Bipolar DNA Translocation Contributes to Highly Processive DNA Unwinding by RecBCD Enzyme*[S]

Mark S. Dillingham[‡§1], **Martin R. Webb**[§], **and Stephen C. Kowalczykowski**[‡2]

From the ‡Sections of Microbiology and of Molecular and Cellular Biology, Center for Genetics and Development,
University of California, Davis, California 95616 and the §National Institute for Medical Research,
The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

We recently demonstrated that the RecBCD enzyme is a bipolar DNA helicase that employs two single-stranded DNA motors of opposite polarity to drive translocation and unwinding of duplex DNA. We hypothesized that this organization may explain the exceptionally high rate and processivity of DNA unwinding catalyzed by the RecBCD enzyme. Using a stopped-flow dye displacement assay for unwinding activity, we test this idea by analyzing mutant RecBCD enzymes in which either of the two helicase motors is inactivated by mutagenesis. Like the wild-type RecBCD enzyme, the two mutant proteins maintain the ability to bind tightly to blunt duplex DNA ends in the absence of ATP. However, the rate of forward translocation for the RecB motor-defective enzyme is only ~30% of the wild-type rate, whereas for the RecD motor-defective enzyme, it is ~50%. More significantly, the processivity of translocation is substantially reduced by ~25- and 6-fold for each mutant enzyme, respectively. Despite retaining the capacity to bind blunt dsDNA, the RecB-mutant enzyme has lost the ability to unwind DNA unless the substrate contains a short 5′-terminated single-stranded DNA overhang. The consequences of this observation for the architecture of the single-stranded DNA motors in the initiation complex are discussed.

The RecBCD enzyme of *Escherichia coli* is involved in the repair of double-stranded breaks in DNA (for review, see Ref. 1). This is an important function, because it has recently become apparent that double-stranded breaks occur frequently as a result, among other things, of replication through damaged template DNA (for discussions, see Refs. 2 and 3). The break in the nucleic acid is rescued by homologous recombination, which uses an undamaged copy of the DNA molecule as a template for the repair. Recombinational DNA repair involves several gene products, and the RecBCD helicase/nuclease acts at the initiation step. The enzyme binds tightly to blunt or nearly blunt DNA ends (4, 5). Then, in a reaction that requires ATP hydrolysis, RecBCD rapidly tracks along the duplex DNA, unwinding it as it goes, and predominantly degrading the 3′-terminated single strand into shorter fragments. This destructive, nucleolytic mode of action is modified when the enzyme encounters a correctly oriented DNA sequence called Chi (crossover hotspot instigator 5′-GCTGGTGG). This important regulatory sequence is over-represented in the *E. coli* genome and is particularly found clustered, with appropriate directionality, around the origin of replication (6, 7). Upon Chi recognition, degradation of the 3′-terminated strand is reduced, and degradation of the 5′-strand is up-regulated, although less so, while DNA translocation and unwinding continue (8–10). Consequently, the product of the enzyme is a DNA duplex with a long 3′-terminated ssDNA[3] overhang onto which the enzyme loads RecA protein (11) to create a recombinogenic molecule, ready for the subsequent DNA strand exchange step of homologous recombination (3). In the absence of Chi recognition, RecBCD continues to act as a potent, unaltered helicase/nuclease. This alternative mode of action is essential for the enzyme's auxiliary role of protecting the cell against phage infection by degrading foreign linear DNA species (12).

The RecBCD enzyme has proven to be a DNA helicase of particular interest, because it possesses several unique biochemical characteristics. RecBCD displays a requirement for a blunt DNA end to act as a loading site, consistent with its role in the initiation of the repair of double-stranded breaks. In contrast, the majority of DNA helicases require a short ssDNA overhang from which to initiate unwinding (13). The maximum rate of enzyme movement is measured between 1000 and 1500 bp s^{-1} at 37 °C (4, 14, 15), which is much faster than related enzymes such as *E. coli* Rep and UvrD that translocate and unwind DNA at about 20 bp s^{-1} (16, 17) in bulk phase measurements, but which can move as rapidly as 275 bp s^{-1} in single-molecule experiments (18). Moreover, the rate of translocation is controlled by the Chi sequence, which acts as a molecular throttle, decreasing the rate of forward motion by about one-half (19). Finally, RecBCD enzyme unwinds an average of about 30,000 bp in a single encounter with a DNA molecule (20), making it easily the most processive helicase reported. This again contrasts with related enzymes like UvrD helicase that only unwind 50 bp (bulk phase measurement (16)) or 255 bp (single molecule measurement (18)).

Many of these unusual characteristics are potentially explained by the recent discovery that RecBCD enzyme is a bipolar DNA helicase (21, 22). The enzyme complex contains two subunits that can catalyze a DNA strand separation activity. The RecB and RecD proteins both contain Superfamily 1 (SF1) DNA helicase motifs (23), and both are weak DNA helicases *in vitro* (21, 24). The two DNA motors are of opposite polarity: RecB catalyzes a 3′→5′ helicase reaction, whereas RecD possesses a 5′→3′ directionality. Although it has not been formally demonstrated for these two proteins, comparison to other SF1 DNA helicases (25, 26) implies that the mechanistic basis for their unwinding

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

[1] Current address: University of Bristol, DNA-protein Interactions Group, Dept. of Biochemistry, School of Medical Sciences, Bristol BS8 1TD, UK.

[2] To whom correspondence should be addressed: University of California, Davis, Section of Microbiology, Center for Genetics and Development, One Shields Ave., Briggs Hall 310, Davis, CA 95616-8665. Tel.: 530-752-5938; Fax: 530-752-5939; E-mail: sckowalczykowski@ucdavis.edu.

[3] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; Chi, crossover hotspot instigator; SF1, superfamily 1; SSB, *E. coli* single-stranded DNA binding protein; WT, wild-type.

polarity relates to ATP-dependent unidirectional translocation on ssDNA. This concept led to the development of a model for RecBCD translocation, alternatively described as "bipolar" translocation (21) or "dual-motor" translocation (22), in which the two DNA motors move in the same direction on either strand of the anti-parallel DNA duplex. Such a model has the potential to explain many of the special properties of RecBCD and reconcile them with the behavior of monomeric DNA helicases, together with models developed for their unwinding mechanisms (27). Specifically, the participation of two motor subunits can contribute to the exceptionally high processivity of DNA translocation, because the translocating enzyme would dissociate from the DNA only when both motors are simultaneously dissociated from the DNA track. The use of two DNA motors is potentially capable of generating more force than a single motor. This feature might result in an increased forward translocation rate, particularly under conditions *in vivo*, in which the translocating enzyme may encounter a variety of "roadblocks" (protein-nucleic acid complexes) on its path to a properly aligned Chi sequence; indeed, RecBCD enzyme is sufficiently potent so it can displace nucleosomes from DNA (28).

To test these ideas directly, we studied the DNA translocation and unwinding activities of RecBCD enzymes in which either the RecB or RecD motor is inactivated by mutagenesis. Mutation of a conserved lysine to glutamine in helicase motif I (the Walker A motif) eliminates ATP hydrolysis and, consequently, helicase activity in purified RecB and RecD helicases (21, 29). The rationale behind these experiments is that the RecB^{K29Q}CD and RecBCD^{K177Q} mutant holoenzymes will allow the isolation of the motor activity of either the RecB or RecD subunit and that the biochemical properties of these enzymes will provide insights into the requirement for a bipolar translocation mechanism. It has been shown previously that these single-mutant enzymes, but not the double-mutant RecB^{K29Q}CD^{K177Q} enzyme, retain the ability to catalyze DNA unwinding (21, 22, 30). This observation strongly suggests that the activities of the two motors are at least partially independent.

Here we measure the rates and processivities of these mutant protein complexes. The rapid DNA unwinding reaction can be followed in real-time using a dsDNA-binding fluorescent dye (14). This allows us to extract the contribution of each helicase motor to the activity of the RecBCD complex as a whole, and so develop the model of RecBCD acting through two complementary, but independent, helicases.

EXPERIMENTAL PROCEDURES

DNA Substrates—pBR322 plasmid DNA (4,361 bp) was purified using Qiagen maxiprep kits and cesium chloride density gradient centrifugation. It was linearized using one of the EcoRI, NdeI, EcoRV, or PstI restriction endonucleases, to produce 4-base 5′-overhangs, 2-base 5′-overhangs, blunt ends, or 4-base 3′-overhangs, respectively. This was followed by inactivation either by heating according to manufacturer's instructions (New England Biolabs) or, where appropriate, by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (PstI-substrate). Agarose gel analysis showed that these DNA substrates were efficiently linearized, and they were not further purified. pBR322 plasmid DNA does not contain Chi sequences. λ phage DNA (48,502 bp), which is also devoid of Chi sequences, was purchased from New England Biolabs. Immediately before use, the λ DNA was incubated at 65 °C for 10 min to separate the cohesive ends and then placed on ice. DNA concentrations were determined by absorbance at 260 nm using an extinction coefficient of 6330 M^{-1} (nucleotides) cm^{-1}.

Proteins—RecBCD, RecB^{K29Q}CD, RecBCD^{K177Q}, and single-stranded DNA-binding protein (SSB) were expressed and purified as described (31–34). Purified proteins are shown in supplementary Fig.

S1. RecBCD concentrations were determined by absorbance at 280 nm using an extinction coefficient of 414,640 M^{-1} (heterotrimer) cm^{-1}.

Stopped-flow Dye-displacement Helicase Assay—Experiments were performed in a Hi-Tech SF61 DX2 stopped-flow apparatus with excitation at 366 nm, and emission was measured after a 400 nm cut-off filter. Excitation slit widths were set to 4 mm. Note that, unless stated otherwise, all quoted concentrations are final, after mixing of equal volumes in the stopped flow apparatus. Reactions were performed at 37 °C in a buffer containing 25 mM Tris acetate, pH 7.5, 6 mM magnesium acetate, and 1 mM dithiothreitol. The buffer also contained 200 nM Hoechst 33258 dye (Molecular Probes) and 100 nM SSB protein, which are saturating concentrations with respect to the DNA substrate. For experiments where the RecBCD enzyme was pre-bound to the DNA, enzyme, at the final concentration indicated, was incubated with 50 pM DNA molecules (equivalent to 100 pM RecBCD binding sites or 0.43 μM nucleotides) for at least 5 min, and this was then mixed with 2 mM (saturating) ATP to initiate the reaction. When RecBCD was not pre-bound, it was mixed with the DNA and ATP to initiate the reaction. In some experiments, 0.5 mg ml^{-1} heparin (sodium salt, Sigma) was present with the ATP to trap free RecBCD, following its release from DNA. Control experiments demonstrated that this concentration of heparin was sufficient to completely eliminate binding of free RecBCD to DNA ends under the conditions of our experiments (data not shown). Under our standard conditions, unwinding of the pBR322 substrates by saturating RecBCD enzyme is between 95 and 100% complete within 20 s, as determined by comparing the amplitude of the fluorescence decrease with that of a heat-denatured pBR322 control (data not shown). Therefore, a saturated, pre-bound, wild-type RecBCD unwinding trace was obtained and used to calibrate the fluorescence signal to the percentage of DNA unwound by assuming that 100% of the DNA was unwound at 20 s.

Data were analyzed using KinetAsyst 3 (Hi-Tech Scientific) and/or Prism 3.0 (GraphPad Software) software to obtain a rate and amplitude of DNA unwinding for each trace. Unwinding time courses were biphasic. In all cases, unwinding rates were determined by performing a linear fit to the first phase of the reaction. For a highly processive enzyme, this rate is approximately constant for most of the first phase. In a less processive system, the rate of unwinding decreases with time due to significant enzyme dissociation, so in the case of RecB^{K29Q}CD enzyme, the observed rate refers to an initial rate of unwinding (when essentially all of the enzyme that was pre-bound to a DNA end is still associated with the DNA and in the process of unwinding it). All unwinding rates (bp s^{-1} per binding site) quoted on graphs are obtained by dividing the observed unwinding rate by 2 to account for the fact there two RecBCD binding sites per DNA molecule. Thus, this value represents the turnover number for DNA translocation and unwinding (bp s^{-1} RecBCD^{-1}) when the RecBCD concentration is saturating. The enzyme affinity for DNA ends (K_d) was determined by performing quadratic single site binding equation fits to the data for the relationship between total enzyme concentration (E) and observed unwinding rate (v) using the program GraphPad Prism 3.0 (Equation 1).

$$v = V_{max}\left[\frac{(K_d + E + D) - \sqrt{(K_d + E + D)^2 - 4ED}}{2D}\right] \quad \text{(Eq. 1)}$$

The variable V_{max} is a scaling factor that represents the specific unwinding rate (bp s^{-1} RecBCD^{-1}) of each enzyme. The total concentration of DNA ends (D) is a constant at 0.2 nM. As was appropriate, the total enzyme and DNA end concentrations used in these fits were for the pre-bound complex, rather than the final concentration after mixing.

Conventional Dye-displacement Helicase Assay—Assays were performed essentially as described (14) in a PerkinElmer Life Sciences LS50B fluorometer. Excitation and emission were at 344 and 487 nm, respectively, and slit widths were all at 5 nm. Experiments were performed at 37 °C in a buffer containing 25 mM Tris acetate, pH 7.5, 6 mM magnesium acetate, 200 nM Hoechst 33258, 1 μM SSB, and 1 mM dithiothreitol. For the "low free Mg^{2+} ion" conditions, the buffer contained 1 mM magnesium acetate, which results in a free magnesium ion concentration of 25 μM as calculated using WEBMAXC software (35). Saturating enzyme (5 nM) was pre-bound to 0.1 nM ends (4.85 μM nucleotides) λ DNA or to 1.1 nM ends (5 μM nucleotides) pBR322 plasmid DNA. Reactions were initiated with a final concentration of 2 mM ATP, or 2 mM ATP and 0.5 mg/ml heparin as indicated. The fluorescence amplitude was calibrated with solutions containing no protein (representing 0% unwound), or heat-denatured DNA (representing 100% unwound).

SSB Binding-coupled Helicase Assay—Assays were performed essentially as described previously (4) in a PerkinElmer Life Sciences LS50B fluorometer. Excitation and emission were at 290 and 340 nm, respectively, and slit widths were all at 2.5 nm. Experiments were performed at 37 °C in a buffer containing 25 mM Tris acetate, pH 7.5, 6 mM magnesium acetate, 1 μM SSB, 1 mM dithiothreitol, 2 mM ATP, and 0.2 nM ends (9.7 μM nucleotides) λ DNA. Reactions were initiated with 5 nM enzyme. Fluorescence was calibrated with solutions containing no DNA (0% unwound) or heat-denatured DNA (100%) unwound. A zero protein control was also performed to confirm that unwinding was RecBCD-dependent and to measure photobleaching.

RESULTS

Monitoring Rapid Unwinding of DNA by RecBCD Using a Stopped-flow Dye-displacement Assay—DNA transactions that result in a net increase or decrease of duplex DNA can be assayed using dsDNA binding dyes, such as 4′,6-diamidino-2-phenylindole, thiazole orange, and Hoechst 33258, that display large fluorescence increases upon binding duplex DNA. Such an assay was useful in characterizing the helicase activity of RecBCD (14). Because RecBCD-catalyzed DNA unwinding can also be assayed independently by following SSB binding to the ssDNA products of the reaction, it was shown that the presence of the dye does not inhibit the enzyme translocation and that the assay provides an accurate measure of both the rate and amplitude of DNA unwinding by RecBCD enzyme (14). The assay was originally designed for use in a standard fluorometer, and one weakness of this approach is that the exceptionally rapid translocation activity of RecBCD means that it is difficult to study reactions on relatively short DNA, or at temperatures above about 20 °C, because the first round of the translocation reaction (i.e. the first turnover with respect to enzyme associated with the DNA substrate) is complete within a few seconds. To allow us to study the first round of DNA translocation, we have slightly modified the dye displacement assay for use in a stopped-flow apparatus (see "Experimental Procedures").

In our first experiments, RecBCD enzyme was pre-bound to linearized pBR322 plasmid DNA (4361 bp) that was saturated with Hoechst 33258 dye. The DNA substrate contains two free ends, which represent two binding sites for RecBCD enzyme (4, 5), and does not contain Chi sequences. The unwinding reaction was initiated by rapid mixing of the protein-DNA complexes with 2 mM ATP. The K_m for ATP during DNA unwinding is about 150 μM (Refs. 4 and 36, and data not shown), and so this concentration of ATP is saturating. Single-stranded DNA-binding protein (SSB) was included in the reactions as a trap for the ssDNA products of the reaction, although, in practice, its presence makes only a small difference to the observed kinetics when the RecBCD enzyme
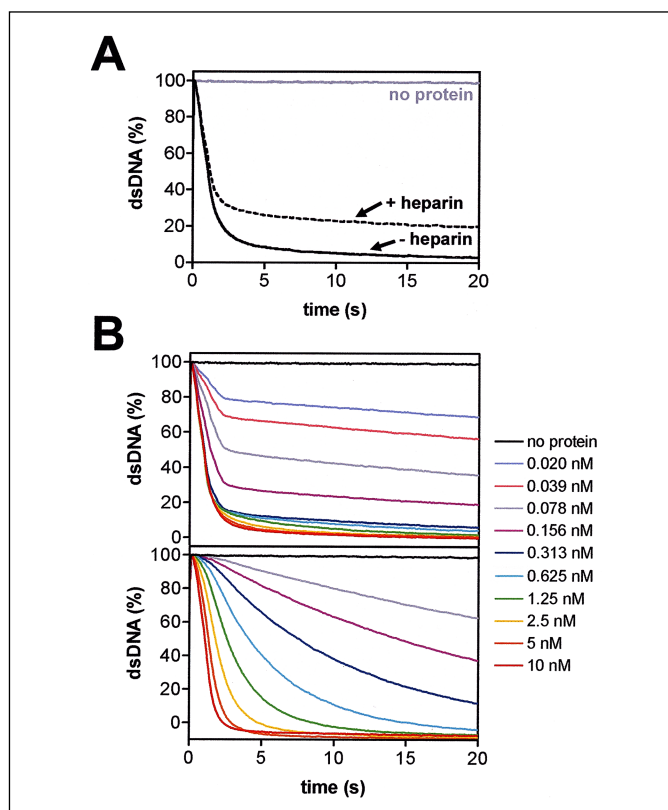

FIGURE 1. **Rapid and processive unwinding of plasmid DNA by RecBCD observed using a dye-displacement assay.** *A,* saturating quantities (10 nM) of RecBCD enzyme were pre-bound to the free ends (0.1 nM) of NdeI-linearized plasmid DNA (4361 bp) and then mixed rapidly with ATP (*solid line*) or ATP and heparin trap (*dotted line*). Unwinding of the DNA was observed as a decrease in fluorescence due to displacement of Hoechst dye that was bound to the dsDNA. The *gray line* is a control reaction in which RecBCD was not present. In *B, top panel*, RecBCD enzyme at the concentration indicated was pre-bound to 0.1 nM free ends of NdeI-linearized plasmid DNA and then rapidly mixed with ATP to initiate DNA unwinding; *bottom panel*, RecBCD enzyme at the concentration indicated was rapidly mixed with 0.1 nM free ends of NdeI-linearized plasmid DNA and ATP.

concentration is saturating with respect to DNA ends (37, and data not shown). At the highest concentration of RecBCD used (10 nM), the reaction proceeded in two phases (Fig. 1*A*, *solid black line*). About 80–90% of the DNA was rapidly unwound at an approximately constant rate, and then the remaining DNA was unwound at a slower rate. The first phase of the reaction is interpreted as rapid unwinding of the DNA substrate mediated by the RecBCD enzymes that are productively bound to the substrate at time zero. The small amount of DNA that is unwound more slowly is interpreted as a fraction of the DNA that is not competent for unwinding at time zero, perhaps because RecBCD enzyme is bound in a non-productive manner. This unwinding is not due to re-initiation of unwinding on partially degraded DNA molecules (*i.e.* a second round of unwinding), because RecBCD cannot initiate unwinding on partially unwound DNA molecules (38). In the absence of enzyme or ATP (not shown), no significant decrease in fluorescence is observed indicating that the reduction in fluorescence is due to RecBCD-catalyzed helicase activity and that photobleaching of the DNA-binding dye is not significant on the timescale of these experiments.

This concentration (10 nM) of RecBCD was clearly saturating, because the trace, and in particular the main, rapid phase of the reaction, was not significantly affected until the final RecBCD concentration was <~0.3 nM (Fig. 1*B*, *top panel*). As the RecBCD concentration was decreased further, the rate and amplitude of the rapid unwinding phase
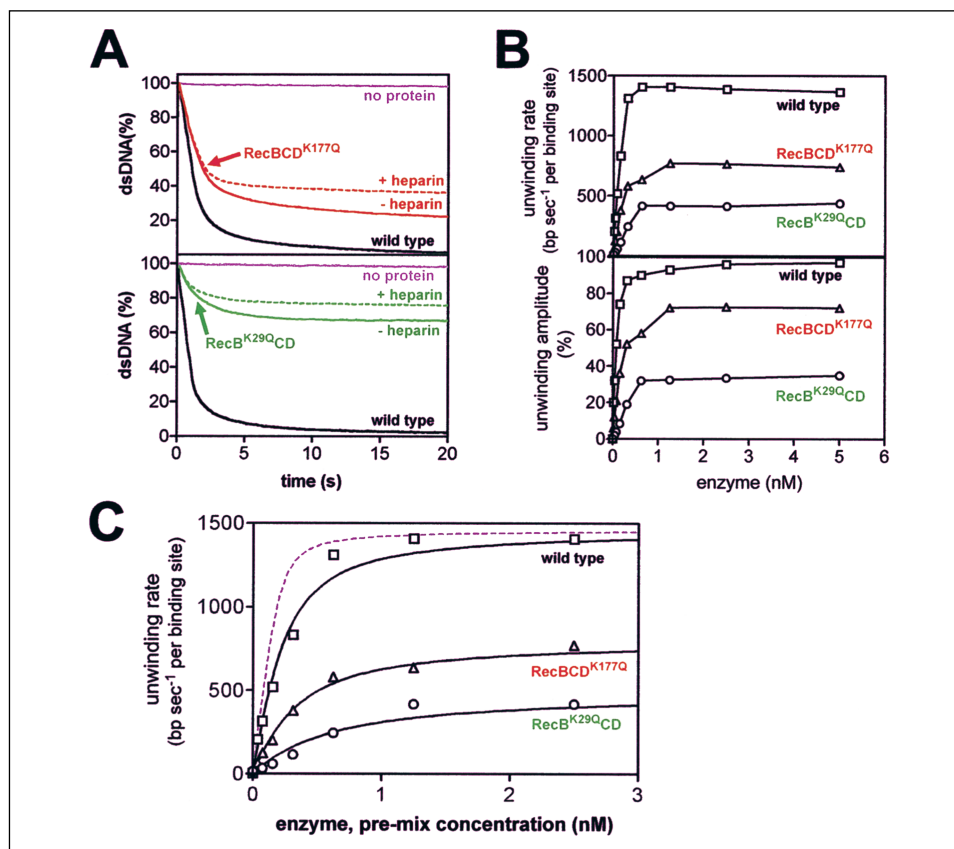
FIGURE 2. **RecBCD motor mutants bind to dsDNA ends tightly, but display reduced rates and amplitudes of plasmid DNA unwinding.** In *A*, *top panel*, saturating quantities (5 nM) of wild-type RecBCD (*black line*) or RecBCD^{K177Q} (*red lines*) enzymes were pre-bound to 0.1 nM free ends of NdeI-linearized plasmid DNA and then rapidly mixed against ATP (*solid lines*) or ATP and heparin (*dotted line*). The *gray line* is a control reaction in which neither enzyme was present. *Bottom panel*, saturating quantities (5 nM) of wild-type RecBCD (*black line*) or RecB^{K29Q}CD (*green lines*) were pre-bound to 0.1 nM free ends of EcoRI-linearized plasmid DNA and rapidly mixed against ATP (*solid lines*) or ATP and heparin (*dotted line*). The *gray line* is a control reaction in which neither enzyme was present. *B*, relationship between enzyme concentration and the observed unwinding rate and amplitude of the first phase of plasmid DNA unwinding for wild-type and mutant RecBCD enzymes. The unwinding rate is expressed as bp s^{-1} per enzyme binding site, and so this represents the turnover number for DNA unwinding (bp s^{-1} RecBCD^{-1}) when the enzyme is saturating. These data can be used to estimate the affinity of the enzymes for the DNA end (*C*). The observed unwinding amplitudes represent the percentage reduction in dsDNA for the first phase approximated from the intercept of linear fits to the two phases. *C*, the relationship between RecBCD enzyme concentration (syringe concentration *before* mixing) and the initial rate of unwinding. For clarity, only the first seven data points are shown. The data are fit to a single site binding equation (see "Experimental Procedures") using 0.2 nM as the total concentration of DNA ends to yield values for the maximum unwinding rate (V_{max}) and the end-binding constant (K_d). These values and their standard errors are shown in TABLE ONE. The *gray dotted line* is a simulation for the wild-type enzyme in which the K_d is forced to be 5-fold tighter ($K_d = 0.022$ nM) than that measured ($K_d = 0.11$ nM), demonstrating that this is a poor fit to the data ($R^2 = 0.87$ compared with $R^2 = 0.98$).

dropped concomitantly, consistent with these values being determined by the amount of RecBCD enzyme bound to the DNA ends at time zero. Even at such a low concentration of DNA ends (0.2 nM *before* mixing with ATP) the binding is close to stoichiometric, but these data can be used to estimate an affinity of RecBCD for DNA ends of 0.11 nM (Fig. 2*C*), in agreement with values in the range 0.1 to 1 nM published previously (4, 5). The experiments described below are generally performed at 5 nM pre-bound RecBCD, which is saturating. Under these conditions, in which the DNA translocation rate and processivity can be studied independently of DNA association kinetics, the net observed DNA unwinding rate is 2,700 bp s^{-1}. Because there are two RecBCD binding sites in each DNA molecule, this corresponds to an observed forward translocation/unwinding rate of 1,350 bp s^{-1} RecBCD^{-1}. Note that, because the observed rate does not take into account the fact that a fraction of the DNA ends may be bound in non-productive complexes (discussed above), it may be a slight underestimate of the true unwinding and translocation rate.

Heparin has been shown to act as a passive trap for any RecBCD that is free at time zero, or that becomes free following dissociation after the first encounter with the DNA substrate (30, 39). Therefore, if a pre-bound RecBCD-DNA complex is mixed with ATP and heparin it is possible to observe a single turnover of the RecBCD with respect to its

association with the DNA substrate. As expected, the rapid phase of the unwinding reaction is largely unaffected by the use of this trap (Fig. 1*A*), supporting the argument that this unwinding is due to the processive activity of RecBCD molecules. Importantly, this result formally eliminates the possibility that any substantial portion of the unwinding occurs by a mechanism involving complete dissociation of RecBCD followed by re-initiation of unwinding. There is a small but significant decrease in the unwinding amplitude with the use of a heparin trap, and there are several possible reasons for this. Firstly, the experiments are performed with enzyme pre-bound to DNA ends at concentrations ~50-fold above the K_d value. Consequently, a few percent of the DNA ends are not bound at time zero, and these cannot act as initiation sites in the presence of heparin. Secondly, the use of heparin in the reactions results in a loss of function of SSB protein, and this could result in a small decrease in the apparent unwinding amplitude by RecBCD due to the re-annealing of ssDNA products. However, the presence or absence of SSB was shown to make little difference to the observed kinetics and does not affect the ability of heparin to function effectively as a trapping agent (data not shown). Finally, there is evidence that a fraction of the RecBCD may be bound in non-productive complexes, which could be heparin-trappable (*i.e.* the "isomerization" to a productive complex may render RecBCD enzyme susceptible to binding and competition by heparin).

We also performed experiments in which the RecBCD was not pre-bound to the DNA, but was instead mixed with the DNA substrate and ATP simultaneously (Fig. 1*B*). At the highest RecBCD concentration used (10 nM), the maximum observed rate of DNA unwinding was similar to that observed in the pre-bound experiment, although a small lag was still present at the start of the trace, presumably due to binding. At lower RecBCD concentrations, the observed rate of DNA unwinding was clearly limited by the kinetics of RecBCD-DNA association.

Mutation of Helicase Motif I in Either RecB or RecD Reduces the Observed Rate and Amplitude of Plasmid Unwinding Catalyzed by the RecBCD Holoenzyme—The main aim of this work was to determine the effect of eliminating ATP hydrolysis in either of the two helicase motors of RecBCD enzyme on DNA translocation. A lysine to glutamine mutation in helicase motif I of either the RecB (K29Q) or RecD (K177Q) polypeptide was shown to eliminate ATP hydrolysis and, consequently, the associated helicase activity in each of the isolated helicase subunits. We have studied the activities of holoenzymes incorporating the same mutations with the rationale that this would isolate the activity of the functional motor. The double mutant enzyme (RecB^{K29Q}CD^{K177Q}) is inactive as both an ATPase and a DNA helicase (22, 29). However, both of the single mutant enzymes, RecB^{K29Q}CD and RecBCD^{K177Q}, are known to retain rapid and processive helicase activities (21, 22), although these activities were not characterized quantitatively.

Both the rate and amplitude of unwinding of linear pBR322 plasmid DNA were reduced for the RecBCD^{K177Q} enzyme, which only has an active RecB helicase subunit (Fig. 2*A*). At saturating enzyme concentrations (5 nM, Fig. 2*B*), the observed rate of DNA unwinding was 1500 bp s^{-1}, corresponding to a forward translocation/unwinding rate of 750 bp s^{-1} RecBCD$^{K177Q\ -1}$, ~2-fold slower than the wild-type enzyme. There is also a decrease in the amplitude of DNA unwinding that is indicative of a decreased processivity. The fact that <100% of the DNA was unwound even in the absence of heparin (*solid line*, Fig. 2*A*) demonstrated that the mutant enzyme cannot reinitiate unwinding on partially unwound DNA molecules; the unwinding of all of the DNA by the wild-type enzyme confirms the quality of the substrates, showing that all of the DNA can be unwound. The final unwinding amplitude of ~80% is equal to 1.7 kbp unwound per enzyme. This value is directly related to, but is significantly less than, the processivity of the enzyme, because the unwinding of the pBR322 DNA was nearly complete. Consequently, determination of the processivity from this amplitude value was associated with a large error. Below, we will examine the unwinding amplitude on longer DNA substrates.

Inactivation of the RecB subunit has a more severe effect on both the rate and amplitude of DNA unwinding (Fig. 2*A*). At saturating RecB^{K29Q}CD, the observed rate of unwinding was 880 bp s^{-1}, corresponding to a forward translocation/unwinding rate of 440 bp s^{-1} RecB^{K29Q}CD^{-1}, ~3-fold slower than the wild-type enzyme. The amplitude of pBR322 DNA unwinding was ~35%, which represents 750 bp unwound per enzyme. Note the more curved nature of the unwinding trace that is also consistent with a reduced processivity. Finally, for both mutants and the wild-type enzyme, a similar amount of the unwinding amplitude is lost when the reaction is challenged with heparin, showing that a similar fraction of non-productive complexes are present at zero time.

Like wild-type RecBCD, both mutant enzymes bind to DNA ends with a sub-nanomolar affinity (Fig. 2*C*). Fitting of the data suggests that RecB^{K29Q}CD and RecBCD^{K177Q} bind to DNA ends with affinities of 0.49 and 0.23 nM, respectively, values that are only slightly less tight than the wild-type affinity (TABLE ONE). Competitive binding experiments for DNA ends (see below) also show that RecBCD and RecB^{K29Q}CD have similar affinities for DNA ends.

TABLE ONE

DNA unwinding parameters for wild-type and mutant RecBCD enzymes

Enzyme	Maximum rate of plasmid DNA unwinding (k_{cat})	DNA end binding affinity (K_d)	Observed amplitude of λ DNA unwinding[a]
	bp s^{-1}	*nM*	*kbp*
RecBCD	1460 ± 50	0.11 ± 0.03	20.7 ± 0.1[b]
RecB^{K29Q}CD	480 ± 40	0.49 ± 0.15	1.2 ± 0.2
RecBCD^{K177Q}	800 ± 20	0.23 ± 0.03	4.9 ± 1.0

[a] The value shown for the λ DNA unwinding amplitude is the mean ± S.D. for three observations.
[b] The value 20.7 kbp for wild-type RecBCD enzyme is an underestimate of the processivity, because the unwinding amplitude is limited to a significant extent by the finite length of the DNA substrate. Modeling of this λ DNA unwinding amplitude using a model where two RecBCD molecules act independently from each end yields a processivity value of 39 kbp (data not shown), which is in reasonable agreement with the literature value of ~30 kbp.

The RecB^{K29Q}CD Enzyme Will Only Initiate Unwinding From a Short 5′-ssDNA Overhang Structure—The helicase activity of RecB^{K29Q}CD was previously demonstrated using λ phage DNA and NdeI-linearized plasmid DNA (21, 22), both substrates that contain short 5′-terminated ssDNA overhangs. Previous analyses of RecB^{K29Q}CD enzyme had suggested that the enzyme did not possess ATPase activity on duplex DNA substrates and, consequently, that it was probably not a DNA helicase (29, 33). However, those studies only employed plasmid DNA substrates that had been linearized with restriction enzymes that generate blunt ends or short 3′- overhangs. For this reason, we tested the activity of wild-type and mutant RecBCD enzymes on linearized plasmid substrates with a variety of different end structures.

Wild-type RecBCD displays very similar unwinding traces, especially with respect to the first, rapid unwinding phase of the reaction, regardless of the restriction enzyme used to create the binding site of the enzyme (Fig. 3). Likewise, RecBCD^{K177Q} enzyme displays broadly similar unwinding rates and amplitude on all four substrates tested, although there is an indication of a slight preference for a 3′-terminated overhang. In contrast, the behavior of the RecB^{K29Q}CD mutant is strikingly different on the four substrates. DNA substrates with short 3′-overhangs or blunt duplex ends are barely unwound at all (Fig. 3, *green traces*), in agreement with the published observations (29, 33). However, the presence of just two additional ssDNA nucleotides on the 5′-strand (NdeI-cut substrate) is sufficient to reveal a rapid and processive helicase activity that is further enhanced if there are four ssDNA nucleotides on the 5′-terminated strand (EcoRI-cut substrate). Note that this is the strand upon which the 5′→3′ helicase activity of RecD acts, and this is the only active helicase subunit in the RecB^{K29Q}CD mutant. The inability of RecB^{K29Q}CD to unwind blunt and 3′-overhang substrates is not due to an inability to bind the substrate, because the mutant enzyme acts as a potent inhibitor of unwinding by wild-type RecBCD if it is pre-bound to the DNA end (Fig. 4). Excess RecB^{K29Q}CD mutant enzyme was pre-bound to DNA, and then equimolar wild-type RecBCD was added. This was then rapidly mixed with ATP to initiate unwinding after a variety of different time delays. The wild-type enzyme exchanges with the pre-bound mutant at a rate of 0.3 min^{-1} until, at equilibrium, the observed unwinding rate and amplitude recover to approximately one-half of the expected values for wild-type alone. This indicates that the wild-type and mutant enzymes have similar affinities for the DNA ends.

Mutation of Helicase Motif I in Either RecB or RecD Severely Reduces Processivity of Translocation by RecBCD—Accurate determination of unwinding processivity is most simply achieved by using DNA substrates that are much longer than the average number of base pairs unwound (i.e. the processivity) by the enzyme. In that case, the average
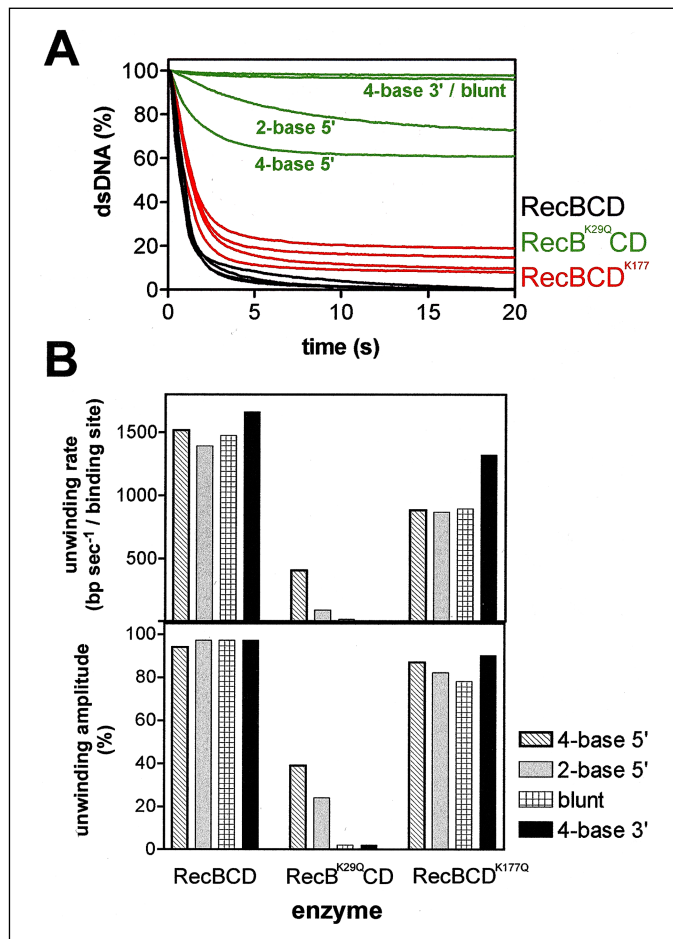
FIGURE 3. **RecB^{K29Q}CD enzyme will only initiate unwinding on DNA with short 5'-overhangs.** *A*, DNA unwinding traces for pBR322 DNA that was linearized with four different restriction endonucleases to produce DNA ends with different structures. Saturating (5 nM) wild-type RecBCD (*black lines*), RecB^{K29Q}CD (*green lines*), or RecBCD^{K177Q} (*red lines*) were pre-bound to 0.1 nM DNA ends and mixed against ATP. *B*, histogram comparing the rate and amplitude of plasmid DNA unwinding by wild-type and mutant enzymes on DNA with different end structures from the data in *A*. The observed unwinding amplitudes represent the percentage reduction in dsDNA for the first phase approximated from the intercept of linear fits to the two phases.

FIGURE 4. **RecB^{K29Q}CD binds tightly to 3'-ssDNA overhang and blunt-ended DNA substrates.** *A*, unwinding traces for 5 nM wild-type RecBCD (*black*) and 5 nM RecB^{K29Q}CD (*gray*) enzymes pre-bound to blunt and 3'-overhang substrates, showing that RecB^{K29Q}CD does not unwind these substrates. To see if RecB^{K29Q}CD was bound to these substrates, we tested its ability to inhibit wild-type RecBCD by competing for the DNA end. The remaining four traces are for an experiment in which the DNA was pre-bound to 5 nM RecB^{K29Q}CD enzyme, before equimolar RecBCD was added and allowed to exchange with the mutant for the indicated times before mixing with ATP. RecB^{K29Q}CD clearly inhibits the wild-type enzyme, with the unwinding rate recovering to nearly one-half of its wild-type value over several minutes. This indicates that the wild-type and mutant enzyme bind to the DNA ends with a similar affinity. *B*, relationship between the duration of the exchange time and the observed unwinding rate. The data are fit to single exponentials to yield apparent dissociation rates for RecB^{K29Q}CD from DNA of 0.32 min^{-1} (*blunt-end*) and 0.33 min^{-1} (*3'-overhang*).

amount of DNA unwound per enzyme is a good approximation of the actual processivity of the enzyme (20). Although we are able to detect a lower processivity in the two mutant proteins and, in theory, to quantify it using plasmid length DNA substrates, the error on these estimates can be large because a substantial proportion of the plasmid is unwound. Phage λ DNA provides a convenient linear DNA substrate that is ~49 kbp in length and that does not contain Chi sequences. It has short 5'-overhangs and is consequently a substrate for the RecB^{K29Q}CD enzyme as has been shown previously (22).

Wild-type RecBCD unwinds 85% of the λ DNA, which represents 20.7 kbp per RecBCD enzyme (Fig. 5, *top panel*, and TABLE ONE). Due to the exceptional processivity of the wild-type enzyme, the λ DNA unwinding amplitude still somewhat underestimates processivity, because a significant proportion of enzymes are limited by the finite length of the DNA substrate. Modeling (not shown) of this λ DNA-unwinding amplitude, using a model wherein two independent RecBCD enzymes unwind from each end, suggests that the enzyme processivity is equal to 39 kbp, which is in reasonable agreement with the literature value of 30 kbp (20). Unwinding of the phage λ DNA by the mutant proteins is substantially less than the substrate-limited maximum of 24.3 kbp per RecBCD (Fig. 5, *top panel*, *gray bars*). Therefore the total
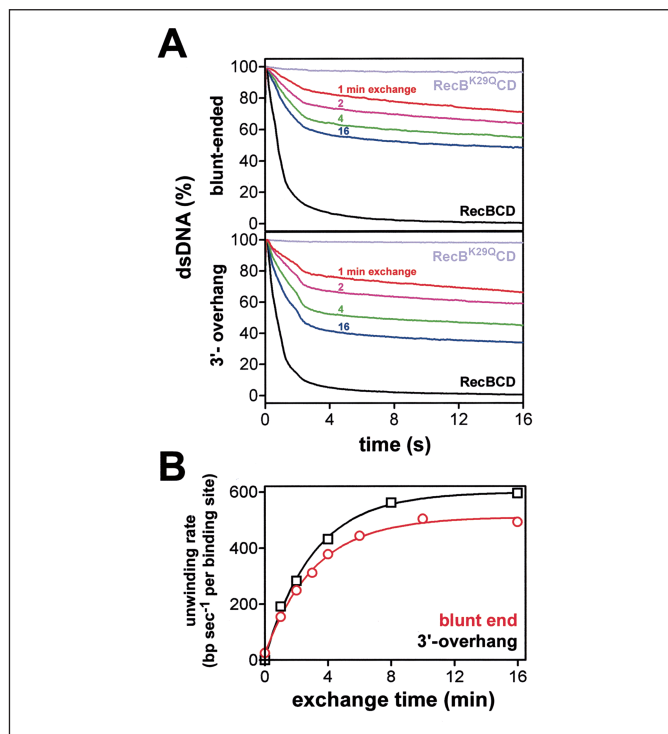
amount of DNA unwound by each enzyme, 1.2 kbp per RecB^{K29Q}CD enzyme and 4.9 kbp per RecBCD^{K177Q} enzyme, is essentially equal to the processivity value (TABLE ONE). These measurements are consistent with the plasmid DNA unwinding amplitudes and demonstrate that the measured unwinding amplitudes are not limited by factors such as strongly bound non-productive protein-DNA complexes, but that they reflect true processivity defects. Inclusion of a heparin trap reduces the unwinding amplitude by ~30% (Fig. 5, *top panel*); as explained above, we suggest that this fraction may mainly represent enzyme that can unwind DNA but must first undergo an isomerization to become resistant to heparin and also competent for DNA unwinding.

To confirm that the reduced processivity of the mutant proteins is not due to mutant-specific inhibition of DNA translocation by the DNA-binding dye, we performed similar experiments using an alternative assay based on the binding of SSB protein to the ssDNA products of the reaction (4). Although we observed moderately increased levels of unwinding for both mutant proteins, their processivities were still much lower than the wild-type enzyme (Fig. 5, *top panel*, *striped bars*).

All of the experiments described above have compared the activities of wild-type and mutant RecBCD enzymes under conditions in which there is a low millimolar free magnesium ion concentration, as is the case in *Escherichia coli* cells (40). The accompanying report (41) demonstrates that the behavior of the mutant proteins is differentially affected by the free magnesium concentration in a complex manner.
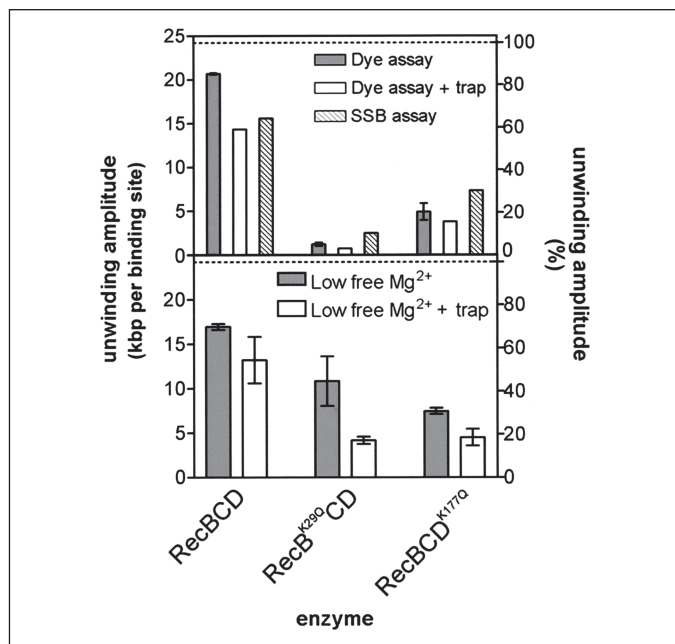
FIGURE 5. **RecBCD motor mutants display severely reduced processivity.** *A*, comparison of the unwinding amplitude of λ DNA (48.5 kbp) by wild-type and mutant RecBCD enzymes. Saturating enzyme was pre-bound to λ DNA (0.1 nm end concentration), and unwinding was initiated by the addition of ATP. Amplitude is quoted in kbp per binding site, and this is equivalent to the average number of base pairs unwound per active enzyme. Experiments were performed using the dye-displacement assay (*gray*), the dye-displacement assay with a heparin trap (*white*) or the SSB-binding assay (*gray stripes*). *B*, same as in *A* but experiments were performed under conditions of low free Mg^{2+} ion ($\sim$25 μM) either in the absence (*gray*) or presence (*white*) of a heparin trap. All *error bars* represent a standard deviation about a mean for three independent experiments. The *dotted lines* represent the substrate-limited maximum unwinding amplitude of 24.3 kbp per enzyme.

This is perhaps expected because the divalent cation is required as a co-factor for ATP hydrolysis by each motor subunit, for nuclease activity on both trailing single-strands, and may affect association/dissociation from the DNA track. Although all three proteins display optimal unwinding rates under conditions of high free magnesium (data not shown), the net plasmid DNA unwinding amplitude for RecB^{K29Q}CD protein is increased in low free Mg^{2+} ion conditions (41). For this reason, we investigated the amplitude of λ DNA unwinding under conditions of limited free Mg^{2+} ion ($\sim$25 μM). Remarkably, although the wild-type and RecBCD^{K177Q} enzymes display broadly similar levels of unwinding regardless of the free Mg^{2+} ion concentration (Fig. 5), RecB^{K29Q}CD enzyme is able to unwind about half of the λ DNA in the absence of a heparin trap (10.8 kbp compared with 1.2 kbp in high free Mg^{2+} conditions). However, this unwinding amplitude is reduced to an exceptional extent (62%) by the inclusion of a heparin trap, suggesting that, under these conditions, substantial amounts of unwinding may be occurring by re-initiation events. Alternatively, it could be possible that most of the RecB^{K29Q}CD enzyme is bound in non-productive complexes under these conditions. However, the observation that RecB^{K29Q}CD unwinds $\sim$80% of pBR322 DNA in the presence of a heparin trap (data not shown) argues that this is not the case, and that the processivity of RecB^{K29Q}CD enzyme is on the order of several thousand base pairs under limited free Mg^{2+} ion conditions.

DISCUSSION

In this work we have analyzed the DNA translocation and unwinding activities of wild-type and mutant RecBCD enzymes. Inactivation of either of the two ssDNA motor subunits encoded by the RecB and RecD subunits does not eliminate DNA translocation and unwinding. Instead, either motor is able to drive forward translocation of the enzyme, and

each can act as an autonomous DNA helicase within the context of the holoenzyme. However, our data show that the dual motor activity of the wild-type enzyme is required for the maximum rate and processivity of DNA translocation (TABLE ONE). The RecB^{K29Q}CD and RecBCD^{K177Q} enzymes, which function with a single DNA motor, track along DNA at a rate that is 3- or 2-fold slower than the wild-type enzyme. Importantly, the processivity of translocation is reduced by 25- and 6-fold, respectively, compared with the wild-type enzyme. The RecBCD^{K177Q} enzyme has been shown previously to produce partially unwound DNA intermediates of between 2 and 5 kbp in length (30), consistent with the reduced processivity observed here. Likewise, the RecB^{K29Q}CD enzyme is shown to produce partially unwound DNA intermediates from 3′-labeled plasmid DNA substrates in the accompanying report (41). Moreover, the RecB^{2109}CD enzyme (which contains a mutation in helicase motif VI of RecB) also displays a reduced processivity (42). Our study has employed mutants of RecBCD that eliminate active ssDNA translocation in each subunit, but which are not expected to eliminate ssDNA binding activity (43). It should be noted that the lysine to glutamine substitution used here appears to prevent ATP binding rather than ATP hydrolysis *per se* (32), and that this may influence ssDNA binding allosterically. Electron-microscopic analyses of the unwinding intermediates formed by these mutant proteins suggest that the inactive motor remains bound to the non-translocated strand close to the initiation site for unwinding (22). It may well be that mutations that eliminated ssDNA binding in the helicase subunits would have had more drastic effects on the rate and/or processivity of translocation.

The RecB^{K29Q}CD mutant only initiates unwinding on DNA ends that possess short 5′-overhangs, and RecBCD^{K177Q} shows a slight preference for DNA with 3′-overhangs. The results point to a model for the architecture of the initiation complex in which the RecB and RecD helicase subunits are located on the 3′- and 5′-terminated single strands, respectively, as suggested previously (44). Moreover, the critical requirement for a short 5′-overhang for initiation of unwinding by the RecD subunit suggests that the RecD helicase may be located "behind" the RecB subunit with respect to the translocation direction and the register of the base pairs (Fig. 6). This model is fully consistent with crystallographic data for a RecBCD-DNA initiation complex (45). Binding of a blunt-ended duplex DNA causes fraying of the final four complementary base pairs, such that they are bound within separate ssDNA tunnels in the enzyme complex, each associated with one of the two ssDNA motors. While the 3′-terminated strand reaches the motor domains of RecB, the 5′-terminated strand falls just short of the RecD helicase.

Rates of RecBCD-catalyzed DNA Unwinding: Which DNA Motor Is Faster?—Our results suggest that the RecB motor is faster than RecD under our experimental conditions. However, this assumes that the motors are independent of one another (i.e. that the activities of the wild-type subunits in the mutant complexes are representative of their activities in the wild-type complex). Given that the RecBCD^{K177Q} protein unwinds DNA at rates that approach those of the wild-type, it would be reasonable to suggest that the RecB motor is the fast motor in the holoenzyme under these conditions. This conclusion is in agreement with the observation (39) that RecBC enzyme unwinds DNA at rates that are similar to the holoenzyme. However, in apparent contradiction, elegant microscopic analysis of the unwinding intermediates of RecBCD-catalyzed DNA unwinding demonstrated that the RecD motor moves over 2-fold faster than RecB (22). This behavior results in the production of a "loop-2-tails" structure containing a long 5′-terminated ssDNA tail, and a loop possessing a shorter 3′-terminated tail associated with the RecB motor. However, these experiments were performed under conditions designed to reduce nuclease activity and to allow the
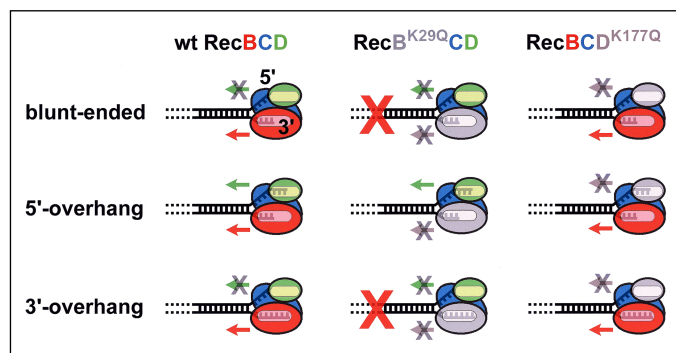
FIGURE 6. **Model to explain the behavior of RecBCD mutant enzymes that possess defective motor subunits.** The model illustrates the inability of RecB^{K29Q}CD to unwind blunt and 3′-overhang DNA substrates. The RecBCD subunits are *color coded,* and *gray* represents an inactive subunit that is not capable of catalyzing ssDNA translocation. The RecD helicase subunit is located "behind" the RecB helicase subunit with respect to the DNA translocation. Binding of wild-type (*wt*) RecBCD to blunt dsDNA ends (*top left complex*) results in the melting of 4 bp of duplex DNA (45). In this initiation complex, RecB protein contacts the 3′-ssDNA strand immediately adjacent to the ssDNA-dsDNA junction, but the 5′-strand does not reach the RecD subunit. In other words, the two helicase motors are not positioned directly opposite each other, but rather the RecD motor is "behind" RecB relative to the direction of translocation and the register of the base pairs. This would mean that wild-type (or RecBCD^{K177Q}) enzyme would be able to initiate unwinding regardless of the exact structure of the DNA end. In contrast, RecB^{K29Q}CD enzyme would only initiate unwinding on substrates with a 5′-overhang of sufficient length to reach the catalytic site for the RecD ssDNA translocation activity in the initial binding event. This model may also explain the slight preference of the RecBCD^{K177Q} enzyme for substrates with a 3′-ssDNA tail, as this may bind more fully to the motor domains of RecB as illustrated.

observation of intact DNA intermediates. This required raising the ATP concentration substantially above the total Mg^{2+} concentration (to reduce free Mg^{2+} and consequently the Mg^{2+}-dependent nuclease activity), as well as introducing Ca^{2+} ions to directly inhibit the nuclease activity. These conditions are substantially sub-optimal for the RecBCD^{K177Q} enzyme (41, and data not shown), and so the difference in results probably reflects the differential effects of solution conditions on the activities of the two motor components.

Implications for General Models of SF1 Helicase Activity— There has been considerable discussion in the literature as to the functional oligomeric state of helicases and how this relates to mechanism. Helicases are a disparate group of enzymes encompassing three superfamilies and two smaller families (23) that function in a variety of DNA-processing events and, as such, they are not necessarily expected to have identical protein architectures and/or biochemical mechanisms. However, within the SF1 class of enzymes, even highly related enzymes such as *Bacillus stearothermophilus* PcrA, *E. coli* Rep and UvrD, and phage T4 Dda have been proposed to function by fundamentally different mechanisms. PcrA and Dda can act as monomers and are proposed to function using an "inchworm"-type mechanism (27, 46). However, dimers or cooperatively acting monomers of RepD, UvrD, and Dda are required to observe maximal DNA helicase activity *in vitro* (17, 47, 48). In distinct contrast, RecBCD is active as a heterotrimer containing a single copy of each of the three constituent polypeptides (5), two of which (RecB and RecD) contain SF1 helicase motifs and function independently as DNA helicases. Before this had been appreciated, the enzyme was thought to be driven solely by the RecB motor, and it had been proposed that the RecBCD system provided evidence against a general requirement for an oligomeric arrangement of identical helicase subunits (5). The recent observation, by ourselves and others (21, 22), that RecBCD contains two helicase subunits invalidates this conclusion. However, the bipolar translocation model that was proposed to explain the presence of a second DNA motor subunit envisions the two activities as being autonomous. In other words, both the RecB and RecD subunits are independent, fully viable "monomeric" DNA motors that drive DNA unwinding by translocating ssDNA in an ATP-dependent fashion. This view is borne out by the observation that RecBCD remains a potent helicase if either, but not both, of the two motors are inactivated by mutagenesis. In fact, the mutant enzymes are at least an order of magnitude more processive and faster than PcrA, Rep, or UvrD. Therefore, these studies clearly indicate that the essential functional unit required for DNA helicase activity catalyzed by a SF1 enzyme can be encoded by a single set of SF1 helicase motifs. —It is also of interest that the observed unwinding rates and processivities of the RecB and RecD helicases are very much poorer when in isolation, compared with when they are found in the context of the holoenzyme (21, 24, 49).[4] This suggests that the architecture of the complete heterotrimeric molecular machine is vital to harness the ssDNA motor activity of each subunit and translate it into effective DNA unwinding. This concept is borne out by the crystal structure of RecBCD, which shows how each motor is presented to the ssDNA strands as they pass through tunnels in the complex (45). It is probably significant that the DNA helicase activities of many purified SF1 DNA helicases, including Rep, UvrD, and PcrA, are strongly stimulated by interactions with protein partners (50–52).

Why Does RecBCD Enzyme Employ a Bipolar DNA Translocation Mechanism?—In this report, we provide direct experimental evidence that bipolar DNA translocation is an important determinant of highly processive DNA unwinding. The RecC subunit also plays a role, because the RecBC enzyme is a fast and processive helicase, whereas RecB is poorly processive (49). The crystal structure of RecBCD suggests an obvious structural basis for this stimulatory effect of RecC; the RecC subunit forms tunnels for each of the unwound ssDNA strands as they pass through the RecBCD complex (45). However, the processivity of RecBC has previously been shown to be only a few thousand base pairs (53), similar to that of the RecBCD^{K177Q} mutant as might be expected (30, 53; and this work) but about an order of magnitude reduced relative to the wild-type (20, 30, 53; and this work).

These observations raise the question of why RecBCD enzyme needs to be so processive. The answer probably relates to the need to reach the regulatory Chi sequences that transform the enzyme from a destructive helicase/nuclease into a recombinogenic multifunctional enzyme. Although Chi sequences are substantially over-represented in the *E. coli* genome (6, 7), they are nevertheless about 5000 bp apart, on average. Consequently, the RecBCD enzyme must be capable of highly processive translocation to reach a Chi sequence. To have a probability of 0.9 or better for reaching a Chi sequence that is 5000 bp away, RecBCD must possess a processivity in excess of ~50 kbp per binding event (20).[5] Based on our experimental data, the RecB^{K29Q}CD and RecBCD^{K177Q} enzymes would have a probability of only ~1 and 40%, respectively, of reaching a Chi sequence. Furthermore, our experiments were performed on naked DNA, whereas *in vivo* the translocating RecBCD enzyme may expect to encounter a variety of protein roadblocks and topological obstacles. Moreover, *in vitro* Chi sequence recognition is only ~30–40% efficient (54, 55). Therefore, the problems for a poorly processive enzyme may be even more acute than is suggested here. Interestingly, AddAB enzyme, the functional analogue of RecBCD enzyme in *Bacillus subtilis* (56), apparently contains a single translocation motor. It will be of interest to see if this enzyme is less processive than RecBCD, perhaps more comparable to RecBC enzyme, and whether this lower processivity correlates with the shorter (and more frequent) 5-bp Chi sequence that AddAB enzyme recognizes. Other

---

[4] M. S. Dillingham and S. C. Kowalczykowski, unpublished observations.
[5] M. S. Dillingham, M. R. Webb, and S. C. Kowalczykowski, unpublished calculations.

possible functions for the bipolar translocation organization may include the ability to bypass gaps or damage in either DNA strand or it may be a determinant of the blunt-end binding specificity. We have also suggested that the nuclease polarity switch at Chi results from a switch in motor subunit usage. The accompanying report (41) demonstrates that this is not the case as, surprisingly, the RecBCD^{K177Q} mutant recognizes and responds to Chi, by switching its nuclease activity in the same manner as the wild-type enzyme. It is also demonstrated that RecBCD^{K177Q} enzyme restores the UV resistance of recBCDΔ cells, showing that, under the conditions used, the motor activity of RecD is dispensable for repair of UV-induced damage *in vivo*.

Nevertheless, the evolutionary conservation of RecD helicase function highlights the importance of bipolar translocation for some aspects of *in vivo* function, because the use of two DNA motors must represent a considerable energetic cost. RecBCD has been shown to hydrolyze ~2 ATP molecules per base pair unwound under optimal conditions, whereas the RecBC and RecBCD^{K177Q} enzymes hydrolyze a little more than 1 ATP per base pair (30, 57, 58). These results suggest the possibility that each SF1 motor subunit hydrolyzes 1 ATP per base translocated, as has been suggested for the PcrA helicase (26, 27). This supposition is fully consistent with the structural organization of RecBCD enzyme (45), which seems to be an excellent example of a molecular machine, whose overall function relies on the interplay of several autonomous biochemical activities encoded by protein modules.

REFERENCES

1. Spies, M., and Kowalczykowski, S. C. (2005) in *The Bacterial Chromosome* (Higgins, N. P., ed) pp. 389–403, ASM Press, Washington, D. C.
2. Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J., and Marians, K. J. (2000) *Nature* **404,** 37–41
3. Kowalczykowski, S. C. (2000) *Trends Biochem. Sci.* **25,** 156–165
4. Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2863–2873
5. Taylor, A. F., and Smith, G. R. (1995) *J. Biol. Chem.* **270,** 24451–24458
6. Burland, V., Plunkett, G., 3rd, Daniels, D., and Blattner, F. R. (1993) *Genomics* **16,** 551–561
7. Cardon, L. R., Burge, C., Schachtel, G. A., Blaisdell, B. E., and Karlin, S. (1993) *Nucleic Acids Res.* **21,** 3875–3884
8. Dixon, D. A., and Kowalczykowski, S. C. (1991) *Cell* **66,** 361–371
9. Dixon, D. A., and Kowalczykowski, S. C. (1993) *Cell* **73,** 87–96
10. Anderson, D. G., and Kowalczykowski, S. C. (1997) *Genes Dev.* **11,** 571–581
11. Anderson, D. G., and Kowalczykowski, S. C. (1997) *Cell* **90,** 77–86
12. Benzinger, R., Enquist, L. W., and Skalka, A. (1975) *J. Virol.* **15,** 861–871
13. Lohman, T. M., and Bjornson, K. P. (1996) *Annu. Rev. Biochem.* **65,** 169–214
14. Eggleston, A. K., Rahim, N. A., and Kowalczykowski, S. C. (1996) *Nucleic Acids Res.* **24,** 1179–1186
15. Bianco, P. R., Brewer, L. R., Corzett, M., Balhorn, R., Yeh, Y., Kowalczykowski, S. C., and Baskin, R. J. (2001) *Nature* **409,** 374–378
16. Ali, J. A., and Lohman, T. M. (1997) *Science* **275,** 377–380
17. Cheng, W., Hsieh, J., Brendza, K. M., and Lohman, T. M. (2001) *J. Mol. Biol.* **310,** 327–350
18. Dessinges, M. N. Lionnet, T., Xi, X. G., Bensimon, D., and Croquette, V. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 6439–6444
19. Spies, M. Bianco, P. R., Dillingham, M. S., Handa, N., Baskin, R. J., and Kowalczykowski, S. C. (2003) *Cell* **114,** 647–654
20. Roman, L. J., Eggleston, A. K., and Kowalczykowski, S. C. (1992) *J. Biol. Chem.* **267,** 4207–4214
21. Dillingham, M. S., Spies, M., and Kowalczykowski, S. C. (2003) *Nature* **423,** 893–897
22. Taylor, A. F., and Smith, G. R. (2003) *Nature* **423,** 889–893
23. Gorbalenya, A. E., and Koonin, E. V. (1993) *Curr. Opin. Struct. Biol.* **3,** 419–429
24. Phillips, R. J., Hickleton, D. C., Boehmer, P. E., and Emmerson, P. T. (1997) *Mol. Gen. Genet.* **254,** 319–329
25. Morris, P. D., and Raney, K. D. (1999) *Biochemistry* **38,** 5164–5171
26. Dillingham, M. S., Wigley, D. B., and Webb, M. R. (2000)) *Biochemistry* **39,** 205–212
27. Velankar, S. S., Soultanas, P., Dillingham, M. S., Subramanya, H. S., and Wigley, D. B. (1999) *Cell* **97,** 75–84
28. Eggleston, A. K., O'Neill, T. E., Bradbury, E. M., and Kowalczykowski, S. C. (1995) *J. Biol. Chem.* **270,** 2024–2031
29. Chen, H. W., Randle, D. E., Gabbidon, M., and Julin, D. A. (1998) *J. Mol. Biol.* **278,** 89–104
30. Korangy, F., and Julin, D. A. (1992) *J. Biol. Chem.* **267,** 3088–3095
31. Bianco, P. R., and Kowalczykowski, S. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6706–6711
32. Korangy, F., and Julin, D. A. (1992) *J. Biol. Chem.* **267,** 1727–1732
33. Hsieh, S., and Julin, D. A. (1992) *Nucleic Acids Res.* **20,** 5647–5653
34. LeBowitz, J. (1985) *Biochemical Mechanism of Strand Initiation in Bacteriophage Lambda DNA Replication.* Ph.D. thesis, Johns Hopkins University, Baltimore, MD
35. Patton, C., Thompson, S., and Epel, D. (2004) *Cell Calcium* **35,** 427–431
36. Lucius, A. L., and Lohman, T. M. (2004) *J. Mol. Biol.* **339,** 751–771
37. Anderson, D. G., and Kowalczykowski, S. C. (1998) *J. Mol. Biol.* **282,** 275–285
38. Taylor, A. F., and Smith, G. R. (1985) *J. Mol. Biol.* **185,** 431–443
39. Lucius, A. L., Jason Wong, C., and Lohman, T. M. (2004) *J. Mol. Biol.* **339,** 731–750
40. Alatossava, T., Jutte, H., Kuhn, A., and Kellenberger, E. (1985) *J. Bacteriol.* **162,** 413–419
41. Spies, M., Dillingham, M. S., and Kowalczykowski, S. C. (2005) *J. Biol. Chem.* **280,** 37078–37087
42. Eggleston, A. K., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **231,** 621–633
43. Soultanas, P., Dillingham, M. S., Velankar, S. S., and Wigley, D. B. (1999) *J. Mol. Biol.* **290,** 137–148
44. Ganesan, S., and Smith, G. R. (1993) *J. Mol. Biol.* **229,** 67–78
45. Singleton, M. R., Dillingham, M. S., Gaudier, M., Kowalczykowski, S. C., and Wigley, D. B. (2004) *Nature,* **432,** 187–193
46. Nanduri, B., Byrd, A. K., Eoff, R. L., Tackett, A. J., and Raney, K. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 14722–14727
47. Maluf, N. K., Fischer, C. J., and Lohman, T. M. (2003) *J. Mol. Biol.* **325,** 913–935
48. Byrd, A. K., and Raney, K. D. (2004) *Nat. Struct. Mol. Biol.* **11,** 531–538
49. Yu, M., Souaya, J., and Julin, D. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 981–986
50. Arai, N., and Kornberg, A. (1981) *J. Biol. Chem.* **256,** 5294–5298
51. Mechanic, L. E., Frankel, B. A., and Matson, S. W. (2000) *J. Biol. Chem.* **275,** 38337–38346
52. Soultanas, P., Dillingham, M. S., Papadopoulos, F., Phillips, S. E., Thomas, C. D., and Wigley, D. B. (1999) *Nucleic Acids Res.* **27,** 1421–1428
53. Korangy, F., and Julin, D. A. (1993) *Biochemistry* **32,** 4873–4880
54. Taylor, A. F., and Smith, G. R. (1985) *Cell* **41,** 153–163
55. Dixon, D. A., and Kowalczykowski, S. C. (1995) *J. Biol. Chem.* **270,** 16360–16370
56. Chedin, F., and Kowalczykowski, S. C. (2002) *Mol. Microbiol.* **43,** 823–834
57. Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2873–2881
58. Korangy, F., and Julin, D. A. (1994) *Biochemistry* **33,** 9552–9560