Monomeric RecBCD Enzyme Binds and Unwinds DNA*

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The RecBCD enzyme (EC 3.1.11.5) is a large ATP-dependent enzyme that is involved in recombination and repair of DNA in Escherichia coli (reviewed in Ref. 1). It is encoded by the recB, recC, and recD genes, whose gene products have Mr of 134,000, 129,000, and 67,000, respectively, as inferred from DNA sequence data and N-terminal peptide analysis (2–5). The enzyme has a potent ATP-dependent exonuclease that is active on either ds or ss DNA and a weak ATP-stimulated endonuclease activity that acts only on ss DNA. It can use the energy of ATP hydrolysis to unwind ds DNA, either transiently or permanently, in a highly processive reaction (6, 7). The enzyme is active on linear, but not circular, ds DNA and thus requires a ds terminus for its unwinding or nuclease activity (8, 9).

Published reports suggest that RecBCD enzyme can exist in either a monomeric (B2C2D2) or a dimeric (B1C1D1) form.2 The native Mr of the enzyme was initially reported to be about 250,000 (9), as estimated by gel gradient centrifugation, consistent with the enzyme molecule containing one copy each of the RecB, RecC, and RecD polypeptides (with a predicted Mr of 330,000). A higher Mr form of the enzyme, apparently dimeric, was observed in sonicates of E. coli (10), together with the previously reported form, but was lost during subsequent purification steps. RecBCD enzyme purified from a strain that overproduced the enzyme had a native Mr of 655,000 but which decreased to about 270,000 in the presence of 0.5 M NH4Cl (11). The higher Mr form was not observed in a subsequent purification (12). More recently, RecBCD enzyme has been produced either by overproduction of the three subunits within E. coli or by mixing of purified subunits (5). Mr estimations by gel filtration or native polyacrylamide gel electrophoresis are consistent with the enzyme from these sources being a monomer, but the specific activity of the reconstituted material was only a few percent of that of the native enzyme (5).

Interest in the subunit structure of RecBCD enzyme was rekindled by a “rolling dimer” model of helicase action which relies on a symmetric, dimeric enzyme structure (13). In that model, monomer A of the dimeric helicase binds (already unwound) ss DNA behind the enzyme, and monomer B binds ds DNA immediately ahead. Monomer A releases its ss DNA and binds to ds DNA ahead of monomer B, which melts its bound ds DNA and remains bound to the ss DNA so produced. Translocation and unwinding thus result from the cycle of alternating binding and unbinding.

Unwinding of ds DNA by RecBCD enzyme has been studied by electron microscopy (6, 14). The enzyme unwinds DNA processively, in the presence of SSB, with the production of either asymmetric structures (a ss loop and two ss tails) or apparently symmetric structures (two ss loops). Both types of structure travel along the DNA at ≥300 bps, while the loops grow at about 100 nt/s. The relative abundance of the two structures is determined by the concentration of SSB (15), suggesting that they arise by a common mechanism (6).

We proposed that, as a minimal model, RecBCD enzyme need contact only one strand of ds DNA and could produce the observed DNA structures by assimilating the DNA ahead of itself and releasing it behind itself at a slower rate (6). Roman and Kowalczykowski (16) proposed that unwinding by RecBCD enzyme results from the action of two helicases, acting at different rates on the two strands of the DNA, and suggested that the helicases may reside in the RecB and RecD subunits of the enzyme. Ganesan and Smith (17) combined the latter model with the “rolling dimer” model of Lohman (13) and suggested that a dimeric form of RecBCD enzyme unwinds DNA. Two copies of the RecB subunit were proposed to act in tandem to translocate along one strand of the DNA, while a pair of (RecC + RecD) complexes translocated along the other strand. The two complexes were postulated to travel at different rates and hence produce the ss loops observed by electron microscopy. The choice of subunits in this latter model was prompted by the UV-cross-linking patterns of the subunits on the ends of ds DNA (17), by the abilities of the RecB and RecD subunits to bind ATP (18), and by the ATPase activity of

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1 The abbreviations used are: ds, double-stranded; ss, single-stranded; bp, base pair(s); nt, nucleotide(s); SSB, E. coli single-stranded DNA binding protein; MOPS, 4-morpholinepropanesulfonic acid.

2 We have previously referred to these two forms as “trimer” and “hexamer” (17) but will henceforth refer to enzyme molecules containing one or two copies each of the RecB, RecC, and RecD polypeptides as monomers and dimers, respectively.
isolated RecB protein (19).

During the course of purifying RecBCD enzyme we observed, purified, and characterized both monomeric and dimeric forms of RecBCD enzyme. We report here that stable complexes were formed between ds DNA and the monomeric form of RecBCD enzyme and that such complexes were competent for unwinding. These results establish the active form of RecBCD enzyme as a monomer and question the physiological relevance of the dimer.

**EXPERIMENTAL PROCEDURES**

**RecBCD Enzyme**

RecBCD enzyme was purified from strain V182 (20), which carries the recB, recC, and recD genes on a 18-kilobase fragment of E. coli DNA cloned in pBR322. Lysis and purification to produce Fraction IV were as described elsewhere (8, 21). All experiments, except where noted and the dynamic light scattering determination, used the first purification described next. Fraction IV was applied to an Affi-Gel heparin column (Bio-Rad) in buffer C (22) containing 0.15 M NH₄Cl. The column was washed with the same buffer and eluted with a 0.15–0.5 M NH₄Cl gradient. Most of the enzyme did not bind to the column and was reappplied at 0.05 M NH₄Cl and eluted with a 0.05–0.6 M NH₄Cl gradient. Enzyme that eluted from the second Affi-Gel heparin column (at 0.1–0.2 M NH₄Cl) was concentrated by binding to a 10-ml column of DEAE-Sepharose and eluting with a gradient of NH₄Cl. Peak fractions were pooled and designated Fraction V-B, to distinguish it from Fraction V, the eluate of the heparin agarose column (Life Technologies, Inc.), employed both in previous purifications (21) and in the second purification described below. Fractions VI-M (the monomeric form of the enzyme) and VI-D (an apparent mixture of monomeric and dimeric forms) are described more fully under “Results.”

In the second purification, Fraction V was purified as previously described (8, 21) and concentrated and purified on a DEAE-Sepharose column, as above, to yield Fraction VI, which lacks detectable contaminants as judged by SDS-polyacrylamide gel electrophoresis. Units of activity in the purified enzyme are defined as those that release 1.0 nmol of dATP, dCTP, dGTP, and TTP, and purified by polyacrylamide gel electrophoresis, using the Klenow fragment of DNA polymerase I in the presence of [α-32P]ATP (New England Nuclear; 3000 Ci/mmol). After digestion with Aval and heat inactivation of the restriction enzyme, the DNA (two labeled Aval-DdeI fragments, of 157 and 1495 bp) was ligated with a self-complementary hairpin-shaped oligonucleotide terminated with an Aval site (5’-CCGAGTCTAGGGCGCTATGGCCCTCTAGAC-3’; Oligos, Etc.). The oligonucleotide bore a 5’-hydroxyl and hence formed a covalent bond on only one strand. The mixture of ligation products was purified on an 8% polyacrylamide gel in Tris acetate-EDTA buffer (25). These closely spaced radioactive bands of about 170 bp were resolved. The desired product, the middle band, was a 157-bp fragment of pBR322 bearing a 3’-Phospho label on the DdeI end and a hairpin-shaped oligonucleotide cap on the other end, with a nick in one strand adjacent to the cap. Three closely spaced radioactive bands were run at 4°C for 1 h at 100 V, prior to changing the buffer and loading the samples, and then run with periodic replacement of the electrophoresis buffer. After electrophoresis, gels were fixed in 40% methanol, 10% acetic acid, the proteins were stained with Fast Stain (Zylon Research, Allston, MA), and the gels were dried between acetate sheets, or on Whatman DEAE paper, prior to autoradiography.

**Nondenaturing Polyacrylamide Gel Electrophoresis**

Gels were run in Haeffer Mighty Small slab gel electrophoresis units, using alumina backplates and 0.75-mm spacers. Gels used an acrylamide-bisacrylamide ratio of 37.5:1 and were run at 4°C. Glycerol (10%, v/v, final concentration) and bromphenol blue (0.04%) were added to samples prior to loading. The continuous buffer system gels were run at 4°C for 1 h at 100 V, prior to changing the buffer and loading the samples, and then run with periodic replacement of the electrophoresis buffer. After electrophoresis, gels were fixed in 40% methanol, 10% acetic acid, the proteins were stained with Fast Stain (Zylon Research, Allston, MA), and the gels were dried between acetate sheets, or on Whatman DEAE paper, prior to autoradiography.

**Determination of Dissociation Constants**

DNA substrates were the 5’-end-labeled BamHI hairpin-shaped oligonucleotide or its derivatives, as described above. For some experiments, dilutions of the DNA were mixed with constant amounts of RecBCD enzyme (Fraction VI-M) in 10 μl of a solution containing 50 mM MOPS-KOH (pH 7.0), 1 mM EDTA, 100 μg/ml each of bovine serum albumin and polyvinylpyrrolidone K-60. After 10 min at 20°C, samples were run on minigels (4–15% polyacrylamide gradient gels in 50 mM MOPS-KOH (pH 7.0), 1 mM EDTA) as described above. For other experiments, reaction mixtures were prepared as above, but with 25 pmol DNA and varying concentrations of RecBCD enzyme, and analyzed on 5% polyacrylamide gels. Incubation and gel electrophoresis were in the presence of 3 mM Mg(OAc)₂ or 2 mM EDTA. The free and retarded radioactive DNA bands were detected and quantitated by PhosphoImager analysis. Results were analyzed by nonlinear regression for varying DNA concentration or by Hill plots for varying enzyme concentration (26).

**Gel Assay for Unwinding by RecBCD Enzyme**

RecBCD enzyme was incubated with the nicked hairpin DNA described above, with ATP omitted, for 10 min at 20°C to allow binding to the end of the DNA. Reactions were started by the addition of ATP (which was mixed, in some reactions, with an agent to prevent multiple rounds of reaction), incubated for 10 s at 20°C in a final volume of 20 μl, and stopped by addition of EDTA (10 mM final concentration), SDS (0.1%), sucrose (10%), and tracking dyes (0.04%). Final concentrations, after addition of enzyme, ATP, and DNA, were: 20 mM MOPS-KOH (pH 7.0), 4 mM Mg(OAc)₂, 100 μg/ml polyvinylpyrrolidone K-60, 5 mM ATP, and 1 mM labeled DNA substrate. Samples were analyzed on an 8% polyacrylamide gel in Tris acetate-EDTA buffer, and the gel was dried prior to exposure to film (27). Final concentrations of inhibitory agents were: 0.13% Sarkosyl (Iuron), 0.13% hirin (Sigma), or 40 mM ds DNA molecules (a DdeI digest of phage λ DNA).

**Dynamic Light Scattering**

The translational diffusion coefficient of RecBCD enzyme was measured by dynamic light scattering, using a DynaPro-801 instrument (Protein Solutions Inc., Charlottesville, VA). The enzyme was dialyzed into 20 mM potassium phosphate (pH 6.8), 0.1 mM EDTA, 0.1 mM dithiothreitol, immediately before measurement. The AutoPro software of the machine calculated the hydrodynamic radius of the protein and its predicted Mₐ, based on data for typical globular proteins.
Two Forms of RecBCD Enzyme—As a final step in its purification, RecBCD enzyme was sedimented through a glycerol gradient (Fig. 1A). The enzyme loaded onto the gradient was >95% pure, as judged by Coomassie staining of SDS-polyacrylamide gels (not shown), yet the protein sedimented as two peaks on the glycerol gradient. The M_r of the two species were estimated by comparison with the sedimentation of known proteins in a parallel tube (28). The estimated M_r of the slower sedimenting material was 313,000 ± 34,000 (Table I), consistent with it comprising one copy each of RecB, RecC, and RecD (expected M_r of 330,000). The slower sedimenting peak fractions from this and other gradients were pooled as Fraction VI-M (for “monomer”). The faster sedimenting material, with an estimated M_r of 560,000 ± 690,000, was similarly pooled, as Fraction VI-D (for “dimer”), as its M_r suggested that it was a dimer of the slower sedimenting material.

Material equivalent to Fractions VI-M and VI-D, obtained from a previous glycerol gradient purification, was analyzed in the same experiment. The monomeric fraction from that gradient contained very little of the “dimer” species (Fig. 1B), while the “dimer” material contained approximately equal weights of the two forms of the enzyme (Fig. 1C). The origin of the monomer-sized material in this “dimer” fraction is unclear: it may have arisen from inefficient purification in the previous glycerol gradient, or it may have resulted from instability of the dimer form of the enzyme. Observations with nondenaturing gels (see below) suggest that the dimeric material also returns to the monomeric state during electrophoresis, suggesting that the dimer may indeed be unstable.

To estimate the relative numbers of copies of each polypeptide in the two forms of RecBCD enzyme, fractions VI-M and VI-D were analyzed by SDS-polyacrylamide gel electrophoresis, and the Coomassie-stained gels were quantitated by densitometry. The observed relative intensities for the three polypeptides were within 15% of those predicted for a protein with one subunit each of 134,000, 129,000, and 67,000 (Table II), as previously reported (7, 29) for purified enzyme. While the relative Coomassie staining abilities of the RecB, RecC, and RecD polypeptides have not been measured, the relative staining abilities of several proteins are proportional to the number of positively charged amino acids they contain (30). As shown in Table II, the three polypeptides of RecBCD enzyme contain similar densities of positively charged amino acids. Hence, the equimolar staining of the three polypeptide bands is consistent with RecBCD enzyme containing equal numbers of copies of the RecB, RecC, and RecD polypeptides. This result, together with the estimated M_r of monomeric enzyme, shows that monomeric enzyme contains one copy of each polypeptide.

Quantitation of a Coomassie-stained native polyacrylamide gel showed that >60% of the protein in Fraction VI-D was in the dimeric form and that 95% of Fraction VI-M was in the monomeric form. Hence, the observation of equal ratios of polypeptides in the two forms (Table II) implies that the dimeric form in Fraction VI-D also contained equal numbers of each subunit. The faster sedimenting form of RecBCD enzyme must thus be a simple dimer of the monomeric form, with two copies each of the RecB, RecC, and RecD polypeptides.

Estimation of M_r by Dynamic Light Scattering—The hydrodynamic radius of the protein in Fraction VII from the second purification of RecBCD enzyme was measured by dynamic light scattering. Fraction VII was >95% pure, as assayed by SDS-polyacrylamide gel electrophoresis and contained ~90% monomer as assayed on a native polyacrylamide gel. Monomeric RecBCD enzyme had a hydrodynamic radius of 6.6 (± 0.2) nm, with an inferred M_r of 282,000 ± 18,000. Fraction VI-D had an estimated M_r of 282,000 ± 18,000. Fraction VI-M had an estimated M_r of 330,000 ± 30,000. (Table I, as previously reported (7, 29) for purified enzyme. While the relative Coomassie staining abilities of the RecB, RecC, and RecD polypeptides have not been measured, the relative staining abilities of several proteins are proportional to the number of positively charged amino acids they contain (30). As shown in Table II, the three polypeptides of RecBCD enzyme contain similar densities of positively charged amino acids. Hence, the equimolar staining of the three polypeptide bands is consistent with RecBCD enzyme containing equal numbers of copies of the RecB, RecC, and RecD polypeptides. This result, together with the estimated M_r of monomeric enzyme, shows that monomeric enzyme contains one copy of each polypeptide.

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### Table I

| Sample     | Fraction no. | M_r x 10^3 | Fraction no. | M_r x 10^3 |
|------------|--------------|------------|--------------|------------|
| Fraction V-B | 13           | 622        | 20           | 313        |
| Fraction VI-M | 14           | 564        | 19           | 345        |
| Fraction VI-D | 13           | 622        | 19           | 345        |
| Fraction V-B | 13.5         | 592        | 20           | 313        |
| Fraction V-B | 12           | 686        | 19           | 345        |

### RESULTS

Monomeric RecB, RecC, and RecD polypeptides have not been measured; the relative Coomassie staining abilities of the RecB, RecC, and RecD polypeptides (Table II), as previously reported (7, 29) for purified enzyme. While the relative Coomassie staining abilities of the RecB, RecC, and RecD polypeptides have not been measured, the relative staining abilities of several proteins are proportional to the number of positively charged amino acids they contain (30). As shown in Table II, the three polypeptides of RecBCD enzyme contain similar densities of positively charged amino acids. Hence, the equimolar staining of the three polypeptide bands is consistent with RecBCD enzyme containing equal numbers of copies of the RecB, RecC, and RecD polypeptides. This result, together with the estimated M_r of monomeric enzyme, shows that monomeric enzyme contains one copy of each polypeptide.

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apparent hydrodynamic radius of 11.2 nm; the instrument was unable to resolve the contributions from the monomeric and dimeric species in the sample and hence could not estimate their separate $M_r$.

The $M_r$ of the monomer form estimated by this method is consistent with those measured by glycerol gradient centrifugation (preceding section) and by native gel electrophoresis (next section). These results confirm that the major form of the enzyme is indeed a monomer.

Estimation of $M_r$ by Native Polyacrylamide Gel Electrophoresis—Proteins migrate in nondenaturing polyacrylamide gels as a function of both their size and their charge. We investigated gel conditions which would separate the monomeric and dimeric forms of RecBCD enzyme, both to confirm the estimates of their $M_r$s and to allow easier analysis of multiple samples, including those in the “gel shift” experiments described below. In a high pH nondenaturing discontinuous 5–20% gradient gel system (31), all of the protein standards migrated as a smooth function of their $M_r$s (Fig. 2). Monomeric and dimeric RecBCD enzyme migrated with apparent $M_r$s of 305,000 and 650,000, respectively (Fig. 2, lanes 2 and 3). In this system, with a resolving gel at pH 9.5, all of the proteins should be highly charged, and hence their migration should be principally a function of their $M_r$s. RecBCD enzyme was unstable in this gel system: faster migrating species were seen, most of which did not appear in the other gel systems examined (Fig. 3 and data not shown). Their apparent $M_r$s were 220,000, 170,000, 160,000, 140,000, 130,000, and 120,000; we suppose that they arose from various combinations of the subunits of the enzyme.

In a nondenaturing 4–15% polyacrylamide gradient gel, at pH 7.5, the protein standards migrated as a smooth function of their $M_r$s (data not shown). The apparent $M_r$s of the monomer were 230,000, and that of the dimer was about 670,000, suggesting that RecBCD enzyme may be more highly charged than the standards at pH 7.5. The isoelectric points of the proteins, as calculated from their primary sequences using the “ Isoelectric” program of the GCG Software package (32), were consistent with this interpretation. RecBCD enzyme had a calculated pI of 5.0, while those of the protein standards were higher: thyroglobulin, 5.4; ferritin, 5.5; catalase, 6.9; and bovine serum albumin, 6.0.

For many experiments, 5% polyacrylamide gels were used and were run at the pH (7.0) used in the DNA binding and unwinding experiments reported below. Such gels easily separated the monomeric and dimeric forms of RecBCD enzyme, as shown in Fig. 3, although the apparent $M_r$s of the enzyme fractions, relative to the protein standards, were considerably lower (about 600,000 for the dimer and about 150,000 for the monomer), again probably due to the higher relative charge, at pH 7.0, on RecBCD enzyme than on the standards.

A significant fraction of the protein migrated faster than RecBCD enzyme in these gels (Fig. 3, lanes 3, 6, 10, and 12). This material may have arisen from enzyme molecules lacking the RecD subunit, as has been observed by others (5). It was not detectable in the glycerol gradient purified enzyme (Fraction VI-M; Fig. 3, lane 4), showing that it was a separate species and not the result of instability of the enzyme during gel electrophoresis. The proportion of dimeric RecBCD enzyme in Fraction VI-D appears higher in Fig. 3 (lane 2) than in Fig. 2 (lane 3), probably due to the instability of the enzyme at the higher pH in the gel in Fig. 2. All purified dimer fractions prepared by glycerol gradient centrifugation appeared, on analysis in gels similar to that shown in Fig. 3, to contain similar amounts of monomeric RecBCD enzyme. It is thus plausible that the different samples of dimeric material contained principally dimeric RecBCD enzyme, but that the dimeric enzyme was un-

### Table I

| Polypeptide | Positive charge density | Relative Coomassie staining intensity |
|-------------|-------------------------|--------------------------------------|
| RecB        | (1)                     | (1)                                  |
| RecC        | 0.87                    | 0.89                                 |
| RecD        | 1.48                    | 1.04                                 |

| Polypeptide | Relative molar abundance of the three polypeptides in monomeric and dimeric RecBCD enzyme |
|-------------|------------------------------------------------------------------------------------------|
| RecB        | 1.18 0.94 1.16                                                                           |
| RecC        | 0.87 0.89 0.95                                                                           |
| RecD        | (1) (1) (1)                                                                               |

### Notes

1. A significant fraction of the protein migrated faster than RecBCD enzyme in these gels (Fig. 3, lanes 3, 6, 10, and 12).

2. This material may have arisen from enzyme molecules lacking the RecD subunit, as has been observed by others (5). It was not detectable in the glycerol gradient purified enzyme (Fraction VI-M; Fig. 3, lane 4), showing that it was a separate species and not the result of instability of the enzyme during gel electrophoresis. The proportion of dimeric RecBCD enzyme in Fraction VI-D appears higher in Fig. 3 (lane 2) than in Fig. 2 (lane 3), probably due to the instability of the enzyme at the higher pH in the gel in Fig. 2. All purified dimer fractions prepared by glycerol gradient centrifugation appeared, on analysis in gels similar to that shown in Fig. 3, to contain similar amounts of monomeric RecBCD enzyme. It is thus plausible that the different samples of dimeric material contained principally dimeric RecBCD enzyme, but that the dimeric enzyme was un-
stable both during glycerol gradient centrifugation (Fig. 1) and during gel electrophoresis (Fig. 3). Origin of the Dimeric Form—Examination of the different stages of purification of RecBCD enzyme suggests that at least some of the dimeric species arose during purification (Figs. 2 and 3). In Fig. 2, lane 4, it appears that Fraction IV of the first purification contained no detectable RecBCD enzyme dimer. However, when this fraction was subsequently analyzed on the pH 7 gel system (in which RecBCD enzyme is more stable), some material was visible that comigrated with the dimer (Fig. 3, lane 3). It is unclear which gel is a more accurate reflection of the true makeup of Fraction IV. Dimer might not have been detected in Fig. 2, due to its instability in that gel system. The band comigrating with dimer in Fig. 3 might be a contaminant rather than RecBCD enzyme or it might be dimeric RecBCD enzyme produced on storage of Fraction IV. Nonetheless, it is clear that, while there may have been a small amount of dimer present in Fraction IV of the first purification (Fig. 3, lane 3), the proportion present in Fraction V-B is considerably greater (Fig. 1A).

In the second purification, protein comigrating with the dimer was barely detectable in Fraction IV (Fig. 3, lanes 10 and 12). Dimer was not detectable in Fraction V (lane 8), perhaps due to the small amount of material that could be loaded on the gel, but was detected when a gel similar to that in Fig. 3 was stained (data not shown). In Fraction VII (lane 6), dimer was clearly present at a higher concentration, relative to the monomeric form of RecBCD enzyme, than it was in Fraction IV. Dimer was thus apparently generated during the late stages of both purifications. It seems plausible that the greater concentrations of RecBCD enzyme achieved in the latter stages of purification may be responsible for production of the dimer.

End-specific DNA Binding by Monomeric RecBCD Enzyme—With purified monomeric form of RecBCD enzyme in hand, we tested whether this form is competent to bind and unwind DNA, as described in the following sections. The migration of a DNA molecule in a nondenaturing polyacrylamide gel is typically retarded when it is bound by a protein molecule, a phenomenon known as “gel retardation.” In such experiments the migration position of unbound enzyme can be determined by staining of the protein in the gel. If the DNA molecule is short, such that its $M_r$ is much less than that of the protein, the migration of the protein may not be affected noticeably by the DNA bound to it. In the experiment shown in Fig. 4, the migration positions of the free monomeric and dimeric forms of RecBCD enzyme were identified by staining the protein. Unbound and bound DNA molecules were detected by autoradiography.

dsDNA molecules with two ends can bind a RecBCD enzyme molecule at each end and are retarded to the position of dimeric RecBCD enzyme (data not shown). Thus, DNA bound at one end by dimeric RecBCD enzyme, or by two monomers, cannot be distinguished from DNA molecules with monomeric RecBCD enzyme molecules bound at each end.

To test whether the initiation complex of RecBCD enzyme on a ds DNA end involved more than one RecBCD enzyme monomer, we used a ds DNA substrate with one terminus masked by a ss loop, called a “hairpin,” as shown in Fig. 4. A dimer of that substrate, with no strand ends, was used to test whether binding was end specific. ds DNA with such ss loops at its termini is resistant to digestion or cleavage by RecBCD enzyme (27, 33). About half of the hairpin DNA with one ds end (1 nM total concentration) was retarded by 0.3 nM monomeric RecBCD enzyme (lane 10), and essentially all of it was retarded by 1 nM RecBCD enzyme (lane 9). Very little of the endless DNA was retarded at any RecBCD enzyme concentration (lanes 2–5), showing that the binding of RecBCD enzyme was indeed to the ends of DNA molecules. The great majority of the retarded DNA molecules were retarded to the position of free monomeric RecBCD enzyme. A monomer of RecBCD enzyme is thus sufficient to form a complex at the end of a ds DNA molecule.

At the highest RecBCD enzyme concentrations (1 and 3 nM), a small fraction of the endless hairpin DNA was retarded to the position of RecBCD enzyme monomers (lanes 4 and 5), presumably from end-independent binding by RecBCD enzyme. At 3 nM RecBCD enzyme, 15% of the retarded hairpin DNA (with one ds end) was retarded to the position of dimeric RecBCD enzyme, as measured by PhosphorImager analysis of the gel in Fig. 4 (lane 6). As 95% of the RecBCD enzyme in Fraction VI-M was monomeric (see above), this retardation to the dimer position presumably resulted from one enzyme molecule binding to the end of hairpin DNA and a second molecule binding elsewhere.

Quantitation of the Binding of RecBCD Enzyme to ds DNA Ends—DNA bound to RecBCD enzyme is separated from unbound DNA in nondenaturing polyacrylamide gels (Fig. 4). Titration of the DNA concentration, at a constant protein concentration below the $K_D$ (the concentration at which half of the maximal amount of DNA is bound) and of the concentration of protein that is active for binding to DNA (26). These measurements were performed with DNA molecules with one ds DNA end bearing a 4-nt 5′-overhang and were made in the absence of Mg$^{2+}$ ions (Table III). The $K_D$ for this DNA was about 100–200 pm. All of the RecBCD enzyme in Fraction VI-M, as inferred from its $A_{260}^{nm}$, was competent for binding to a ds DNA end.

We next investigated the effects of Mg$^{2+}$ ions and the nature of the ds DNA end on the ability of RecBCD enzyme to bind DNA. Hairpin DNA with 5′-overhangs of 0–4 nt were mixed with RecBCD enzyme and analyzed by gel retardation in the presence or absence of Mg$^{2+}$ ions. The DNA concentration was kept constant and the protein concentration was varied. Analysis of Hill plots showed that the $K_p$ for DNA with a 4-nt 5′-overhang was 0.56 nM (Table IV), in reasonable agreement with the data in Table III in which the DNA concentration was varied. Since the estimates of $K_p$ in Tables III and IV depend

![Fig. 4. End-specific binding by RecBCD enzyme monomers.](image-url)
TABLE III
Concentration of active binding protein and $K_D$ for the binding of RecBCD enzyme to ds DNA ends

In each experiment the noted concentrations of RecBCD enzyme (Fraction VI-M) were incubated with varying concentrations of labeled DNA in 1 mM EDTA, and the fraction of the DNA bound by RecBCD enzyme was determined by gel electrophoresis, as described under "Experimental Procedures." The $K_D$ and concentration of active binding protein were calculated by nonlinear regression (26).

| Expt. | DNA | Total RecBCD | Active binding protein | $K_D$ |
|-------|-----|--------------|------------------------|-------|
|       |     |              |                        |       |
| 1     | 5’-Overhang | 25 | 31 | 110 |
| 1     | 5’-Overhang | 50 | 61 | 150 |
| 1     | 5’-Overhang | 100 | 170 | 100 |
| 2     | 5’-Overhang | 25 | 30 | 150 |
| 2     | 5’-Overhang | 50 | 60 | 100 |
| 3     | 5’-Overhang | 25 | 55 | 210 |
| 3     | Blunt     | 200 | 490 | 1800 |

TABLE IV
Binding of RecBCD enzyme to ds DNA ends: effects of 5’-overhang length and Mg$^{2+}$ ions

Hairpin shaped ds DNA (25 pM) bearing the noted number of unpaired nucleotides at the 5'-end, was incubated with varying concentrations of monomeric RecBCD enzyme, and $K_D$ values were determined as described under "Experimental Procedures."

| S’-Overhang | $K_D$ 3 mM Mg$^{2+}$ | $K_D$ 2 mM EDTA |
|-------------|----------------------|-----------------|
| nt          |                      |                 |
| 4           | 0.08                 | 0.56            |
| 3           | 0.30                 | 0.75            |
| 2           | 0.28                 | 2.6             |
| 1           | 0.37                 | 6.8             |
| 0           | 0.65                 | 6.6             |

Monomeric RecBCD Enzyme Unwinds DNA—We have shown above that a single RecBCD enzyme monomer can form a complex with a ds DNA end. We wished to determine whether such a complex was competent to unwind DNA, or whether it had to recruit additional enzyme molecules in order to unwind DNA, as had been suggested by the dimer model for helicase action (13). We used agents that would prevent the formation of new RecBCD-DNA complexes without interfering with preexisting complexes or inhibiting the action of the enzyme.

In the experiments presented in Fig. 5, RecBCD enzyme was allowed to bind to the end of DNA, in the presence of Mg$^{2+}$ but the absence of ATP, and the reaction started by the addition of a mixture of ATP and one of the inhibitory agents. The DNA substrate was a 170-bp ds DNA, blocked at one end with a hairpin oligonucleotide. The substrate had a nick adjacent to the hairpin, to allow separation of the strands by RecBCD enzyme without the need for nuclease activity. Heparin (34), Sarkosyl (35), or excess unlabeled ds DNA molecules were used to prevent reinitiation by RecBCD enzyme. Each of the agents was effective at preventing unwinding: addition of the agent prior to RecBCD enzyme prevented detectable unwinding when ATP was subsequently added (lanes 2, 6, and 8). When added after the enzyme, unlabeled DNA had little effect on the extent of unwinding (lanes 7 versus 10), while Sarkosyl decreased unwinding slightly (lanes 9 versus 10). Heparin inhibited unwinding by about 50% at 2 nM RecBCD enzyme about half as much unwound DNA was produced in the presence of heparin as in its absence (lanes 4 versus 10), but the inhibition was overcome by doubling the RecBCD enzyme concentration (lanes 5 versus 10).

Complexes were formed between 1 nM nicked (170 bp) hairpin DNA molecules and 1, 2, or 4 nM RecBCD enzyme, and a portion of each was analyzed on a nondenaturing gel (Fig. 4, lanes 13-15). With 4 nM RecBCD enzyme, all of the DNA was retarded, while with 2 nM enzyme, approximately half of it was. The retarded DNA migrated more slowly than monomeric RecBCD enzyme but faster than the dimeric form. The 35-bp DNA-RecBCD enzyme complex comigrated with unbound monomeric enzyme (Fig. 4, lane 8), while the 70-bp DNA-RecBCD enzyme complex was slightly retarded (lane 5) and the 170-bp DNA-RecBCD enzyme complex more retarded (lanes 14 and 15). The retardation was thus length-dependent, and all of the complexes contained one monomeric RecBCD enzyme molecule per DNA.

The remainder of the complexes were allowed to react, by addition of ATP and the blocking agents, and unwinding monitored by gel assay (Fig. 5). With 2 nM RecBCD enzyme half of the DNA was retarded (and hence in a complex; Fig. 4, lane 14), and approximately half of it was unwound (Fig. 5, lane 7) when unlabeled DNA was used to prevent reinitiation by RecBCD enzyme. Hence, nearly all of the complexes appear competent for unwinding.

With 4 nM RecBCD enzyme, all of the DNA was bound to RecBCD enzyme (Fig. 4, lane 15), but only about half of it was unwound when reaction was initiated by addition of ATP plus heparin (lane 5). However, as noted above, heparin appeared to
inhibit unwinding by about 50%, and so it would again appear that all the complexes were competent to unwind.

In summary, the initiation complex between RecBCD enzyme and a ds DNA end contained one RecBCD enzyme monomer per DNA end, and virtually every such complex appeared to be competent to unwind DNA.

**DISCUSSION**

**Monomeric and Dimeric Forms of RecBCD Enzyme—**The evidence presented here and by others (9, 11, 12) indicates that one form of RecBCD enzyme contains one each of the RecB, RecC, and RecD polypeptides (M_r = 134,000, 129,000, and 67,000, respectively) (2–4); we refer to this form (B_1C_1D_1) as a monomer. The molar ratios of the polypeptides are close to 1:1:1, as estimated by Coomassie staining of the polypeptides separated by electrophoresis in an SDS-gel and adjusted for the positive charge densities of the polypeptides (Table II). Summing the M_r of the polypeptides, deduced from the nucleotide sequences of their genes (2–4), gives 330,000 for the monomer. We measured the M_r of the monomer to be 345,000 by sedimentation rate (Table I), 282,000 by dynamic light scattering (see "Results"), and 305,000 by native gel electrophoresis at high pH (Fig. 2 and "Results"). A fourth method, gel filtration, gave 345,000 (5). Although each of these methods has assumptions (28), their agreement indicates that the form designated monomer is indeed B_1C_1D_1.

We (Fig. 1) and others (10, 11) observed a second form of RecBCD enzyme with higher M_r. This form has the same molar ratio of the three polypeptides (1:1:1) as the monomer (Table II). Its M_r, estimated by sedimentation rate to be about 600,000 (Table I) or 655,000 (11) or by native gel electrophoresis at high pH (Fig. 2 and "Results"). Thus, this form appears to be a simple dimer (B_2C_2D_2) of the monomer. The form of RecBCD enzyme observed in cell lysates (10) may arise from the binding of one monomer to each end of ds DNA fragments generated during lysis (see below). The dimer observed in purified enzyme may stem from the high concentration achieved during purification (Fig. 3). These considerations, plus the low specific activity of the dimeric form (Fig. 1), make us doubt that the dimeric form is physiologically relevant.

**Binding of RecBCD Enzyme to DNA Ends—**We characterized the binding of RecBCD enzyme to ds DNA by gel mobility-shift assay (26), but used two enhancements to the assay to enable us to study the stoichiometry of binding of RecBCD enzyme to ds ends. The migration of enzyme-DNA complexes was compared to that of monomeric and dimeric RecBCD enzyme on the same gel, to determine the multimeric state of the enzyme bound to DNA ends. The DNA substrate used was a linear ds DNA with one flush (or nearly flush) end but with the other end masked by a ss loop. This enabled us to unambiguously detect complexes between one RecBCD enzyme molecule and one DNA end, without the complication of DNA molecules bearing a RecBCD enzyme molecule at each end, which would have migrated at the position of dimeric RecBCD enzyme. The results (Tables III and IV) show that RecBCD enzyme bound most tightly (K_D ~ 100 pM) to DNA with a 4-nt 5'-overhang and that binding was enhanced by Mg^{2+} ions, but not absolutely dependent on them.

**Binding of ds DNA to RecBCD enzyme—**As measured by filter binding, is dependent on Mg^{2+} ions (17) but the binding measured by gel retardation reported here occurred without Mg^{2+} ions. Presumably Mg^{2+} ions are required for the binding of the protein to the filter, rather than for the binding of the DNA to the protein. The Hemophilus influenzae homolog of RecBCD enzyme does not require Mg^{2+} ions for filter binding and has a K_D, as measured by the ratio of dissociation to association rate constants, of 0.5 pm (36), about 100-fold lower than that reported here. The K_D for the binding of RecBCD enzyme to flush-ended ds DNA has been measured, by filter-binding assays, to be 7 pm (29). The reason for the 250-fold difference between the values obtained by filter-binding and by gel-shift assays (Table III, last line) is unknown. Wilcox and Smith (35) also concluded, for the Hemophilus enzyme, that the complex contained one enzyme molecule per DNA end, based on the noncooperative nature of the binding, but were unable to determine unambiguously whether the active form of the enzyme was a monomer or a dimer.

One ds DNA break in E. coli would produce about 4 nM ds DNA ends (37). Essentially all such breaks would thus be bound by the available RecBCD enzyme, as the enzyme concentration (~100 nM) and the concentration of ends are both significantly above all estimates of the K_D. RecBCD enzyme is thus likely to attack efficiently any ds DNA end introduced into the cell.

**Monomeric RecBCD Enzyme Can Unwind DNA—**We wished to test the hypothesis that a dimer of RecBCD enzyme molecules is required to unwind DNA (17). We constructed a DNA substrate longer than the known footprint of the enzyme on ds DNA ends (16–20 nt) (17), so that the enzyme would have to translocate along ds DNA in order to separate its strands. Essentially all the complexes between a RecBCD monomer and a ds end were competent to unwind DNA. The possibility that a second RecBCD enzyme might be recruited onto the initiation complex was considered. Three different techniques, each of which blocked the unwinding of DNA by RecBCD enzyme, were used to prevent such recruitment. None of them prevented the preformed monomeric enzyme-DNA complex from unwinding the DNA. As the three agents used were so different (unlabeled DNA, heparin, and a detergent), it is very likely that at least one of them, especially DNA, would have blocked the recruitment of a second monomer onto the existing DNA-enzyme complex.

We thus conclude that a monomer of RecBCD enzyme can bind to the end of ds DNA and translocate along and unwind the DNA. The simple form of the dimeric unwinding model for RecBCD enzyme (17) based on that of Lohman (13) thus cannot be correct. As RecBCD enzyme is a large multisubunit enzyme, one might consider combinations of existing helicase models. The RecBCD model of Roman and Kowalczykowski (16) utilizes two helicases, one acting on each strand, and the helicase model of Lohman (13) requires two (quasi) equivalent binding sites for each helicase. Hence, for a monomer of RecBCD enzyme to unwind DNA by such a mechanism, two sets of quasi-equivalent binding sites would be required. However, only one putative ATP binding site has been identified in the RecB and RecD subunits, and there is no evidence for repeated structures in any of the enzyme subunits (2–4). It thus seems unlikely that RecBCD enzyme unwinds DNA via the Lohman (13) dimer model.

Knowledge of the minimal complex necessary for unwinding to occur should facilitate determining the mechanism by which RecBCD enzyme unwinds DNA. Electron microscopy of RecBCD enzyme and further characterization of its complex with a ds DNA end may yield important clues in this quest.

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