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RECONSTITUTION OF HOMOLOGOUS PAIRING ACTIVITY DEPENDENT UPON THE COMBINED ACTIVITIES OF PURIFIED *E. COLI* RECA, RECBCD, AND SSB PROTEINS[1]

Stephen C. Kowalczykowski and Linda J. Roman

Department of Molecular Biology
Northwestern University Medical School
Chicago, Illinois 60611

ABSTRACT The recA and recBCD proteins are essential components of the recBCD-pathway of homologous recombination in *E. coli.* Using two different *in vitro* reactions, we can demonstrate homology-dependent DNA heteroduplex formation requiring the concerted action of recA protein, recBCD enzyme, and (depending on the DNA substrates used) SSB protein. DNA heteroduplex formation proceeds in three kinetically distinct steps. The first step is unwinding of the linear dsDNA by recBCD enzyme. The second step requires trapping, by either recA or SSB proteins, of the ssDNA produced. The third step involves recA protein-dependent homologous pairing of the DNA intermediates: for reactions involving circular ssDNA and linear dsDNA, renaturation activity is required; for linear dsDNA and supercoiled dsDNA, joint molecule formation is required. These *in vitro* results suggest that the helicase activity of recBCD enzyme can initiate homologous pairing and are consistent with *in vivo* data which indicate that recBCD enzyme may act early (as well as late) in genetic recombination. Development of a novel assay to characterize the helicase activity of recBCD enzyme facilitated mechanistic analysis of the recA- and recBCD-dependent homologous pairing reaction. Under optimal conditions, recBCD enzyme can unwind dsDNA as fast as 930 bp/sec/functional recBCD enzyme; the K_m for dsDNA ends is approximately 1 nM; and 2-3 ATP molecules are hydrolyzed per bp unwound. The processivity of unwinding was also determined. An average of 25,000 bp is unwound before dissociation of the active recBCD enzyme; the relevance of this observation to activation at χ sites will be discussed.

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INTRODUCTION

Homologous recombination in wild-type *E. coli* proceeds primarily *via* the recBCD pathway (1,2). Genetic studies have established the need for recA protein, recBCD enzyme, SSB protein, DNA gyrase, DNA ligase, and DNA polymerase I in this pathway (3,4,5,6,7). These individual proteins have been studied extensively and defensible ideas exist for the role they might play in the *in vivo* process. However plausible any of these hypotheses might be, experimental demonstration is essential. Here we describe two *in vitro* reaction systems that demonstrate the formation of heteroduplex DNA which is dependent on recA, recBCD, and (in one case) SSB proteins. Through these biochemical studies, we also determine a mechanistic function for each protein in these reactions. Our results establish experimentally that the helicase activity of recBCD enzyme can produce ssDNA which can be both trapped effectively and used productively by recA protein as a substrate in homologous pairing reactions.

In vitro, the recA protein possesses numerous enzymatic activities (8,9,10). It has DNA-dependent ATPase activity, ATP-stimulated DNA renaturation activity, ATP-dependent DNA strand exchange activity, and ATP- and DNA-dependent repressor cleavage activity. A common requirement in all of these reactions is ssDNA. In particular, recA protein-catalyzed DNA strand exchange cannot occur unless one of the DNA substrates contains single-stranded DNA which is homologous to a region of the other dsDNA substrate. Of all the reactions promoted by recA protein, the DNA renaturation and strand exchange activities are of special relevance to the genetic recombination process since heteroduplex DNA is formed in these reactions.

The recBCD enzyme is also multifunctional, possessing a number of enzymatic activities: ATP-dependent dsDNA exonuclease, ATP-dependent ssDNA exonuclease, ATP-dependent ssDNA endonuclease, DNA-dependent ATPase, and DNA helicase activities (1,11). From genetic studies, it is inferred that the recBCD enzyme can act in both early (12,13) (*i.e.*, initiation) and late (14) (*i.e.*, resolution) steps of the recombination mechanism. The specific role of each biochemical activity in the recombination process is not yet clear; however, it is reasonable to expect that the nuclease activities are used to produce either the ssDNA or the dsDNA breaks necessary to initiate exchange events or to resolve Holliday junctions. The DNA helicase activity might be involved in the production of ssDNA necessary for recA protein function or in the extension of regions of DNA heteroduplex (*i.e.*, branch migration).

A specific model incorporating the known biochemical and genetic properties of the recA and recBCD proteins into a mechanism for the initiation of homologous recombination has been advanced (15). In brief, the model proposes that recBCD enzyme uses its helicase activity to travel through dsDNA, producing characteristic "twin-loop" structures. Upon encountering a χ site, recBCD enzyme nicks the ssDNA just 3' to the χ site, producing a ssDNA tail which is now a suitable substrate for recA protein action. RecA protein binds to this ssDNA tail and promotes the invasion of a homologous dsDNA region leading to the exchange of DNA strands.

We have designed biochemical experiments to test an important premise of the model proposed by Smith and co-workers; namely, that the helicase activity of recBCD enzyme can create ssDNA that, through recA protein's homologous pairing

activity, can be used to produce heteroduplex DNA. The approach is to use a pair of DNA substrates that are not suitable substrates for recA protein alone, but one of which is a substrate for recBCD helicase activity. Thus, by virtue of the need for DNA unwinding by recBCD enzyme, DNA heteroduplex formation requires the coordinate action of recA and recBCD proteins. To completely understand the molecular events that underlie recA- and recBCD-dependent (hereafter abbreviated recABCD-dependent) DNA heteroduplex formation, it was necessary to more fully understand the enzymatic properties of recBCD helicase activity. To that end, a novel helicase assay was developed. Below, we describe results from the following studies:

1) DNA heteroduplex formation between ssDNA and dsDNA that is dependent on recBCD enzyme helicase and recA protein renaturation activities (16).
2) Joint molecule formation between two different dsDNA molecules that is dependent on recBCD enzyme helicase and recA protein strand invasion activities (17).
3) Enzymatic characterization of the helicase activity of recBCD enzyme using a fluorescent DNA helicase assay (18).
4) The relationship of ATP hydrolysis to the mechanism of recBCD enzyme helicase activity (19).
5) Characterization of the processivity of recBCD helicase activity and its possible relationship to χ sites (20).

RESULTS

DNA Heteroduplex Formation Dependent on RecA and RecBCD Proteins.

The pair of substrates shown in Figure 1 (top) were used to examine whether heteroduplex DNA could be formed in a reaction that required both recA protein and recBCD enzyme activities (16). Although these DNA substrates are routinely used to study recA protein-dependent DNA strand exchange, under appropriate experimental conditions (*e.g.*, low Mg^{+2} concentrations and/or 25° C), catalysis by recA protein (alone) is very poor. Since the linear dsDNA molecule is a substrate for recBCD enzyme unwinding activity, it was anticipated that DNA heteroduplex formation would occur as a consequence of DNA unwinding by recBCD enzyme, followed by recA protein-catalyzed renaturation of the resultant ssDNA. Although these DNA substrates are somewhat simplistic, the reaction pathway will illuminate an unexpected rate-limiting step in enzymatic DNA heteroduplex formation by recA and recBCD proteins.

Because the ssDNA is uniformly ^{3}H-labeled, DNA heteroduplex formation would result in the formation of S1 nuclease-resistant labeled dsDNA. A reaction time course is shown in Figure 2. Formation of heteroduplex DNA is dependent on the presence of both recA and recBCD proteins, DNA sequence homology, and a dsDNA molecule which is a substrate for recBCD enzymatic unwinding. Maximum heteroduplex DNA formation (35%) occurs after approximately 60 minutes. The slow rate of formation was surprising since, under these conditions, unwinding of the dsDNA by recBCD enzyme occurs in approximately 2 minutes (see below; (18)); this

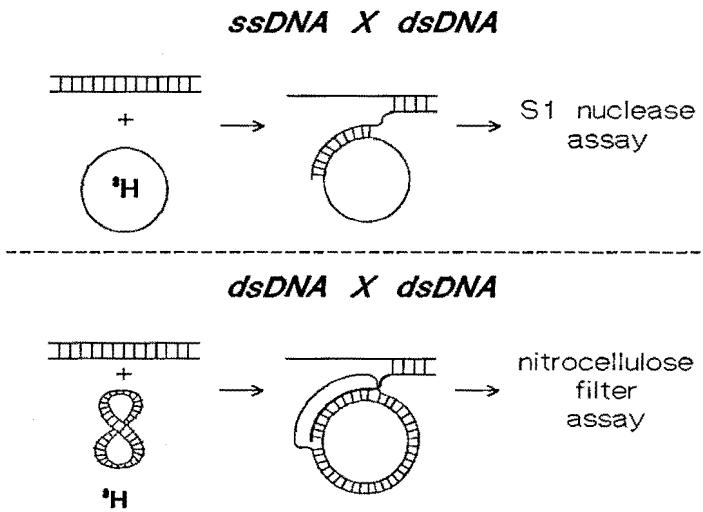

FIGURE 1. Subtrates and assays used for the ssDNA X dsDNA reaction (top) and for the dsDNA X dsDNA reaction (bottom).

implies that the rate-limiting step is not enzymatic unwinding of DNA. Consistent with this conclusion, changing the recBCD enzyme concentration (which is subsaturating relative to the concentration of DNA ends) by 2-fold in either direction affected the observed rate of DNA heteroduplex formation by less than 10%.

Since most recA protein enzymatic properties are dependent on the ratio of protein to ssDNA, an effect of recA protein concentration was expected. Surprisingly, the optimal concentration of recA protein in the recABCD-dependent reaction was independent of DNA concentration (see Figure 3; at 25°C, the optimum is ≈ 3 μM). This suggests that an intrinsic property of recA protein alone (rather than of the recA protein-ssDNA complex) is responsible for the rate-limiting step in this reaction.

Insight into the nature of the rate-limiting step comes from a comparison of the rate of recA protein-catalyzed renaturation to that of recABCD-dependent heteroduplex DNA formation (Figure 3). RecA protein-catalyzed DNA renaturation is approximately 8-9 fold faster than recABCD-dependent heteroduplex DNA formation. Thus, the ssDNA produced by recBCD enzyme is utilized 8-9 fold more poorly by recA protein than identical ssDNA produced by heat denaturation. This implies that recA protein is unable to efficiently trap the ssDNA tails produced by recBCD enzyme unwinding before they reannealed, thereby requiring repeated unwinding by recBCD enzyme.

To verify that recABCD-dependent heteroduplex DNA formation required recA protein renaturation activity, the effect of SSB protein was examined. SSB protein generally stimulates joint molecule formation by recA protein but inhibits DNA renaturation (22). Consistent with our expectation, concentrations of SSB protein which exceed approximately 20% of the amount required for saturation of the total amount of ssDNA produced, completely inhibit recABCD-dependent heteroduplex

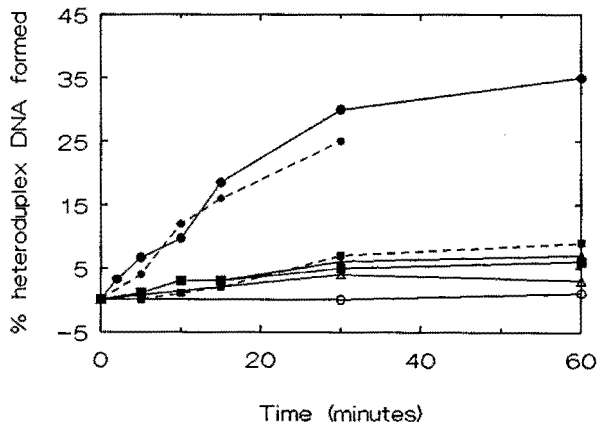

FIGURE 2. The formation of heteroduplex DNA by the combined actions of recA and recBCD proteins. Circles: standard reaction containing 10 μM linear dsDNA, 5 μM circular ssDNA, 5 μM recA protein and 13.9 nM recBCD enzyme; triangles: standard reaction without recA protein; squares: standard reaction without recBCD enzyme; open circles: standard reaction with circular dsDNA (M13 RF) instead of linear dsDNA; open triangles: standard reaction with nonhomologous linear dsDNA instead of linear M13 dsDNA. The solid and dashed lines represent data at 25° C and 37° C, respectively.

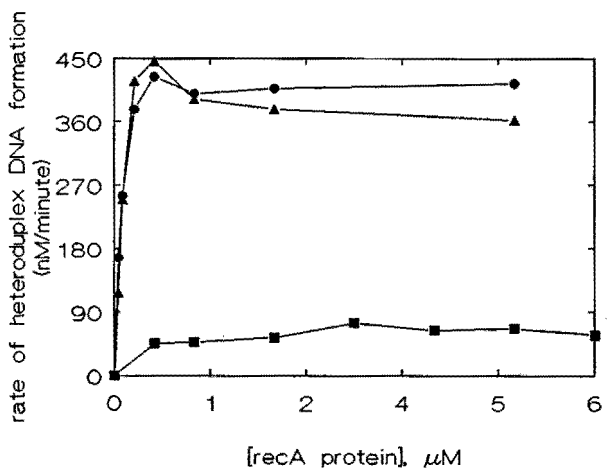

FIGURE 3. RecA protein concentration dependence of renaturation and heteroduplex DNA formation at 25° C. Circles: renaturation of heat denatured DNA in the buffer described previously (21) (25 mM Tris HCl, pH 7.2, 10 mM magnesium chloride, 200 μM ATP, 5% glycerol, and an ATP regenerating system); triangles: renaturation in standard buffer (25 mM Tris acetate, pH 7.5, 1 mM each of dithiothreitol, ATP, magnesium acetate, 2 mM spermidine acetate and an ATP regenerating system); squares: recABCD-dependent heteroduplex DNA formation in standard reaction buffer. Reactions contained 10 μM dsDNA, 5 μM tritium-labeled ssDNA, and 13.9 nM recBCD enzyme, when present.

DNA formation. Inhibition of recA protein-catalyzed DNA renaturation occurs at a similar SSB protein:DNA ratio, confirming that recABCD-dependent heteroduplex DNA formation proceeds *via* recA protein-catalyzed DNA renaturation.

To determine whether the behavior of recA protein in this *in vitro* reaction paralleled its behavior *in vivo*, the ability of several mutant recA proteins to form recABCD-dependent heteroduplex DNA was examined. In all cases, there is a correspondence between *in vivo* phenotype, *in vitro* recA protein-dependent DNA renaturation, and *in vitro* recABCD-dependent DNA heteroduplex formation (Table 1).

TABLE 1
BEHAVIOR OF MUTANT RECA PROTEINS[a]

		% of wild-type protein rate			
RecA Protein	Phenotype	Renaturation	ssDNA X dsDNA	Strand Exchange	dsDNA X dsDNA
wt	+	100	100	100	100
441	+	70	133	100	65
430	+/-	41	55	45	30
142	-	<2	<5	<2	<5
1	-	<2	<5	<2	<5

[a]The column headings represent: "recA protein", the allele designation; "phenotype", recombination phenotype; "renaturation", recA protein-dependent DNA renaturation activity; "ssDNA X dsDNA", recABCD-dependent DNA heteroduplex formation; "strand exchange", recA protein-dependent DNA strand exchange activity; "dsDNA X dsDNA", recABCD-dependent joint molecule formation. See references (16,17) for experimental conditions and citations to mutant recA protein properties.

A model for recABCD-dependent heteroduplex DNA formation that incorporates these results is shown in Figure 4. The three essential elements of the model are: 1) recBCD enzyme unwinds the linear dsDNA to produce a ssDNA substrate for recA protein function (note that, although depicted as full length, the dsDNA is nicked approximately once every 800 bp (23)); 2) recA protein traps the unwound ssDNA tails before they reanneal; and 3) heteroduplex DNA is produced as a consequence of the DNA renaturation activity of recA protein. An unexpected outcome of the experimental work is that step 2 is the rate-limiting step; *i.e.*, the trapping of the unwound ssDNA tails by recA protein is relatively inefficient. The data suggest that recBCD enzyme must unwind a dsDNA molecule 8-9 times, on average, before heteroduplex formation occurs. We estimate, that about half of the time, the unwound ssDNA tails simply reanneal by themselves while the remainder reanneal due to recA protein-dependent renaturation. Thus, coordination of recBCD enzyme unwinding and recA protein renaturation activities is relatively poor for this reaction.

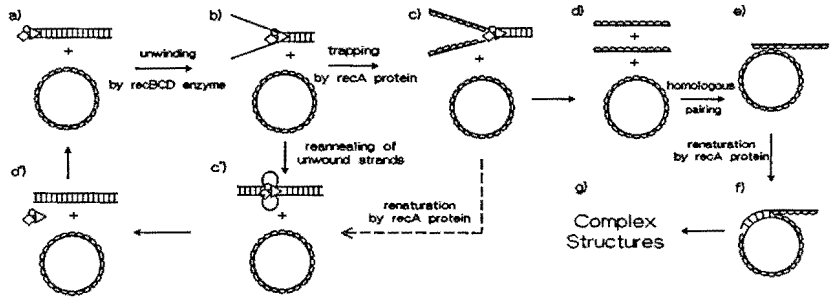

FIGURE 4. Model for heteroduplex DNA formation catalyzed by recBCD and recA proteins. Ovals: recA protein; diamond/triangle/circle: recBCD enzyme.

Joint Molecule Formation Dependent on RecA, RecBCD, and SSB Proteins.

The second pair of DNA substrates used is shown at the bottom of Figure 1 (17). These substrates differ from the previously discussed pair in that replicative form (RF) dsDNA is substituted for the homologous circular ssDNA. Because there is no region of homologous ssDNA in either of these DNA substrates, recA protein alone is unable to promote homologous pairing (24). However, the linear dsDNA (but not the RF dsDNA) can be unwound by recBCD helicase activity to produce ssDNA. Under suitable conditions, recA protein promotes invasion of the RF dsDNA by homologous ssDNA to form a joint molecule. Since the RF dsDNA is ^{3}H-labeled, joint molecule formation can be detected using a nitrocellulose filter assay.

Figure 5 shows a time course for joint molecule formation using the substrates illustrated in Figure 1. Joint molecule formation occurs only when both recA protein and recBCD enzyme are present and requires DNA sequence homology and linear dsDNA. Formation of joint molecules is also evident when the reaction products are run on agarose gels, confirming the nitrocellulose filter assay. Joint molecules form rapidly, peaking at 1-2 minutes, and then decline (as observed in recA protein-dependent reactions due to processive unwinding of the RF dsDNA (25)). The initial rate of recABCD-dependent joint molecule formation is faster than the rate observed with recA protein alone when comparable DNA substrates (homologous ssDNA and RF dsDNA) are used. Thus, in contrast to the ssDNA X dsDNA reaction described above, ssDNA produced by recBCD enzyme in the dsDNA X dsDNA reaction is utilized very effectively by recA protein. We presume this enhancement, which will be discussed below, is due primarily to the presence of SSB protein.

Formation of recABCD-dependent joint molecules displays saturation behavior with regard to recA and recBCD proteins (not shown; (17)). Since linear dsDNA is unwound to produce ssDNA in the recABCD-dependent reaction, the stoichiometry of recA protein utilization should be defined relative to the linear dsDNA concentration and should agree with that obtained for recA protein-dependent joint molecule formation. The amount of recA protein necessary for saturation is determined by the linear dsDNA concentration and not by the RF dsDNA concentration (17).

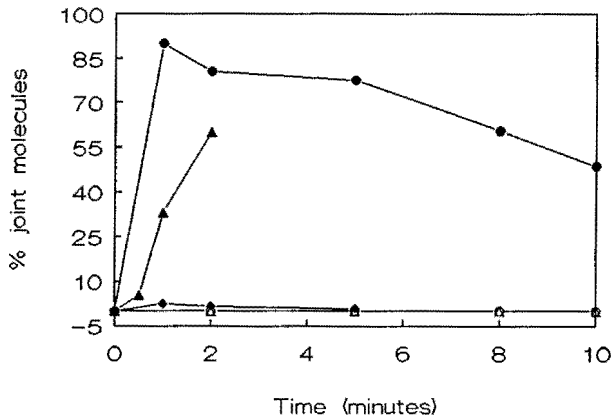

FIGURE 5. The formation of joint molecules by the combined actions of recA, recBCD, and SSB proteins. Closed circles: standard reaction containing 10 μM linear dsDNA and 5 μM circular dsDNA; open circles: standard reaction without recA protein; open triangles: standard reaction without recBCD enzyme; closed diamonds: standard reaction with nonhomologous linear pBEU41 dsDNA rather than linear M13 dsDNA; closed triangles: reaction in the absence of recBCD enzyme using heat-denatured linear dsDNA. Reactions were at 37° C and contained 1 μM SSB protein and, where included, 5 nM recBCD enzyme and 5 μM recA protein.

Saturation of joint molecule formation in this recABCD-dependent reaction occurs at approximately 1 recA protein monomer per 3-4.5 nucleotides of linear dsDNA; optimal recA protein-dependent DNA strand exchange occurs at an identical ratio (26). Saturation by recBCD enzyme is also defined with respect to the linear dsDNA concentration; approximately 2 functional recBCD molecules are required for each linear dsDNA molecule (*i.e.*, one recBCD molecule per dsDNA end). Thus, recABCD-dependent joint molecule formation is maximal when the rate of enzymatic unwinding by recBCD enzyme is also maximal.

SSB protein stimulates the rate of recA protein-dependent joint molecule formation up to an optimum concentration but, at higher SSB protein concentrations, a reduction is observed (27,26). The effect of SSB protein on recABCD-dependent joint molecule formation is nearly identical, with an optimum at approximately 1 SSB protein monomer per 10 nucleotide residues. Confirmation that the joint molecule forming activity of recA protein is required also comes from the effects of NaCl and magnesium acetate on both recA protein-dependent and recABCD-dependent joint molecule formation; for both reactions, parallel effects of these salts are observed.

The DNA substrates used here are believed to be representative of the DNA molecules involved in the recBCD-pathway of homologous recombination *in vivo*. If so, then the behavior of mutant recA proteins in this *in vitro* recABCD-dependent reaction should correlate with their *in vivo* phenotypes. Table 1 shows this to be the case. Thus, the molecular events occurring in this recABCD-dependent reaction are likely to be representative of those occurring *in vivo*.

The molecular steps occurring during recABCD-dependent joint molecule formation in the dsDNA X dsDNA reaction are depicted in Figure 6. These steps are

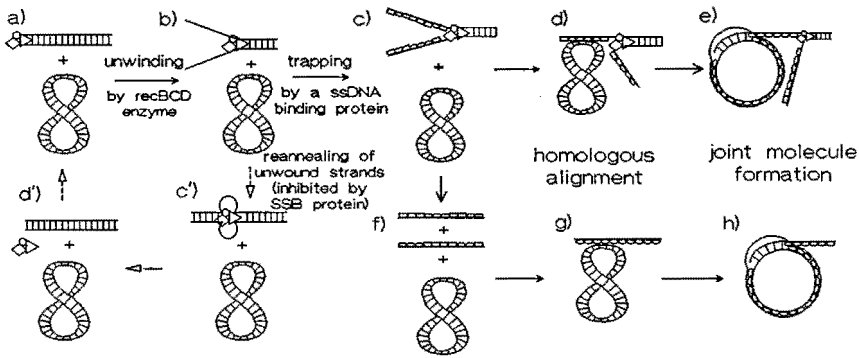

FIGURE 6. Model for joint molecule formation catalyzed by recBCD and recA proteins. Ovals: recA protein; squares: SSB protein; diamond/triangle/circle: recBCD enzyme.

similar to those shown in Figure 4, but both the rate-limiting step and the nature of the recA protein-catalyzed step differ. The sequence is: 1) the linear dsDNA is unwound by recBCD enzyme (as mentioned before, the linear dsDNA is nicked approximately every 800 bp (23)); 2) the unwound ssDNA is trapped by both recA and SSB proteins; and 3) recA protein catalyzes joint molecule formation between the ssDNA and the RF dsDNA. In contrast to the ssDNA X dsDNA reaction, trapping of the ssDNA produced by recBCD enzyme is no longer rate-limiting. This results from the presence of SSB protein in these reactions; as shown below, SSB protein is very effective at trapping the ssDNA produced by recBCD enzyme. This ssDNA is also complexed with recA protein, resulting in mixed recA protein-SSB protein-ssDNA complexes. Since recA protein can displace SSB protein from ssDNA under these conditions (28,29), some net displacement of SSB protein occurs. Thus, the coupling of dsDNA unwinding by recBCD enzyme to joint molecule formation by recA protein is made very efficient by the highly effective ssDNA trapping ability of SSB protein.

Characterization of the DNA Unwinding Activity of RecBCD Enzyme using a Novel DNA Helicase Assay.

Complete mechanistic understanding of the recABCD-dependent pairing reactions requires full knowledge of the enzymatic characteristics of the recBCD helicase activity. Previously, this property of recBCD enzyme was studied using only electron microscopy (30,31). Unfortunately, electron microscopy is not suited for routine enzymatic characterization. Consequently, we developed a helicase assay that is rapid, continuous, and quantitative. This assay is based on the fact that the intrinsic fluorescence of SSB protein is quenched upon binding to ssDNA (32). Thus, when dsDNA is unwound by recBCD enzyme in the presence of SSB protein, the observed protein fluorescence will decrease in a time-dependent manner due to binding of SSB protein to the ssDNA produced (18). The fluorescence decrease is proportional to the

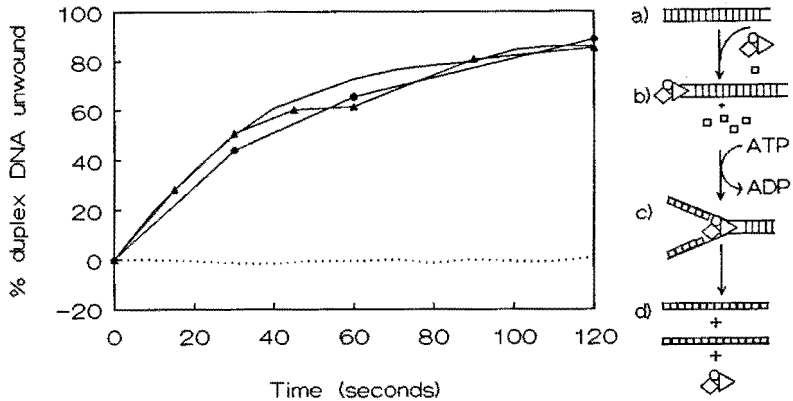

FIGURE 7. LEFT: Comparison of recBCD enzyme helicase activity using different assays. Solid line: fluorometric helicase assay with linear dsDNA; circles: agarose gel assay with linear dsDNA; triangles: S1 nuclease assay with linear dsDNA; dotted line: fluorometric assay with supercoiled RF M13 DNA. All assays contained 0.69 nM dsDNA molecules, 2 μM SSB protein and 8.5 nM recBCD enzyme; temperature was 25 °C. **RIGHT:** Fluorometric detection of recBCD enzyme helicase activity. RecBCD enzyme (triangles, diamonds and circles) binds to the end of a dsDNA molecule (b) and, accompanied by the hydrolysis of ATP, unwinds the duplex molecule. The ssDNA produced is immediately bound by SSB protein (rectangles) (c), resulting in a decrease in the intrinsic protein fluorescence of SSB protein. Unwinding continues until the DNA is completely single-stranded (d). This diagram oversimplifies the types of DNA molecules produced by recBCD enzyme. "Loop tails" and forked molecules are both observed in the electron microscope (30,31).

extent of DNA unwinding and, by comparison to the fluorescence quenching obtained with an equivalent amount of heat denatured DNA, the extent of dsDNA unwinding can also be determined.

A typical time course is shown in Figure 7. This rate of dsDNA unwinding by recBCD enzyme is the same when the new fluorescent assay is compared to agarose gel or S1 nuclease assays, and is independent of either SSB protein or T4-coded gene 32 protein concentration when excess binding protein is used. The final extent of unwinding is the same at all enzyme concentrations, demonstrating that the enzyme acts catalytically. Under optimal conditions, nearly 100% of the linear M13 dsDNA is unwound by recBCD enzyme.

As for any enzymatic reaction, increasing the recBCD enzyme concentration, the dsDNA concentration, or the ATP concentration increases the rate of DNA unwinding. From such data (18), the kinetic parameters (K_m and k_{cat}) for recBCD enzyme helicase activity were determined under a variety of conditions. These values, corrected for the amount of functional recBCD enzyme present, are summarized in Table 2. The rate of DNA unwinding varies from 250 to 470 bp/s/functional recBCD molecule at 25 °C and increases to 930 bp/s/functional recBCD molecule at 37 °C. The rate of unwinding is decreased when Ca^{+2} is present or when the ATP concentration exceeds the divalent ion concentration (not shown). In addition, k_{cat} values determined from the fluorescent assay are similar to the molecular unwinding rates obtained from the electron microscopic assay when identical experimental conditions are used, further confirming the validity of the fluorescence assay (30,31).

TABLE 2
ENZYMATIC PARAMETERS FOR RECBCD ENZYME HELICASE AND ATPASE ACTIVITIES[a]

	helicase activity		ATPase activity		
[NaCl], mM	app K_m, nM	k_{cat}, s^{-1}	app K_m, nM	k_{cat}, s^{-1}	ATP/bp
0	0.6	250	0.1	740	3.0
50	0.7	340	0.2	830	2.4
100	1.5	470	0.2	630	1.3
150	2.1	410	0.3	790	1.9
200	1.8	290	0.2	680	2.4
0[b]	1.1	930	nd	nd	nd

[a]Data are from (18,19) and represent the activity of a functional recBCD enzyme; the apparent K_m is in units of linear dsDNA molecules and the k_{cat} is in bp unwound/s/functional recBCD enzyme or ATP hydrolyzed/s/functional recBCD enzyme for the helicase or ATPase activities, respectively. The column, "ATP/bp", is the ratio of k_{cat} values for ATPase and helicase activities; "nd" is not done.
[b]Determined at 37° C and 0 mM NaCl.

Table 2 also shows that the apparent K_m for dsDNA (molecules) is 0.6-2.3 nM. Since the concentration of a single dsDNA end in *E. coli* is calculated to be approximately 1 nM, the K_m values are in a physiologically significant range. This result is consistent with the presumption that the DNA helicase activity of recBCD enzyme is functionally important *in vivo*.

Relationship of ATP Hydrolysis to RecBCD Enzyme Helicase Activity.

By definition, all DNA helicases are DNA-dependent ATPases. However, the specific mechanism by which this important class of enzymes transduces the free energy of ATP hydrolysis to DNA unwinding is unknown. As a first step, it is essential to determine the stoichiometry of ATP hydrolysis per base pair of DNA unwound (19). Therefore, the enzymatic parameters for ATP hydrolysis by recBCD enzyme were determined under conditions identical to those used in the helicase assays (Table 2). The ratio of the catalytic rate constant (k_{cat}) for ATP hydrolysis to that for DNA unwinding represents the catalytic efficiency of DNA unwinding. For recBCD enzyme, this value is approximately 2 ATP molecules per base pair unwound.

The result that recBCD enzyme hydrolyzes 2 molecules of ATP for each base pair unwound, together with other experimental data, suggests a model for recBCD helicase action. The additional facts are that both the recB and recD polypeptides are predicted to possess an ATP binding site (33,34); this has been experimentally confirmed using 8-azido-ATP crosslinking (35). Also, recBCD enzyme displays no polarity with regard to DNA unwinding (*i.e.*, it does not require a ssDNA tail to initiate unwinding) (36); in fact, ssDNA tails longer than approximately 25 nucleotides pre-

vent unwinding by recBCD enzyme (36). This is consistent with the ATPase assays which show that k_{cat} for ssDNA is approximately 16-fold lower than that for dsDNA and suggests that recBCD enzyme must contact both strands of dsDNA for full activation of ATPase activity. This result, in turn, implies that recBCD enzyme translocates poorly along ssDNA (if one assumes that translocation requires ATP hydrolysis).

All of these facts are readily accommodated by a model that envisions recBCD enzyme as being composed of two distinct translocating subunits bound to opposing strands of ssDNA. The DNA is probably contacted *via* the recB and recD subunits, whereas the recC subunit is important for χ site recognition/cutting (37). Consequently, when recBCD enzyme is traveling through a dsDNA molecule, each subunit must be translocating along a single DNA strand with opposite translocation polarities. If hydrolysis of one ATP molecule is required to advance each subunit by one nucleotide residue, then the observed stoichiometry of 2 ATP molecules hydrolyzed per base pair unwound is readily understood. An additional aspect of recBCD helicase activity can also be explained: namely, the formation of looped DNA structures during unwinding. If both translocating subunits move at the same rate, then only forked structures are obtained. But if, instead, one of the subunits moves at a rate which is 100 bp/s slower than the other, then a ssDNA loop is generated which appears to grow at 100 bp/s, as previously observed (30). This proposed model for recBCD enzyme action makes some experimentally verifiable predictions and should be useful in guiding future mechanistic studies.

Processivity of RecBCD Helicase Activity.

Another parameter of recBCD helicase activity that is important to biological function is its processivity of unwinding. The processivity of recBCD enzyme helicase activity can be measured using the fluorescent helicase assay (Figure 7) by taking advantage of a property mentioned above (20). RecBCD enzyme is unable to initiate unwinding on dsDNA substrates that possess ssDNA overhangs which are longer than approximately 25 nucleotides (36). As a consequence, if recBCD enzyme dissociates from a dsDNA molecule that has been unwound by more than 25 nucleotides (which cannot reanneal due to bound SSB protein), then that partially unwound DNA molecule can not be unwound further (by a second enzyme molecule). Because of this property, a dsDNA molecule that is greater than 2-fold longer than the average length of processively unwound DNA will not be completely unwound by recBCD enzyme. In the fluorescent helicase assay, this would be manifest as a reduction in the maximum extent of fluorescence quenching relative to controls using (completely) heat-denatured DNA.

To test this prediction, bacteriophage N4 DNA (72 kb in length) was used in a helicase assay; only 70%, on average, of each DNA molecule was unwound (agarose gels confirm that all of the DNA molecules are partially unwound by recBCD enzyme and not that just a sub-population of DNA is 100% unwound). Since unwinding can occur from each dsDNA end, approximately 25 kb (72 kb x 0.7 ÷ 2) was unwound per dsDNA end. Cleavage of the N4 DNA to produce approximately 50 kb and 20 kb fragments results in nearly complete unwinding in the fluorescence assay as expected.

These results agree with the electron microscopic observation that recBCD enzyme can fully unwind bacteriophage T7 (40 kb) (31,36).

The rate of recBCD helicase activity is sensitive to NaCl concentration. Figure 8 demonstrates the average number of base pairs unwound per binding event, ***N***, is also salt sensitive. To further verify that ***N*** is independent of the DNA used, bacteriophage λ dsDNA (48 kb) was also tested. Identical values of ***N*** are obtained for both N4 and λ DNA, particularly at 280 mM NaCl where the shorter length of λ DNA is not limiting the observed processivity value.

The processivity, P, is defined as the probability of unwinding another base pair relative to the probability of enzyme dissociation. Thus, a simple expectation is that the processivity should decrease when the rate of unwinding is decreased. For example, decreasing the ATP concentration causes ***N*** to decrease in a hyperbolic manner (with an apparent K_N of 40 μM) to as low as 2.5 kb/end at 10 μM ATP.

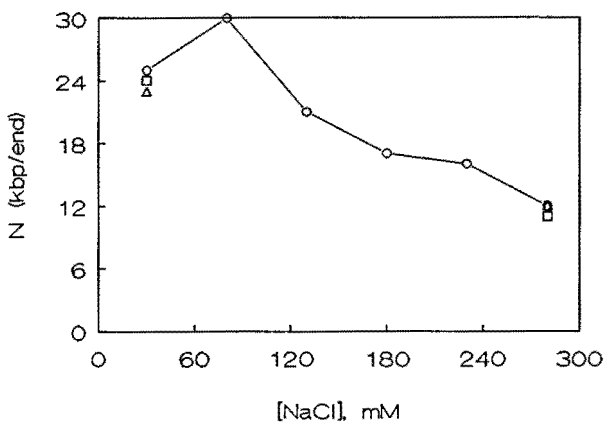

FIGURE 8. Number of kbp unwound per recBCD enzyme molecule as a function of sodium chloride concentration. Concentrations of dsDNA and recBCD enzyme were 1.4 nM ends and 7.6 nM, respectively. The concentration of SSB protein was 20.6 μM or 13.3 μM for N4 and λ DNA, respectively. Standard buffer conditions were used except that NaCl was added as indicated. Circles: N4 dsDNA at 25 °C; squares: λ dsDNA at 25 °C; triangles: N4 dsDNA at 37 °C.

DISCUSSION

We have established and characterized a pair of *in vitro* reactions in which DNA heteroduplex formation is dependent upon the combined activities of both recA and recBCD proteins; with one set of substrates, SSB protein is also important. For both reactions, DNA heteroduplex formation requires three kinetically discrete steps: first, the dsDNA is unwound by recBCD enzyme to produce ssDNA tails; second, these ssDNA tails must be trapped by a ssDNA binding protein (either recA or SSB protein) before they reanneal; and third, heteroduplex DNA formation is catalyzed by recA protein. The reaction pathways differ primarily with regard to which recA protein function is required: for the ssDNA X dsDNA reaction, renaturation function is

necessary; for the dsDNA X dsDNA reaction, joint molecule forming function is necessary.

These results provide *in vitro* evidence for the hypothesis that recBCD enzyme can initiate recombination events by producing a ssDNA substrate suitable for subsequent recA protein-dependent action. The DNA substrates used in the dsDNA X dsDNA reaction are representative of the DNA molecules thought to be participating in the recBCD-pathway of homologous recombination in *E. coli* (i.e., a linear dsDNA molecule and the supercoiled chromosomal dsDNA). Thus, it is assumed that the events occurring in these *in vitro* reactions are representative of the events occurring *in vivo* when recA, recBCD, and SSB protein encounter similar DNA molecules. We should add that although the M13 DNA substrates used here contain a χ sequence (at position 4942), its function has not yet been experimentally tested. Under the conditions optimized for these *in vitro* reactions, and based on our view of the reaction steps as depicted in Figures 4 & 6, we do not expect to detect an effect of χ sites on the pairing reactions. However, this does not preclude a potential function for χ sites in such *in vitro* reactions. For example, we might expect that under suitable suboptimal reaction conditions (*e.g.,* when trapping of ssDNA is limiting; see below), then the effect of a χ site might be manifest. Experimental verification of these speculations is currently in progress.

Comparison of the rate-limiting step in each reaction illustrates an unanticipated requirement for proper coordination of recA protein and recBCD enzyme functions in heteroduplex DNA formation. In the ssDNA X dsDNA reaction, the slowest step is the trapping of the ssDNA tails by recA protein; whereas, in the dsDNA X dsDNA reaction, trapping is no longer rate-limiting, primarily due to the presence of SSB protein. The difference in the trapping abilities of these two proteins most likely originates from the fact that the binding of SSB protein to ssDNA is very rapid (probably diffusion controlled), whereas the binding of recA protein to ssDNA is limited by a kinetically slow nucleation step (38) (of unknown molecular nature). Thus, in contrast to multi-protein systems (*e.g.,* the replication enzymes of bacteriophage T4), coordination of recA, recBCD, and SSB proteins is a matter of "kinetic timing". By this we mean that the first enzyme in the biochemical pathway, the recBCD enzyme, unwinds DNA very rapidly. In fact, it unwinds dsDNA too rapidly for the resultant ssDNA to be trapped by the slow-binding recA protein since the kinetic lifetime of the protein-free ssDNA tails is determined by the rate of their reannealing. Therefore, an intermediary is required; SSB protein serves this function and, by analogy, acts as a middle "gear" to permit proper meshing of the first and third "gears". SSB protein efficiently traps and increases the ssDNA lifetime sufficiently to permit limited binding by recA protein. This is followed by displacement of the SSB protein by recA protein. Thus, functional defects in either recA, recBCD, or SSB proteins which disrupt the lifetime of any of these intermediates are expected to alter the outcome of product formation.

If the dsDNA X dsDNA reaction represents a reasonable model for events occurring during normal recBCD-dependent recombination, then what, if any, is the biological relevance of the ssDNA X dsDNA reaction? We can only speculate. Normally, the presence of free SSB protein and the absence of free ssDNA in *E. coli* under conditions of normal growth will inhibit any heteroduplex DNA formation by a

biochemical mechanism similar to that described for the ssDNA X dsDNA reaction. However, conditions which result in extensive DNA damage (such as UV irradiation) create extensive amounts of ssDNA, which could titrate all of the free SSB protein (SSB protein is not induced for the first few hours after DNA damage (39,40)). Thus, these physiological conditions would favor biochemical conditions which should be compatible with the pathway described for the *in vitro* ssDNA X dsDNA reaction. This speculative conclusion implies that the renaturation activity of recA protein plays primarily a repair function whereas, the joint molecule forming activity is more directly involved in recombination function. It is interesting to note that for the 6 mutant recA proteins which we have characterized, there is an exact correspondence between recombination phenotype and both *in vitro* DNA joint molecule formation and DNA renaturation activities; *i.e.,* those defective in recombination are always defective in both *in vitro* activities. This implies that these functions of recA protein are related and are essential.

Finally, quantitative characterization of recBCD enzyme helicase activity shows that its properties are significant with regard to *in vivo* function. First, the K_m values for its substrates are in physiologically appropriate ranges. For dsDNA, it is approximately 1-2 nM (in dsDNA molecules) and, for ATP, it is 130 μM; the cellular concentrations equal or exceed these values. Second, the unwinding of dsDNA is rapid and efficient; unwinding rates are as high as 930 bp/s/functional recBCD molecule, and about 2-3 ATP molecules are hydrolyzed per bp unwound. Third, unwinding is highly processive (P=0.99996), with an average of approximately 25,000 base pairs unwound (from a dsDNA end) before dissociation of the recBCD enzyme.

This high degree of processivity is of particular significance to activation at χ sites. Since cutting at a χ site requires that recBCD travel through and unwind the dsDNA (41,42), *in vivo* action of recBCD at χ sites distal to the *cos* entry site of bacteriophage λ requires that a recBCD molecule can processively unwind up to 48 kb of DNA. The probability of unwinding at least n base pairs is given by P^n. Using the experimentally obtained value for P (0.99996) predicts that nearly 15% of the recBCD molecules which enter one end of λ DNA are able to traverse the full length of the genome. Thus, the processivity of recBCD helicase activity is sufficiently great as to allow activation at the most distal χ sites in λ (though an attenuation with distance should be observed).

An interesting ramification of this highly processive unwinding is that, by virtue of recBCD enzyme's ability to unwind a substantial portion of λ DNA, a significant depletion of the intracellular free SSB concentration must occur. That is, since the total amount of SSB protein is estimated at approximately 400-800 tetramers per cell (43,44), *all* of the SSB protein could be sequestered by unwinding approximately one-half of the λ genome (assuming an SSB protein site size of 65 nucleotides/tetramer). An important consequence of these calculations (assuming they are reasonable) is that, under physiological conditions, trapping by SSB protein will not be optimal and it is likely that significant reannealing of the dsDNA will occur. Under such conditions, if the ssDNA produced at a χ site is longer lived for any reason, then the mechanistic role of χ sites can be readily appreciated.

In conclusion, the studies described are an initial attempt to reconstitute homologous pairing *in vitro* which requires some of the proteins known to comprise

the recBCD-pathway of genetic recombination. Our results are consistent with an initiation function for recBCD enzyme, followed by recA protein-promoted homologous pairing involving the ssDNA produced by recBCD enzyme. Both the *in vitro* recABCD-dependent pairing reactions and the helicase characterization help establish some of the fundamental properties of wild-type recBCD enzyme function. Further characterization utilizing these assays and variants of these assays should help to establish the biochemical role of other proteins involved in homologous recombination, the effect of χ sites on recBCD enzymatic activities, and the quantitative basis for recombination defects of recBCD mutant proteins, all of which are in progress.

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