mechanism, the DNA is an activator of ATP hydrolysis but is not itself altered during the reaction. The steady-state rate equation for Scheme 2 was derived by the King−Altman method and found to be identical to eq 2, except that the parameters *i*, *j*, *k*, *l*, and *m* depend differently on the rate constants (this derivation is available as supporting information). The equation for Scheme 2 becomes eq 5 when the DNA concentration is varied at a fixed, saturating, ATP concentration:

$$\frac{v_i}{E_T} = \frac{k_p[\text{DNA}]}{(k_{-3}/k_3) + [\text{DNA}]} \quad (5)$$

and $K_m^{app} = k_{-3}/k_3$ and $V_{max}^{app} = k_p E_T$.

The results for RecBCD when the $pd(T)_{12}$ concentration was varied over a wide range, at constant ATP, are indicative of two nonidentical DNA-dependent ATPase active sites (Figure 4). These data were fit to

$$v_i = \frac{V_1[\text{DNA}]}{K_1 + [\text{DNA}]} + \frac{V_2[\text{DNA}]}{K_2 + [\text{DNA}]} \quad (6)$$

where V_1 and V_2 are the apparent V_{max} for sites 1 and 2, and K_1 and K_2 are the apparent K_m^{DNA} for sites 1 and 2. (Each of these individual sites could be described by the mechanism of either Scheme 1 or Scheme 2.)

The results obtained with RecBC (Figure 2) are adequately described by Scheme 1, if all the substrate binding steps are in rapid equilibrium and catalysis (k_p) is relatively slow. The initial rate for this situation (rapid equilibrium, random sequential) is given by Segel (1975, p 275)

$$\frac{v_i}{E_T} = \frac{k_p[\text{ATP}][\text{DNA}]}{\alpha K_{ATP} K_{DNA} + \alpha K_{ATP}[\text{DNA}] + \alpha K_{DNA}[\text{ATP}] + [\text{ATP}][\text{DNA}]} \quad (7)$$

where K_{ATP} and K_{DNA} are the dissociation constants for ATP and DNA ($= k_{-1}/k_1$ and k_{-2}/k_2, respectively, from Scheme 1), and α is the factor by which binding of one substrate alters the affinity for the other substrate. Plots of v_i *vs S*, at a fixed concentration of the second substrate, are hyperbolic, following apparent Michaelis−Menten kinetics (eq 3). The apparent parameters when ATP is varied at constant DNA are $V_{max}^{app} = k_p E_T/(1 + \alpha K_{DNA}/[\text{DNA}])$ and $K_m^{app} = \alpha K_{ATP}(1 + K_{DNA}/[\text{DNA}])/(1 + \alpha K_{DNA}/[\text{DNA}])$. The parameters when DNA is varied at constant ATP are $V_{max}^{app} = k_p E_T/(1 + \alpha K_{ATP}/[\text{ATP}])$ and $K_m^{app} = \alpha K_{DNA}(1 + K_{ATP}/[\text{ATP}])/(1 + \alpha K_{ATP}/[\text{ATP}])$.

Nuclease Reactions. Oligothymidine substrates and the oligo(dT) ladder were 5′-end-labeled using polynucleotide kinase and [γ-^{32}P]ATP. Hexokinase (1 unit) and glucose (10 mM) were then added to remove any unreacted ATP. The labeled oligomers were purified by spun column chromatography (Bio-Spin 6 columns, Bio-Rad). Analysis by thin-layer chromatography (PEI−cellulose; 1 M sodium phosphate, pH 3.5 mobile phase) showed that at most trace amounts of ATP (<5 nM) remained in the purified 5′-end-labeled oligomers.

Nuclease reaction conditions were exactly as for the ATPase reactions, except that the DNA was 5′-^{32}P-labeled and the ATP (when included) was not radioactive. Stock solutions of [5′-^{32}P]pd(T) oligomers for nuclease reactions were prepared by mixing a known amount of nonradioactive 5′-phosphorylated oligomer with a trace amount of [5′-^{32}P]-oligomer. The reaction mixtures were placed at 37 °C and the reaction was initiated by adding the enzyme. Aliquots were removed and quenched by adding to an equal volume of 80 mM EDTA, 80% formamide, and 0.2% bromophenol blue. The quenched samples from nuclease reactions with $pd(T)_{12}$ and $pd(T)_{25-30}$ were analyzed on polyacrylamide gels (20% acrylamide/0.67% bisacrylamide) in 1.5× TBE (Maniatis et al., 1982). Samples from reactions with poly(dT) were analyzed on 1.5% agarose gels in 1× TBE. The gels were dried and exposed to the PhosphorImager, and the radioactivity in each band was quantitated by volume integration.

The concentrations of undegraded $pd(T)_{12}$ or $pd(T)_{25-30}$ and the concentrations of each nuclease product at time *t* were calculated from

$$[\text{pd(T)}_i]_t = [\text{pd(T)}_n]_0 (V_i)_t / (\sum V_i)_t \quad (8)$$

where $[\text{pd(T)}_i]$ is the concentration of the DNA fragment containing *i* thymidine residues, $[\text{pd(T)}_n]_0$ is the initial $pd(T)_{12}$ or $pd(T)_{25-30}$ concentration, $(V_i)_t$ is the integrated volume of fragment *i* at time *t*, and $(\sum V_i)_t$ is the sum of the integration volumes of all the DNA fragments present at time *t*. The initial rate of the nuclease reaction was calculated from the slope of the linear portion of the time course.

Figures showing nuclease reactions (Figures 3 and 7) were prepared from gray-scale printouts made directly from the PhosphorImager. The limits of the display (white-to-black) were chosen so that the product bands were readily visible. For this reason, the initial substrate bands appear overly dark in these prints. Care was taken that the exposure time was short enough that the image itself was not saturated so that the integration results are reliable.

DNA Binding. Binding measurements with $pd(T)_{12}$ were done using nitrocellulose filters (BA85, Schleicher & Schuell) to trap enzyme−DNA complexes and DE81 filters (Whatman) to trap unbound DNA, as described by Wong and Lohman (1993). Binding conditions were the same as those for ATP hydrolysis, with about 0.7 nM [5′-^{32}P]$pd(T)_{12}$ and no ATP. One experiment was done with 10 mM $CaCl_2$ in place of $MgCl_2$. The fraction of DNA bound to the nitrocellulose filter (f) was determined from

$$f = [CPM_{NC} - (\sigma)(CPM_{DE})]/(CPM_{NC} + CPM_{DE}) \quad (9)$$

where CPM_{NC} and CPM_{DE} are the counts bound to the nitrocellulose and DE81 filters, respectively, for a given binding mixture, and $\sigma = CPM_{NC}/(CPM_{NC} + CPM_{DE})$ for a sample from a mixture containing no RecBCD enzyme. [This corrects for the background of DNA which binds nonspecifically to the filter in the absence of protein (Wong & Lohman, 1993).] The dissociation constant (K_d) was determined from (Woodbury & von Hippel, 1983)

$$f = 1 - \frac{(1 + \zeta[\text{RecBCD}]/K_d)}{(1 + [\text{RecBCD}]/K_d)} \quad (10)$$

where ζ is the probability that a DNA molecule with one RecBCD enzyme bound will *not* be retained on the filter.

Binding to $pd(T)_{25-30}$ was done using an electrophoretic mobility shift assay. [The background binding of [5′-^{32}P]-$pd(T)_{25-30}$ to nitrocellulose filters was very high (>50%) in the absence of RecBCD, for unknown reasons. This was observed with two different batches of [5′-^{32}P]$pd(T)_{25-30}$. The background was much lower with no $MgCl_2$ (ca. 10%), but little binding of the enzyme was detected without $MgCl_2$.] Polyacrylamide gels (6% acrylamide, 30:1 acrylamide: bisacrylamide) were prepared in a buffer consisting of 50 mM HEPES, 25 mM Tris base, and 10 mM $CaCl_2$, adjusted to pH = 7.5 with 5 M KOH. The gel was prerun (4 V/cm) for 1 h before use. Binding mixtures were the same as the ATP hydrolysis mixtures, except that 10 mM $CaCl_2$ was substituted for $MgCl_2$, in order to suppress the RecBCD nuclease activity but maintain the divalent cation concentration, and glycerol (5% v/v) was included. [5′-^{32}P]$pd(T)_{25-30}$ was present at about 0.5 nM. Mixtures were placed at 37 °C for 5 min and then 3−5 μL was loaded on the gel. The gel was run at 4 V/cm, dried, and exposed to the PhosphorImager.

RESULTS

ATP Hydrolysis Reactions with $pd(T)_4$−$pd(T)_{16}$. We first tested oligo(dT) substrates of various sizes as cosubstrates for ATP hydrolysis by the RecBCD, RecBC, and RecB enzymes (Figure 1). RecBCD and RecBC show similar dependence on the oligomer size, with significant activity seen with the oligomers longer than $pd(T)_8$ and lower activity with $pd(T)_4$ and $pd(T)_6$. The activity of RecB is much lower than either RecBC or RecBCD with all the substrates under these conditions, similar to previous results (Boehmer & Emmerson, 1992). We have not studied the RecB-catalyzed reaction any further. No activity was observed with any of the enzymes in the absence of DNA. We chose $pd(T)_{12}$ as the substrate for most further investigation.

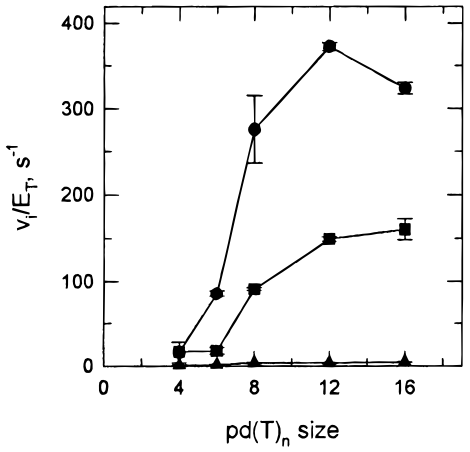


FIGURE 1: ATP hydrolysis by the RecBCD, RecBC, and RecB enzymes with oligothymidines of various sizes. Reaction mixtures (standard conditions) contained 10 μM $pd(T)_n$ of size n nucleotides, 250 μM ATP, and (●) 0.69 nM RecBCD [for $pd(T)_4$ and $pd(T)_6$], and 0.345 nM RecBCD [for $pd(T)_8$−$pd(T)_{16}$], (■) 0.5 nM RecBC, or (▲) 12.5 nM RecB. Error bars are the range of duplicate measurements.

ATP Hydrolysis Catalyzed by RecBC with $pd(T)_{12}$. Steady-state measurements of the rates of $pd(T)_{12}$-stimulated ATP hydrolysis with RecBC were done in which the concentration of one substrate was varied at a fixed concentration (low or high) of the second substrate (Figure 2). Each individual data set fits well to the Michaelis−Menten equation (eq 3) to give the apparent K_m and V_{max} values given in the legend to Figure 2. These apparent parameters and their definitions (see Materials and Methods, eq 7 and following) give a set of simultaneous equations from which we can calculate values for the parameters in eq 7: $K_{ATP} = 212$ μM, $K_{DNA} = 27$ μM, $\alpha = 0.17$, and $k_p = 186$ s^{-1}. These parameters were then used to calculate values for v_i at each [S], and the results are also shown in Figure 2. The fit to the data is good, considering that the data are from four separate, independent experiments. The value of k_p is similar to values we have reported previously for k_{cat} for ATP hydrolysis by RecBC with double-stranded DNA (Korangy & Julin, 1994). Thus $pd(T)_{12}$ is quite effective at stimulating ATP hydrolysis, in spite of its small size. The values of K_{ATP} and K_{DNA} are the predicted dissociation constants for each substrate in the absence of the other. The value of α means that the affinity for one substrate is about 6-fold greater when the other is bound than in its absence, and so the apparent K_ms for each substrate at a fixed, high, concentration of the second are smaller than the fitted K_{ATP} and K_{DNA}. The simplest interpretation of these data, given that RecB is itself an ATPase, is that the RecBC enzyme contains a single DNA-dependent ATP hydrolysis active site, in the RecB subunit.

Reactions Catalyzed by RecBCD and RecBCD-K177Q with $pd(T)_{12}$: Nuclease Reactions with RecBCD on $pd(T)_{12}$. We first checked whether $pd(T)_{12}$ is a nuclease substrate for RecBCD (the nuclease activity of RecBC is much lower than that of RecBCD, and its activity with these oligomers was not tested). $pd(T)_{12}$ is cleaved by RecBCD in both the

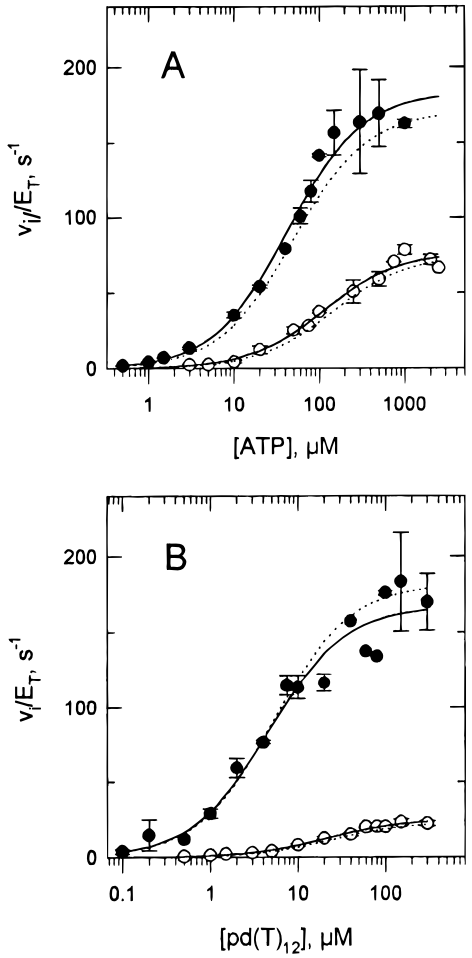


FIGURE 2: ATP hydrolysis by the RecBC enzyme with pd(T)$_{12}$. (A) Reaction mixtures (standard conditions) contained varied ATP and (●) 0.25 nM RecBC enzyme and 50 μM pd(T)$_{12}$ [solid lines are the fits to eq 3 with $K_m^{app} = 43$ μM and $V_{max}^{app}/E_T = 187$ s^{-1}] or (○) 0.5 nM RecBC enzyme and 3 μM pd(T)$_{12}$ [$K_m^{app} = 114$ μM and $V_{max}^{app}/E_T = 77$ s^{-1}]. (B) Reaction mixtures (standard conditions) contained varied pd(T)$_{12}$ and (●) 0.25 nM RecBC and 1.2 mM ATP [$K_m^{app} = 4.5$ μM and $V_{max}^{app}/E_T = 167$ s^{-1}] or (○) 0.25 nM RecBC and 5 μM ATP [$K_m^{app} = 21$ μM and $V_{max}^{app}/E_T = 27$ s^{-1}]. Dotted lines in each plot are calculated from eq 7 using the parameter values given in the text (see Results).

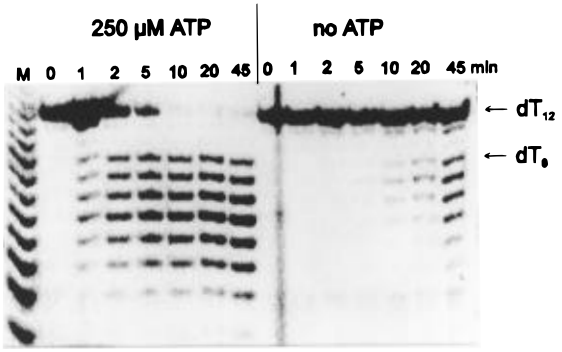


FIGURE 3: Nuclease reactions catalyzed by the RecBCD enzyme with pd(T)$_{12}$. pd(T)$_{12}$ (0.1 μM) was mixed with a trace of [5′-^{32}P]-pd(T)$_{12}$ and treated with 3.5 nM RecBCD enzyme under standard conditions, with 250 μM ATP or no ATP. Samples were removed at the indicated times and loaded on a 20% polyacrylamide gel. M, [5′-^{32}P]pd(T) ladder.

absence and presence of ATP (Figure 3). The reactions are quite slow at the RecBCD and ATP concentrations used in the ATP hydrolysis experiments (the reactions shown in Figure 3 contained 10-fold higher RecBCD concentration than was used in the ATP hydrolysis experiments shown below). Less than 10% of the pd(T)$_{12}$ is cleaved during the typical time of an ATP hydrolysis measurement, with the RecBCD enzyme concentrations used in those experiments ($\leq$0.35 nM), even at the highest ATP concentrations (500 μM ATP). Thus there is little change in the pd(T)$_{12}$ concentration during the ATP hydrolysis time courses (see below), and these time courses are essentially linear for several minutes even at high ATP and low pd(T)$_{12}$.

The nuclease products range in size from a 9-mer to a dimer, indicating that the enzyme acts endonucleolytically on this substrate, cleaving 3−10 nucleotides from the labeled 5′ end. The predominant product is a hexamer. ATP (250 μM) stimulated the rate of disappearance of pd(T)$_{12}$ about 50-fold at low pd(T)$_{12}$ but had much less effect at higher pd(T)$_{12}$ concentrations (Table 1). Comparing the nuclease and ATP hydrolysis rates at 10 μM pd(T)$_{12}$ and 250 μM ATP reveals that several hundred ATP molecules are hydrolyzed for each pd(T)$_{12}$ molecule cleaved. Thus, most of the ATP hydrolysis stimulated by pd(T)$_{12}$ is not coupled to nuclease cleavage.

Kinetics of ATP Hydrolysis by the RecBCD and RecBCD-K177Q Enzymes: Dependence on pd(T)$_{12}$ Concentration. ATP hydrolysis catalyzed by RecBCD and RecBCD-K177Q with varied pd(T)$_{12}$ concentrations, at 2.5, 20, and 500 μM ATP, gave the results shown in Figure 4. The data for RecBCD fit poorly to eq 3, but the fit is better to eq 6 for two independent pd(T)$_{12}$ binding sites (see Materials and Methods). The parameters for the second site appearing at high pd(T)$_{12}$ are very poorly determined, except at 2.5 μM ATP. The data for RecBCD-K177Q were fit well by the Michaelis−Menten equation, for a single active site, and there is no indication of a second site at high pd(T)$_{12}$. The averaged apparent kinetic parameters from several experiments are given in Table 2.

Both the RecBCD and RecBCD-K177Q enzymes have a high-affinity pd(T)$_{12}$ site which, at high ATP, saturates with an apparent K_m about 50-fold lower than the apparent K_m for pd(T)$_{12}$ of RecBC, also at high ATP (i.e., with about 50-fold greater affinity than in RecBC). The kinetic parameters for RecBCD-K177Q are in all cases very similar to those for RecBCD, strongly suggesting that it is the RecB subunit's ATPase site which is stimulated by the high-affinity pd(T)$_{12}$ binding site. Moreover, the mutation in the RecD subunit appears to have had little if any effect on the activity of the ATPase site in RecB. The second, lower affinity site for pd(T)$_{12}$ is not present in RecBCD-K177Q. This suggests that the low-affinity pd(T)$_{12}$ site stimulates ATP hydrolysis by the RecD subunit, since the ATP binding site in RecD has been disabled in the RecBCD-K177Q enzyme (Korangy & Julin, 1992a). [We cannot completely rule out the possibility that the mutation in the RecD subunit has simply knocked out the low-affinity pd(T)$_{12}$ binding site.]

Kinetics of ATP Hydrolysis by the RecBCD and RecBCD-K177Q Enzymes: Dependence on ATP Concentration. Variation of the ATP concentration, at 10 μM pd(T)$_{12}$, a concentration where only the high-affinity site in RecBCD is stimulated, gives results which fit well to the Michaelis−Menten equation for both the RecBCD and RecBCD-K177Q enzymes (Figure 5, ○). The kinetic parameters under these conditions are very similar for the two enzymes (Table 2). The k_{cat} values are also close to those measured previously with double-stranded DNA (Roman & Kowalczykowski,

Table 1: Rates of Nuclease Reactions on $pd(T)_{12}$ and $pd(T)_{25-30}$ Catalyzed by RecBCD[a]

[DNA] (μM oligomer)	[ATP] (μM)	rate ($\pm$std dev) [mol of oligomer cleaved min^{-1} (mol of RecBCD)$^{-1}$] $pd(T)_{12}$	N^b	$pd(T)_{25-30}$	N^b
0.03	0	0.054 (0.016)	3	0.12 (0.04)	3
0.03	500	3.4 (1.8)	4	50 (24)	4
0.1	0	0.18 (0.06)	4	ND[c]	
0.1	250	8 (4)	3	ND	
3	0	9.4 (1)	3	3.6 (0.6)	3
3	500	50 (14)	3	200 (40)	3
10	0	38 (18)	3	ND	
10	250	60	1	ND	

[a] Reactions were done as in Materials and Methods and Figures 3 and 7. [b] Number of determinations. [c] ND, not determined.

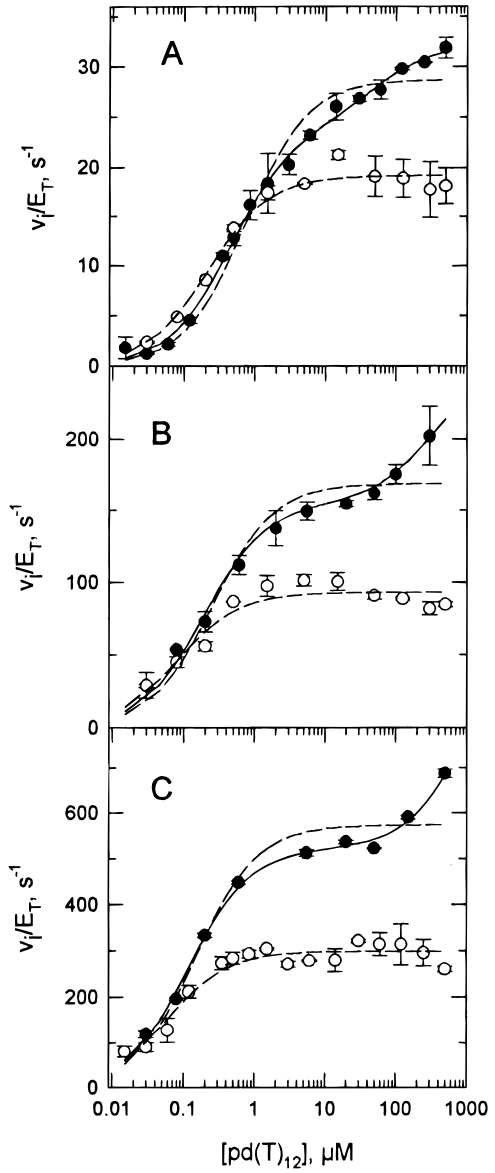


FIGURE 4: ATP hydrolysis by the RecBCD (●) and RecBCD-K177Q (○) enzymes: Variation of $pd(T)_{12}$ at constant ATP. (A) Reaction mixtures contained 2.5 μM ATP and 0.17 nM enzyme. (B) Reaction mixtures contained 20 μM ATP and 0.17 nM enzyme. (C) Reaction mixtures contained 500 μM ATP and 0.35 nM enzyme. Solid lines through the RecBCD data are the best fit to eq 6 (Materials and Methods) for two independent active sites. Dashed lines are best fits to eq 3. The fitted parameters are given in Table 2.

1989b; Korangy & Julin, 1994), particularly the relative values. As observed above for RecBC, the results show that the small DNA oligomer is quite effective at stimulating ATP hydrolysis.

We performed one experiment (data not shown) where the ATP concentration was varied (7−1450 μM) at 500 μM $pd(T)_{12}$ [a concentration of $pd(T)_{12}$ at which both sites should be at least partially activated], to look for more direct evidence for two ATPase sites. The data were fit essentially identically by the single-site Michaelis−Menten equation (eq 3); $K_m^{app} = 76$ μM and $V_{max}^{app}/E_T = 940$ s^{-1}) and by the two-independent-sites model (eq 6); $K_1 = 50$ μM, $K_2 = 195$ μM, $V_1/E_T = 600$ s^{-1}, and $V_2/E_T = 360$ s^{-1}). Thus, two independent sites having similar K_m^{app} values, or different V_{max}^{app} values, are difficult to distinguish in such an experiment.

The results when we varied the ATP at much lower $pd(T)_{12}$ (30 nM) are also shown in Figure 5 (●). These data do not fit well to the Michaelis−Menten equation at low ATP concentrations, for either the RecBCD or the RecBCD-K177Q enzymes (insets to Figure 5). This behavior was observed in four experiments with the wild-type enzyme and three with the RecBCD-K177Q mutant.

This deviation from Michaelis−Menten behavior in Figure 5 (sigmoidicity) is similar to that indicative of positive cooperative interactions between two active sites. However, this type of v_i vs S curve can also arise from the mechanisms in Schemes 1 and 2 (Segel, 1975; Ferdinand, 1966), which have only a single catalytic site. Indeed, the data at low ATP fit better to eq 2 for Schemes 1 or 2. [The parameter values from these fits (i, j, k, l, and m) are not well determined and have no simple physical meaning. We feel that it is more significant that the data can be reproduced reasonably well by eq 2 and are consistent with Scheme 1 or 2.] As argued below (see Discussion), the large magnitude of the reaction rates at high ATP and 30 nM $pd(T)_{12}$ are actually best explained by Scheme 2.

The observation of apparent sigmoidicity with the RecBCD-K177Q enzyme led us to disfavor the possibility of positive cooperativity involving ATPase sites in the RecB and RecD subunits. Recent experiments (Taylor & Smith, 1995) provide evidence that the functional form of RecBCD is a heterotrimer and not a putative hexameric form ($RecB_2C_2D_2$), arguing against the possibility of cooperative interactions between two RecB subunits. We also considered the possibility that the oligomeric structure of the enzyme changes from one of low activity to higher activity, in response to an increase in the ATP concentration. We assumed that a change (decrease or increase) in the enzyme concentration could lead to a similar increase in activity. However, there was no significant change in the specific

Table 2: Steady-State Kinetic Parameters for $pd(T)_{12}$-Stimulated ATP Hydrolysis Catalyzed by the RecBCD and RecBCD-K177Q Enzymes

enzyme	[ATP] (μM)	[$pd(T)_{12}$] (μM oligomers)	V_{max}^{app}/E_T[a] (s^{-1})	K_m(ATP)[a] (μM)	$K_m[pd(T)_{12}]$[a] (μM oligomers)	N[b]
RecBCD	2.5	varied	24		0.46	1
			8[c]		50[c]	
RecBCD-K177Q			20		0.22	1
RecBCD	20	varied	150 (8)		0.185 (0.011)	2
			26 (20)[c]			
RecBCD-K177Q			102		0.112	1
RecBCD	500	varied	600 (120)		0.100 (0.02)	2
RecBCD-K177Q			296 (4)		0.0885 (0.043)	2
RecBCD	varied	0.03	190 (60)	120 (42)[d]		4
RecBCD-K177Q			120 (20)	63 (23)[d]		3
RecBCD	varied	10	940 (110)	124.5 (43)		3
RecBCD-K177Q			620	101.9		1

[a] Apparent steady-state kinetic parameters from fits to eq 3 or 6. Standard deviations are shown in parentheses. [b] Number of determinations. [c] Kinetic parameters for the second site in Figure 4. [d] ATP concentration giving half-maximal velocity.

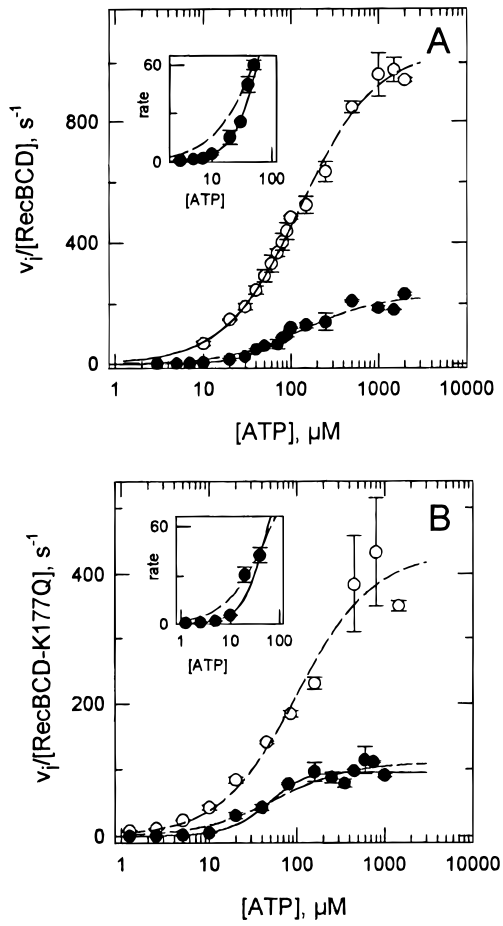


FIGURE 5: ATP hydrolysis by the RecBCD and RecBCD-K177Q enzymes: Variation of ATP at constant $pd(T)_{12}$. Reaction mixtures contained (A) 0.345 nM RecBCD enzyme and 0.03 μM $pd(T)_{12}$ (●) or 10 μM $pd(T)_{12}$ (○) or (B) 0.35 nM RecBCD-K177Q enzyme and 0.03 μM $pd(T)_{12}$ (●) or 0.25 nM RecBCD-K177Q enzyme and 10 μM $pd(T)_{12}$ (○). Dashed lines are fits of the data to eq 3, and solid lines are fits to eq 2. Insets are plots of the data at 0.03 μM $pd(T)_{12}$ and low ATP concentrations.

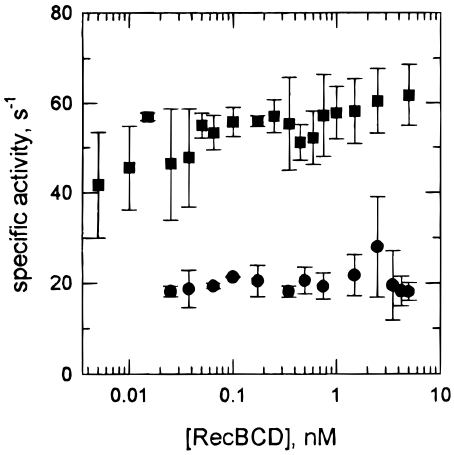


FIGURE 6: Specific ATPase activity of RecBCD as a function of enzyme concentration. The rate of ATP hydrolysis was measured at 30 nM $pd(T)_{12}$ and 20 μM ATP (●) or 50 μM ATP (■), at the indicated RecBCD enzyme concentrations. The initial rate was divided by the enzyme concentration to obtain the specific activity. Error bars are standard deviations of triplicate determinations.

activity (v_i/[RecBCD]) when we varied the RecBCD enzyme concentration in the experiments shown in Figure 6, arguing against this explanation for the deviation from Michaelis–Menten behavior in Figure 5.

Reactions Catalyzed by RecBCD and RecBC with $pd(T)_{25-30}$ and poly(dT): Nuclease Reactions with RecBCD on $pd(T)_{25-30}$ and poly(dT). $pd(T)_{25-30}$ and poly(dT) are much better nuclease substrates in the presence of ATP (500 μM) than is $pd(T)_{12}$. Essentially all of the $pd(T)_{25-30}$ is degraded to products 2–12 nt in size within 2 min (data not shown) at 3.5 nM RecBCD, 0.03 μM $pd(T)_{25-30}$, and 500 μM ATP [reaction conditions similar to those used with $pd(T)_{12}$ in Figure 3]. Similarly, >95% of poly(dT) was degraded in 2.5 min at 0.2 μM (nt) poly(dT) and these ATP and RecBCD concentrations.

Figure 7 shows nuclease reactions with $pd(T)_{25-30}$ with low RecBCD concentrations and with and without ATP. In contrast to the reaction with $pd(T)_{12}$ (Figure 3), the distribution of nuclease products from $pd(T)_{25-30}$ depends on whether ATP is present. The first cleavages in the absence of ATP appear to occur near either end, since both small and large 5′-labeled products are visible, with fewer intermediate-sized bands. In the presence of ATP, products 13–17 nucleotides in size accumulate in relatively greater amounts than in the absence of ATP. These represent about 30–40% of the products in the last time points taken (the overall extents of reaction are about 10% in each case). The 13–17-mers are degraded to smaller products at longer times (data not shown). The rates in the absence of ATP are similar for $pd(T)_{12}$ and $pd(T)_{25-30}$ (Table 1). ATP (500 μM) stimulates the reaction with $pd(T)_{25-30}$ by about 400-fold at 0.03 μM $pd(T)_{25-30}$ and by 50-fold at 3 μM $pd(T)_{25-30}$. These results, and those of other ongoing experiments in our laboratory (Randle and Julin, unpublished observations), suggest that ATP affects the way the DNA is bound relative to the

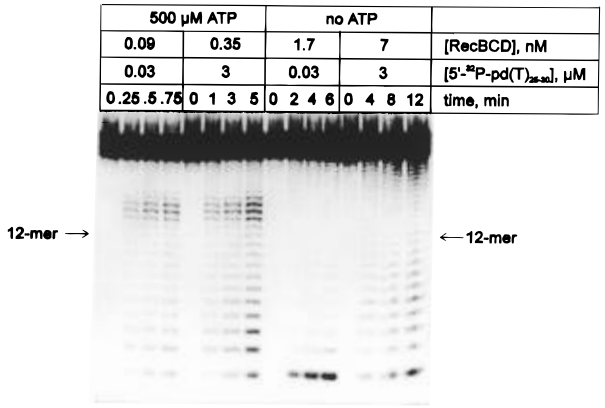


FIGURE 7: Nuclease reactions catalyzed by RecBCD with $pd(T)_{25-30}$. Reaction mixtures contained the indicated concentrations of ATP, RecBCD enzyme, and $pd(T)_{25-30}$. Samples were removed at the indicated times and run on a polyacrylamide gel (20% acrylamide/0.67% bisacrylamide). The position of a [5′-^{32}P]$pd(T)_{12}$ marker (12-mer), from other experiments, is also indicated.

nuclease active site. This effect is seen with $pd(T)_{25-30}$ but not with $pd(T)_{12}$.

ATP Hydrolysis by RecBCD with pd(T)$_{25-30}$ and poly(dT). The rapidity of the nuclease reaction with $pd(T)_{25-30}$ and poly(dT) limits their usefulness as ATPase cosubstrates in these experiments. However, little nuclease activity is seen at low ATP concentrations, and so ATP hydrolysis experiments were done only at 5 μM ATP (Figure 8A). The apparent K_m for $pd(T)_{25-30}$ is about 100-fold lower than that for $pd(T)_{12}$ [K_m[$pd(T)_{25-30}$] = 120 (±65) nM nt = 4.4 (±2) nM oligomers; K_m[$pd(T)_{12}$] = 6.5 (±0.6) μM nt = 620 (±75) nM oligomers], suggesting that the enzyme binds more tightly to the larger oligomer than to $pd(T)_{12}$. Poly(dT) is a poorer substrate than $pd(T)_{25-30}$ in terms of the nucleotide residue concentration (K_m^{app} = 840 nM nt ≈ 0.7 nM molecules), but it is slightly more effective than $pd(T)_{25-30}$ in terms of the number of DNA molecules present. The second, lower affinity site for DNA is also evident in these experiments with $pd(T)_{25-30}$ and poly(dT). The apparent V_{max} values at 5 μM ATP are essentially identical for all three DNA cosubstrates (Figure 8A). We also measured ATPase rates at high DNA (0.8−2.4 mM nt) and 1.2 mM ATP in one experiment, to get an estimate of the V_{max}^{app}/E_T at saturating ATP. The rates were similar for the three DNA substrate [v_i/E_T = 570 s^{-1} for $pd(T)_{12}$, 790 s^{-1} for $pd(T)_{25-30}$, and 900 s^{-1} for poly(dT)].

The difference between $pd(T)_{12}$ and $pd(T)_{25-30}$ seen in the kinetic measurements is also evident in direct binding measurements (Figure 9). Binding of RecBCD to $pd(T)_{12}$ saturates with a K_d of 70 (±25) nM (two determinations) (K_d = 40 nM with 10 mM $CaCl_2$ rather than $MgCl_2$). $pd(T)_{25-30}$ is bound at least 100-fold more tightly than $pd(T)_{12}$. Thus the relative binding affinities for $pd(T)_{12}$ and $pd(T)_{25-30}$ parallel their relative effectiveness as ATPase cosubstrates and as nuclease substrates.

ATP Hydrolysis Catalyzed by RecBC with pd(T)$_{25-30}$ and poly(dT). We then looked at the dependence of ATP hydrolysis by RecBC on the size of the DNA, at saturating ATP (1.2 mM, Figure 8B). $pd(T)_{12}$ and $pd(T)_{25-30}$ give virtually identical kinetic parameters: V_{max}^{app}/E_T = 168 s^{-1} for $pd(T)_{12}$ and V_{max}^{app}/E_T = 164 s^{-1} for $pd(T)_{25-30}$; K_m = 42 μM nt, 3.5 μM oligomers for $pd(T)_{12}$ and K_m = 42 μM nt, 1.5 μM oligomers for $pd(T)_{25-30}$. Thus it is likely that

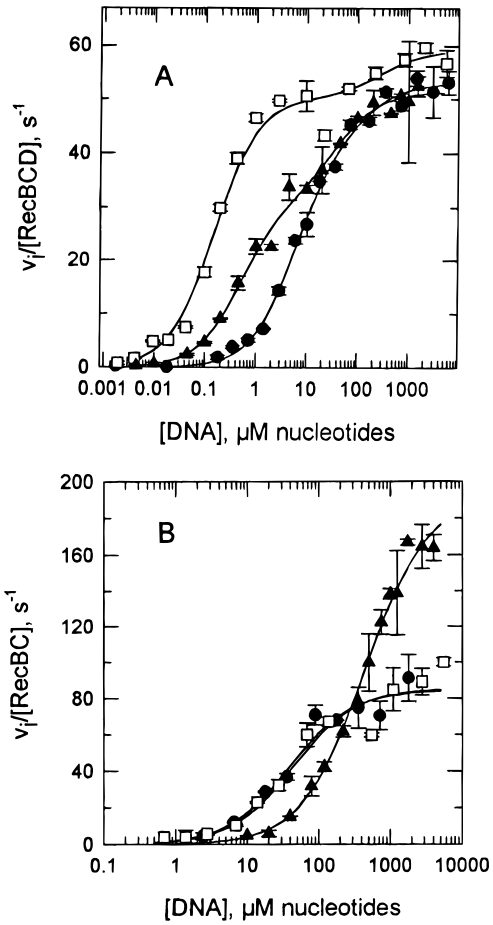


FIGURE 8: ATP hydrolysis by RecBCD and RecBC with $pd(T)_{12}$, $pd(T)_{25-30}$, and poly(dT). (A) Reaction mixtures contained 5 μM ATP, 0.086 nM RecBCD enzyme, and the indicated concentration (in micromolar nucleotides) of $pd(T)_{12}$ (●), $pd(T)_{25-30}$ (□), or poly(dT) (▲). Solid lines are fits to eq (6) (Materials and Methods). (B) Reaction mixtures contained 1.2 mM ATP, 0.25 nM RecBC enzyme, and the indicated concentration (in micromolar nucleotides) of $pd(T)_{12}$ (●), $pd(T)_{25-30}$ (□), or poly(dT) (▲). Solid lines are fits to eq 3 (Materials and Methods).

the rapid equilibrium version of Scheme 1 applies to $pd(T)_{25-30}$ as well as to $pd(T)_{12}$. Unlike RecBCD, RecBC does not have significantly greater affinity for $pd(T)_{25-30}$ than for $pd(T)_{12}$ at this ATP concentration. The apparent K_m in nucleotides for poly(dT) is much greater than for the smaller substrates (K_m = 635 μM nucleotides; ≈ 0.5 μM poly(dT) molecules). The V_{max}^{app}/E_T for the long substrate is also greater than for the small substrates (V_{max}^{app}/E_T = 390 s^{-1}).

DISCUSSION

The observations reported in this paper can be accommodated by the model shown in Figure 10. The results suggest the following: (1) the RecBCD enzyme has two DNA binding sites which interact with a fairly long region of single-stranded DNA. We presume that these sites would interact with the two single strands on a partially unwound duplex DNA molecule. (2) The RecB and RecD subunits both contribute to DNA binding in one site which stimulates ATP hydrolysis by RecB. (3) The second site stimulates ATP hydrolysis by RecD, but the subunit(s) which actually bind the DNA in this second site are not clear from these kinetics experiments. It may be in the RecC and/or the RecD subunits (see below). (4) A small DNA substrate [i.e., $pd(T)_{12}$] can bind and stimulate ATP hydrolysis at high levels,

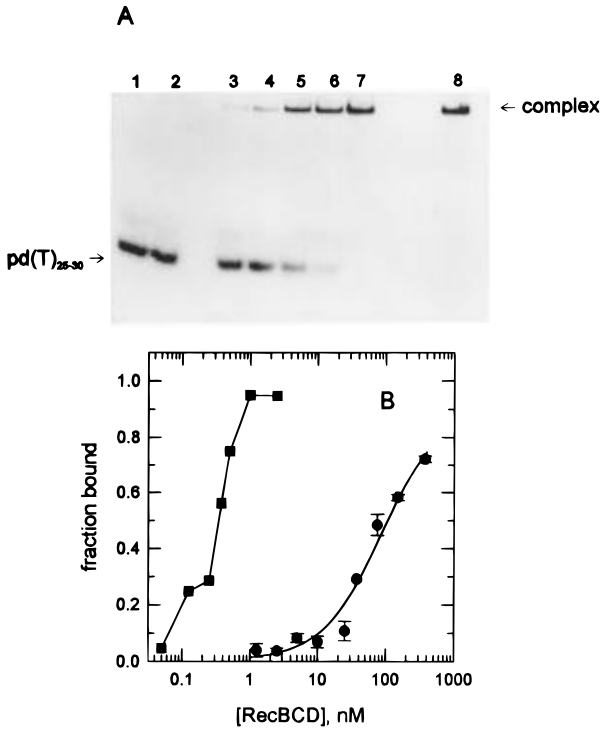


FIGURE 9: Binding of RecBCD to $pd(T)_{12}$ and $pd(T)_{25-30}$. (A) Binding to $pd(T)_{25-30}$ analyzed by electrophoretic mobility shift assay. Binding mixtures contained 50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 0.67 mM DTT, 5% (v/v) glycerol, about 0.5 nM [5′-^{32}P]$pd(T)_{25-30}$, and 0 (lane 1), 0.05 (lane 2), 0.125 (lane 3), 0.25 (lane 4), 0.375 (lane 5), 0.5 (lane 6), 1 (lane 7), and 2.5 (lane 8) nM RecBCD. Samples were run on a 6% polyacrylamide gel prepared as described in Materials and Methods. (B) (●) Binding to [^{32}P]$pd(T)_{12}$ was measured by nitrocellulose filter binding as described in Materials and Methods. Binding mixtures were as in panel A, except they contained 10 mM $MgCl_2$ rather than $CaCl_2$, with about 0.8 nM [^{32}P]$pd(T)_{12}$. The solid line is the fit to eq 10 (Materials and Methods). (■) Data for $pd(T)_{25-30}$ were determined from the gel shown in panel A. The amount of radioactivity in the bound complex was determined as a fraction of the total radioactivity in each lane. This was then plotted for each enzyme concentration.

without being cleaved to a great extent, while longer DNA is cleaved much more rapidly. This leads us to suggest that DNA bound in an ATPase-stimulating site must extend to the nuclease active site where it is cleaved. There may not be a separate DNA binding site for the nuclease activity.

Several previous investigations provide evidence that there are at least two DNA binding sites in the RecBCD enzyme. The enzyme produces large loops of single-stranded DNA, visible by electron microscopy, while it unwinds double-stranded DNA (Taylor & Smith, 1980; Muskavitch & Linn, 1982). The enzyme has been proposed to bind the DNA in two sites at the base of these loops (Taylor & Smith, 1980; Muskavitch & Linn, 1982). All three subunits were photo-cross-linked to the end of double-stranded DNA in the absence of ATP (Ganesan & Smith, 1993), indicating that all three contribute to DNA binding. The RecB protein was cross-linked specifically to the 3′-terminated strand, while both RecC and RecD were cross-linked to the 5′-terminated strand. DNase I footprinting experiments also indicated that the enzyme protects a rather large region at the end of the double-stranded DNA (16−21 base pairs) in these complexes (Ganesan & Smith, 1993).

ATP hydrolysis by RecBC is stimulated by $pd(T)_{12}$ binding to a single site with moderate affinity. The rapid equilibrium,

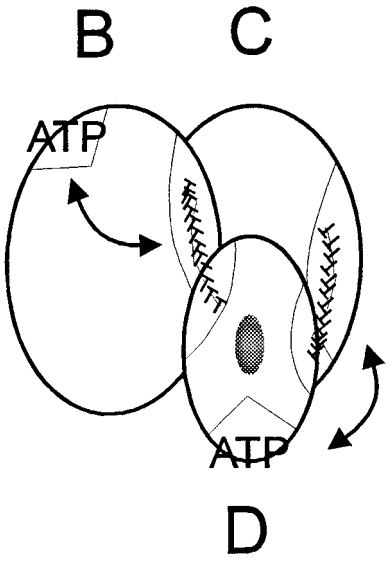


FIGURE 10: Model for the interactions of RecBCD and RecBC with single-stranded DNA oligomers. Arrows indicate interactions between DNA [$pd(T)_{12}$] and ATP binding sites. The nuclease active site is shown as a gray oval. The subunit location of this site is not known. Other details are discussed in the text (Discussion).

random sequential mechanism adequately describes this reaction, and it implies that $pd(T)_{12}$ dissociates readily from RecBC during the ATP hydrolysis reaction. Binding to $pd(T)_{12}$ is about 6−7-fold tighter when ATP is also bound according to these results, but we cannot otherwise conclude that binding or release of the DNA is "coupled" to ATP binding or hydrolysis. Longer DNA substrates [$pd(T)_{25-30}$ or poly(dT)] are not bound significantly more tightly in this site than is $pd(T)_{12}$. The photo-cross-linking experiments mentioned above indicate that both RecB and RecC (in RecBCD) can bind DNA. A DNA binding site in the RecC subunit would not be evident in the ATPase kinetics of RecBC if DNA binding to RecC does not affect ATP hydrolysis by RecB. The RecBC enzyme unwinds double-stranded DNA rapidly and processively (Korangy & Julin, 1994), and thus it probably can bind to both strands of a duplex molecule.

The presence of the RecD subunit (i.e., in RecBCD or RecBCD-K177Q) substantially alters the properties of the DNA binding site which stimulates ATP hydrolysis by the RecB subunit. The affinity for $pd(T)_{12}$ of this binding site is about 50-fold greater in the presence of the RecD subunit. Moreover, the longer substrate [$pd(T)_{25-30}$] is bound much more tightly than $pd(T)_{12}$ when RecD is present. The RecD and RecB subunits might share an elongated DNA binding site, so that RecD contributes directly to binding the DNA molecule which stimulates ATP hydrolysis by RecB. This collaboration between RecB and RecD in binding to single-stranded DNA was not seen in the photo-cross-linking experiments (Ganesan & Smith, 1993), perhaps because they were done using blunt-ended duplex DNA, without ATP. The interaction of RecB and RecD might require unwinding of some number of base pairs before it can occur. It is also possible that RecD has an indirect effect on RecB, perhaps affecting the conformation of RecB so that it has a greater affinity for these DNA substrates, without itself binding to the DNA.

The second DNA binding site is evident in the kinetics of ATP hydrolysis by the RecBCD enzyme (Figure 4). The

second site has much lower affinity for the small DNA oligomers than does the one which stimulates RecB. We believe that ATP hydrolysis by RecD is stimulated by DNA binding at this second site, given the results with RecBCD-K177Q (Figure 4). The function of ATP hydrolysis by RecD in the complex RecBCD-catalyzed reaction is still not clear. It is not required for either DNA unwinding or nuclease activity (Korangy & Julin, 1992b), although the mutation (RecBCD-K177Q) or loss (RecBC) of RecD affected the rate and processivity of these reactions (Korangy & Julin, 1993, 1994). Previous experiments with the RecB-K29Q-CD mutant enzyme (Hsieh & Julin, 1992) provide further evidence that single-stranded DNA can stimulate ATP hydrolysis by RecD and that the ATP hydrolysis can stimulate the nuclease reaction on single-stranded DNA. We are currently studying the ATP-dependent nuclease reactions catalyzed by these mutant enzymes and their responses to χ sequences.

The nuclease reaction on pd(T)$_{12}$ is much slower than ATP hydrolysis and is clearly not coupled directly to the bulk of ATP hydrolysis observed with this DNA substrate. Thus the high-affinity site for pd(T)$_{12}$ which is seen in the ATPase kinetics of RecBCD is probably not where pd(T)$_{12}$ binds for the nuclease activity. The fact that the longer DNAs [pd(T)$_{25-30}$ and poly(dT)] are much better nuclease substrates than is pd(T)$_{12}$, and that pd(T)$_{25-30}$ is bound more tightly leads us to suggest that the nuclease catalytic site is located at one end of an elongated DNA binding site. pd(T)$_{12}$ may be too small to reach from the ATP-stimulatory site to the nuclease catalytic site, but longer substrates can bind with high affinity and can extend to the nuclease active site, and thus they are somewhat better nuclease substrates. The subunit(s) comprising the nuclease active site are not known, and it is possible that more than one subunit contributes to the nuclease activity, either by binding the DNA or contributing active-site residues. The RecBC enzyme has a low level of nuclease activity (Palas & Kushner, 1990; Randle and Julin, unpublished observations), but the presence of RecD is required for high nuclease activity by RecBCD (Chaudhury & Smith, 1984; Amundsen et al., 1986; Masterson et al., 1992). RecC has been reported to be related to nucleases from other organisms (Fraser et al., 1990).

Nuclease activity by RecBCD in the absence of ATP has been observed previously using circular single-stranded DNA substrates (Goldmark & Linn, 1972). Essentially no activity is seen with large linear single-stranded DNA in the absence of ATP, in reactions where the production of acid-soluble DNA fragments is measured (Goldmark & Linn, 1972). The experiments reported here, and ongoing investigations with mixed-sequence oligomers in this laboratory (Randle and Julin, unpublished observations), demonstrate a significant effect of ATP even with very small DNA oligomers. The role of ATP hydrolysis in the nuclease activity is not clear. ATP may enhance the binding of these substrates at the nuclease active site, since the effect of ATP is less at high DNA concentrations. ATP also seems to affect the way the nuclease active site interacts with the larger substrate, since the products of the reaction are quite different when ATP is present.

Kinetic Mechanisms of ATP Hydrolysis. We originally considered the mechanism of Scheme 1 to account for the nonhyperbolic (sigmoidal) curves we observed at low pd(T)$_{12}$ (30 nM) in the experiments shown in Figure 5. However, the rates at high ATP in this experiment are difficult to explain by the mechanism of Scheme 1. If the enzyme were to dissociate from the DNA after every turnover and reassociate before hydrolyzing another ATP molecule, then the rate of DNA binding becomes significant in the overall reaction kinetics at low pd(T)$_{12}$ concentrations. According to Scheme 1, the steady-state ATPase rate at saturating ATP (i.e., let [ATP] $\rightarrow \infty$) is determined simply by the rate of DNA binding to the enzyme·ATP complex, followed by catalysis:

$$\mathrm{E{\cdot}ATP + DNA} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E{\cdot}ATP{\cdot}DNA} \xrightarrow{k_p} \mathrm{E + DNA + ADP + P_i}$$

and the initial rate is given by eq 4 (Materials and Methods). The observed rates allow us to place a lower limit on the magnitude of k_3, assuming first the mechanism of Scheme 1. The value of k_p can be estimated directly from the observed V_{max}^{app} when both substrates are saturating: $k_p = V_{max}^{app}/[\mathrm{RecBCD}] = 940\ s^{-1}$ (Table 2). The observed rate at saturating ATP, with 30 nM pd(T)$_{12}$ and 0.345 nM RecBCD, is $v_i = 0.083\ \mu$M ATP hydrolyzed/s. Using these numbers and eq 4, we calculate that $(k_{-3} + k_p)/k_3 \approx 9 \times 10^{-8}$ M. If $k_p = 940\ s^{-1}$, then $k_3 \geq 10^{10}\ M^{-1}\ s^{-1}$.

This estimated value of k_3 is very large and is likely greater than the diffusion limit for an enzyme binding its substrate (Fersht, 1985). Estimates for the diffusion limit for an enzyme binding to a small DNA molecule are in the range of 5×10^7 to $2 \times 10^9\ M^{-1}\ s^{-1}$ (Record et al., 1991; Surby & Reich, 1996; Rao et al., 1994; Goeddel et al., 1977). We can also estimate an expected upper limit for the RecBCD·pd(T)$_{12}$ association rate constant from $k_{assoc}\ (M^{-1}\ s^{-1}) = 4\pi\kappa af(D_{pd(T)_{12}} + D_{RecBCD})N_0/1000$ (von Hippel & Berg, 1989), where $D_{pd(T)_{12}}$ and D_{RecBCD} are the diffusion constants for pd(T)$_{12}$ and RecBCD enzyme, respectively ($D_{pd(T)_{12}} + D_{RecBCD} \approx D_{pd(T)_{12}}$), a is the sum of the radii of the enzyme and the DNA, in centimeters, κ is the fraction of the surfaces of the enzyme and DNA which are reactive, f is a factor that reflects the increase or decrease in the diffusion constants which could arise from electrostatic interactions between the enzyme and DNA, and N_0 is Avogadro's number. Using the following estimates for these constants, $D_{pd(T)_{12}} \approx 1.5 \times 10^{-6}\ cm^2/s$ (Eimer & Pecora, 1991), $a = 8.7 \times 10^{-7}$ cm [$r_{pd(T)_{12}} \leq 21$ Å = (12 × 3.5 Å)/2 (Camerman et al., 1976) and $r_{RecBCD} \approx 66$ Å (Taylor & Smith, 1995)]; $\kappa \approx 0.1$ [i.e., the pd(T)$_{12}$ binding site on RecBCD occupies only a small percentage of the total surface area of the enzyme], and $f = 1$, then $k_{assoc} \approx 10^9\ M^{-1}\ s^{-1}$. The magnitude of f is unknown, and it could either further reduce or increase the estimate of k_{assoc}. The p*I* of RecBCD enzyme is about 5, from isoelectric focusing measurements (Randle and Julin, unpublished observations), indicating that the enzyme has an overall negative charge at pH = 7.5. Electrostatic effects would thus further reduce the maximum possible value of k_{assoc}.

The modification of Scheme 1 to give Scheme 2 gives a mechanism which more easily accounts for the experimental data. The enzyme remains bound to the DNA (E·DNA, Scheme 2) after hydrolyzing ATP and releasing the ADP and P$_i$ products. There is no requirement that the enzyme rebind the DNA at every turnover. According to Scheme 2, the rate at saturating ATP and low DNA is given by eq 5, and the K_m^{app} for pd(T)$_{12}$ is just the K_d (= k_{-3}/k_3) for pd-

$(T)_{12}$ binding to the enzyme·ATP complex. The K_d of 9×10^{-8} M calculated from eq 5 and the observed reaction rate (see above) is in excellent agreement with the K_m^{app} for pd$(T)_{12}$ measured independently when we varied the pd$(T)_{12}$ concentration at high ATP ($K_m^{app} = 100$ nM, Table 2). There is no requirement that any rate constant (i.e., k_3) be greater than diffusion-controlled.

ATPase Kinetics with Larger DNA Molecules. The conclusion that the RecBCD enzyme remains bound to the DNA during multiple ATP hydrolysis turnovers suggests either of two further possibilities. The pd$(T)_{12}$ could function simply as an activator of ATP hydrolysis, and binding to the DNA is not otherwise affected by ATP hydrolysis. A second possibility is that the enzyme moves from one end to the other of this substrate and that several ATP molecules are hydrolyzed during this movement. We feel that this is unlikely, given the small size of this oligomer, and that movement along this very short DNA molecule would be equivalent to dissociation.

We tested larger DNA molecules [pd$(T)_{25-30}$ and poly(dT)] as ATPase cosubstrates in part to address the question of whether the ATP hydrolysis catalyzed by RecBCD is coupled to movement along the DNA. ATP-dependent movement along single-stranded DNA is hypothesized to be of fundamental importance in the mechanism by which some helicases unwind double-stranded DNA (Geiselmann et al., 1993; Hill & Tsuchiya, 1981). The dependence of steady-state kinetic parameters for ATP hydrolysis (K_m and/or V_{max}) on DNA size has been used to show that some DNA helicases move along single-stranded DNA in an ATP-dependent process (Young et al., 1994a).

It is clear from the binding, ATPase, and nuclease results that RecBCD interacts with pd$(T)_{25-30}$ much differently than with pd$(T)_{12}$. However, the ATPase results with the longer DNAs provide no evidence that RecBCD moves along these single-stranded DNA molecules under the conditions we have tested. Instead, the enzyme appears to bind to the end of the single-stranded DNA substrates, since the ATPase rate depends more closely on the concentration of DNA molecules [pd$(T)_{25-30}$ and poly(dT)] than that of nucleotide residues. It is unlikely that the enzyme dissociates and rebinds often from pd$(T)_{25-30}$, given the very tight binding and low K_m for pd$(T)_{25-30}$. It is more likely that the enzyme remains bound and does not move, as concluded above for pd$(T)_{12}$.

The ATP-coupled movement mechanism discussed by Young et al. (1994a) implies that the enzyme under consideration hydrolyzes ATP processively. It is possible that the RecBCD enzyme is prevented from moving to any extent by the low ATP concentration in these experiments and that an effect of the DNA size on the K_m^{app} could be observed at higher ATP concentrations. However, the enzyme is able to unwind DNA processively at 10 μM ATP (Roman et al., 1992) and has nuclease activity on double-stranded DNA at 5 μM ATP, with 10 mM Mg^{2+} (Korangy & Julin, 1992b). Little unwinding and degradation is seen at even lower ATP (2 μM) (Korangy & Julin, 1992b, 1994). Moreover, our results are consistent with the observation that the RecBCD enzyme is unable to unwind double-stranded DNA with long single-stranded termini at either the 3′- or 5′-termini, under conditions where the nuclease activity is suppressed but unwinding of blunt-ended DNA did occur (5 mM ATP, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) (Taylor & Smith, 1985).

The increase of both V_{max} and K_m with DNA size which we see for RecBC could indicate that this enzyme does move along single-stranded DNA. In particular, a slow step after the enzyme binds the DNA but before it begins to hydrolyze ATP and move could account for this observation (Young et al., 1994a). The enzyme must traverse this slow step frequently with small DNA but much less frequently, compared to the time during which it hydrolyzes ATP, for long DNA. Unwinding by RecBC enzyme on double-stranded DNA substrates containing large single-stranded regions has not been tested.

The RecBCD and RecBC enzymes clearly must move during the reaction with a long double-stranded DNA molecule, since both act processively (Roman et al., 1992; Korangy & Julin, 1993). Both enzymes are able to initiate unwinding from a blunt end, with no requirement for a single-stranded DNA loading site. The enzyme could interact with both the double- and single-stranded regions of a partially unwound DNA molecule, or with both single-stranded ends of such a molecule. The unwinding mechanism would thus involve more than just the enzyme interacting with, and moving along, one strand of the duplex. By using these small DNA oligomers, we may have uncoupled DNA binding sites which are normally bound to parts of the same double-stranded DNA molecule. The reactions of RecBCD with small DNA oligomers thus would not reflect all the interactions of the enzyme, or of individual subunits, with double-stranded DNA.

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SUPPORTING INFORMATION AVAILABLE

Derivation of the steady-state rate equation for Scheme 2 (3 pages). Ordering information is given on any current masthead page.

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