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Enhancement of recA Protein-promoted DNA Strand Exchange Activity by Volume-occupying Agents*

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Polly E. Lavery‡ and Stephen C. Kowalczykowski‡§¶

From the ‡Department of Cell, Molecular, and Structural Biology, Northwestern University Medical School, Chicago, Illinois 60611 and the §Division of Biological Sciences, Section of Microbiology, University of California, Davis, California 95616

To investigate the *in vivo* effects of macromolecular crowding we examined the effect of inert macromolecules such as polyvinyl alcohol and polyethylene glycol on the *in vitro* activity of recA protein. The addition of either of these volume-occupying agents enables recA protein to promote homologous pairing and exchange of DNA strands at an otherwise nonpermissive magnesium ion concentration. In the presence of these macromolecules, both the rate of recA protein association with single-stranded DNA (ssDNA) and the steady-state affinity of recA protein for ssDNA are increased. Consequently, the ability of recA protein to compete with ssDNA-binding protein (SSB protein) is enhanced, and the inhibitory effects of SSB protein on the formation of recA protein-ssDNA presynaptic complexes are eliminated. Because the ability of recA protein to bind to ssDNA-containing secondary structures is also enhanced in volume-occupied solution, joint molecule formation is not greatly reduced when SSB protein is omitted from the reaction. Thus, increased recA protein interactions with ssDNA contribute to enhanced presynaptic complex formation. In addition, polyvinyl alcohol and polyethylene glycol must also affect another property of recA protein, *i.e.* self-association, which is required for synapsis and DNA strand exchange. Our examination of DNA strand exchange in the presence of volume-occupying agents helps to reconcile the requirement for elevated magnesium ion concentrations in recA protein-promoted recombination reactions *in vitro*, with a presumably low magnesium ion concentration *in vivo*.

The recA protein of *Escherichia coli* is necessary for genetic recombination (for reviews see Cox and Lehman, 1987; Radding, 1988; Kowalczykowski, 1991a). *In vitro*, recA protein promotes the unique reaction of DNA strand exchange (Cox and Lehman, 1981). In this reaction one strand of a linear duplex DNA molecule is exchanged for a homologous circular ssDNA[1] molecule, producing a gapped circular heteroduplex DNA product molecule and a displaced linear ssDNA molecule. This reaction is likely to be representative of the cellular recombination process because mutant recA proteins that are capable of at least some level of recombination *in vivo* are able to promote the DNA strand exchange reaction *in vitro* (Menetski and Kowalczykowski, 1990; Lavery and Kowalczykowski, 1990) whereas proteins incapable of recombination *in vivo* are unable to promote DNA strand exchange *in vitro* (Rusche *et al.*, 1985; Kowalczykowski *et al.*, 1989; see Kowalczykowski, 1991b).

Although the *in vivo* concentration of free magnesium ion is generally thought to be quite low, the *in vitro* ability of recA protein to promote heteroduplex DNA formation is dependent on the presence of magnesium ion in excess of 1 mM (Shibata *et al.*, 1979), with optimal DNA strand exchange activity observed at 10 mM magnesium chloride (Cox and Lehman, 1982) or 6–10 mM magnesium acetate (Roman and Kowalczykowski, 1986). At 1 mM magnesium ion, recA protein is displaced from ssDNA by SSB protein, but at higher magnesium ion concentrations it is resistant to this displacement (Kowalczykowski *et al.*, 1987). Thus, the inability of recA protein to form heteroduplex DNA at 1 mM magnesium ion in the presence of SSB protein can be attributed to an inability to form presynaptic complexes (Roman and Kowalczykowski, 1986). However, if this were the sole impediment to heteroduplex DNA formation then the reaction would be expected to occur at 1 mM magnesium ion in the absence of SSB protein, and this is not the case. Additionally, we have shown that although the mutant recA441 protein is not displaced from ssDNA by SSB protein at 1 mM magnesium ion, it is unable to promote DNA strand exchange under these conditions (Lavery and Kowalczykowski, 1990). Thus, elevated magnesium ion concentration appears to fulfill a requirement for DNA strand exchange other than enabling recA protein to compete with SSB protein during the formation of presynaptic complexes. This requirement may reflect the need to form a specific recA protein aggregate (see Lavery and Kowalczykowski, 1990); the formation of this aggregate can be induced by elevated magnesium ion concentration, the presence of spermidine, or, as we will demonstrate in this study, the presence of volume-occupying agents.

In vitro biochemical reactions are typically studied in solutions in which macromolecular species are quite dilute (less than 0.1% by weight) whereas the intracellular concentration of soluble proteins is generally as great as 20% by weight (see Fulton, 1982). Although no single species may be present at high concentration, all macromolecular species combined will occupy a significant volume fraction of the intracellular space, *i.e.* it is "volume-occupied" (Minton, 1981). Minton (1981, 1983) has used statistical mechanical methods to demonstrate that even in the absence of direct interactions between macromolecular species, the volume from which molecules are excluded by others can have a dramatic effect on the ther-

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¶ To whom correspondence should be addressed: Division of Biological Sciences, Section of Microbiology, University of California, Davis, CA 95616. Tel.: 916-752-5938.

[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; etheno M13 DNA, M13 ssDNA containing 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine residues; SSB protein, *E. coli* single-stranded DNA-binding protein; PVA, polyvinyl alcohol; PEG, polyethylene glycol.

modynamic equilibria and kinetics of enzyme-catalyzed reactions. Additionally, he predicts self-association processes to be favored, *i.e.* the aggregation state of self-associating proteins is expected to be increased under volume-occupied conditions.

To approximate physical volume-occupied conditions, Jarvis *et al.* (1990) utilized various presumed inert macromolecules such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), and dextran in their *in vitro* examination of the DNA replication proteins of bacteriophage T4. Solutions containing PEG or PVA have also been shown to promote the association of ribosomal subunits (Zimmerman and Trach, 1988), to favor the binding of DNA polymerases to DNA (Zimmerman and Harrison, 1987), and to increase the efficiency of *in vitro* transposition events (Mizuuchi, 1983; Ichikawa and Ohtsubo, 1990). In this study we report that the presence of 7.5–10% PVA or PEG enables recA protein to promote the DNA strand exchange reaction at the otherwise nonpermissive magnesium acetate concentration of 1 mM. Under these volume-occupied conditions, both the rate of recA protein association with ssDNA and the steady-state affinity of recA protein for ssDNA are increased, which enables recA protein to compete more effectively with SSB protein and secondary structures for ssDNA. Additionally, the protein-protein interactions crucial to DNA strand exchange must also be enhanced by the presence of these volume-occupying agents.

MATERIALS AND METHODS

Chemicals and Buffers—All chemicals were reagent grade, and solutions were made using glass-distilled water. ATP was purchased from Boehringer Mannheim and dissolved as a concentrated stock at pH 7.5. Low molecular weight (10,000–30,000) PVA and PEG 8,000 were purchased from Sigma, dissolved in water as concentrated stocks, and used without further purification; the concentrations of these polymers are reported in weight/unit volume percentages. When PVA was dialyzed against water overnight and used in the DNA strand exchange reaction, no significant differences were observed; thus the effects on DNA strand exchange are not caused by low molecular weight contaminants in the commercial preparations of the polymers. Reactions were performed in TMD buffer (25 mM Tris acetate, 1 mM magnesium acetate, and 1 mM dithiothreitol) and contained PVA or PEG as indicated.

Proteins—recA protein was purified from strain JC12772 (Uhlin and Clark, 1981) using a preparative procedure[2] based on spermidine precipitation (Griffith and Shores, 1985). SSB protein was purified from strain RLM727 as described (LeBowitz, 1985). Protein concentrations were determined using molar extinction coefficients of 2.7×10^4 M^{-1} cm^{-1} for recA protein and 3×10^4 M^{-1} cm^{-1} for SSB protein (Ruyechan and Wetmur, 1976), both at 280 nm.

Lactate dehydrogenase and pyruvate kinase were purchased from Sigma as ammonium sulfate suspensions. Working solutions of these enzymes were made by centrifuging a homogeneous sample of the suspension and dissolving the protein pellet in reaction buffer.

DNA—Single- and double-stranded DNA were purified from bacteriophage M13mp7 using the procedures described by Messing (1983). The duplex DNA was linearized by digestion with *Eco*RI restriction endonuclease. Molar nucleotide concentrations were determined using extinction coefficients of 6,500 M^{-1} cm^{-1} for duplex DNA and 8,780 M^{-1} cm^{-1} for ssDNA, both at 260 nm.

DNA Strand Exchange Assay—The agarose gel assay for DNA strand exchange was conducted as described previously (Cox and Lehman, 1981; Lavery and Kowalczykowski, 1990). This procedure allows the separation of linear duplex DNA substrate molecules, gapped circular heteroduplex DNA product molecules, joint molecule intermediate DNA species, and homology-dependent DNA networks (Menetski and Kowalczykowski, 1989; Lavery and Kowalczykowski, 1990).

Ethidium bromide-stained gels were photographed, and the negatives were scanned using a Zeineh soft laser scanning densitometer, interfaced with a Hewlett-Packard 3390A integrator. The percentages of joint molecule intermediate species, homology-dependent DNA networks, and product molecules formed were determined as the amount of each species present divided by the total amount of dsDNA present at each time point.

Unless otherwise indicated, DNA strand exchange reactions were performed at 37 °C in TMD buffer containing 0.5 mM ATP, 3 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 3 μM recA protein, 0.45 μM SSB protein, 5 μM M13 ssDNA, and 10 μM M13 dsDNA. recA protein was incubated for 1 min in reaction buffer containing ssDNA prior to the addition of SSB protein; the reaction was then started by the addition of dsDNA.

ATPase Assay—The hydrolysis of ATP was monitored using a spectrophotometric assay that couples the production of ADP to the oxidation of NADH (Kreuzer and Jongeneel, 1983; see Kowalczykowski and Krupp, 1987). Reactions were carried out at 37 °C in TMD buffer containing 0.2 mg of NADH/ml, 0.5 mM ATP, 3 mM phosphoenolpyruvate, 30 units/ml each of pyruvate kinase and lactate dehydrogenase, 3 μM recA protein, 0.45 μM SSB protein, and 5 μM M13 ssDNA.

DNA Binding Assay—The binding of recA protein to etheno M13 DNA was monitored as described (Menetski and Kowalczykowski, 1985; Menetski *et al.*, 1988). Salt titrations were carried out at 25 °C by adding aliquots of concentrated NaCl to complexes of recA protein and etheno M13 DNA in buffer (25 mM Tris-HCl, 1 mM magnesium chloride, 0.1 mM dithiothreitol, and PEG as indicated). Reactions contained 1 μM recA protein, 3 μM etheno M13 DNA, and ATP or ADP as indicated. An ATP-regenerating system consisting of 8 units/ml pyruvate kinase and 3 mM phosphoenolpyruvate was present in reactions containing ATP.

Association Experiments—The rate of recA protein association with ssDNA was determined using etheno M13 DNA to monitor binding (Menetski and Kowalczykowski, 1990). Reactions were performed at 25 °C in 25 mM Tris-HCl, 1 mM magnesium chloride, 0.1 mM dithiothreitol, 100 mM NaCl, 0.5 mM ATP, and PEG as indicated; 0.1 μM recA protein was added to 6 μM etheno M13 DNA equilibrated in reaction buffer, and the fluorescence increase upon binding was monitored.

The experimental data were analyzed by the nonlinear least squares routine in the Data Evaluation System 80 program (DAES80), from R and L Software. This program was used to fit the data either to a single exponential equation plus a variable end point of the form

$$F = Ae^{-kt} + B$$

or to a double exponential equation plus a variable end point of the form

$$F = A_{fast}e^{-k(fast)t} + A_{slow}e^{-k(slow)t} + B$$

where F is the fluorescence signal at time t; A_{fast} and A_{slow} are the amplitude of each component at $t = 0$; k_{fast} and k_{slow} are the observed first-order rate constants for each component; and B is the end point.

RESULTS

The Presence of PVA Permits recA Protein to Promote DNA Strand Exchange at an Otherwise Nonpermissive Magnesium Ion Concentration—At 1 mM magnesium acetate, recA protein is unable to promote DNA strand exchange (Cox and Lehman, 1982; Roman and Kowalczykowski, 1986); however, if PVA is added to the reaction buffer, DNA strand exchange occurs (Fig. 1). Fig. 2*A*, which is derived from densitometric scanning of data such as those presented in Fig. 1, displays average time courses for the appearance of joint molecule intermediate DNA species, gapped circular heteroduplex DNA product molecules (form II DNA), and homology-dependent DNA networks. The dependence of DNA strand exchange on elevated magnesium ion concentration is alleviated by the presence of 7.5% PVA; under these conditions the reaction is comparable, though somewhat slower, to that observed at 6 mM magnesium acetate, a condition that is near optimal for form II product DNA production (Fig. 2*B*). Although the addition of PVA eliminates the requirement for elevated magnesium ion concentration, it does not abolish the need for magnesium ion; *i.e.* if magnesium acetate is omitted from the reaction buffer, DNA strand exchange does not occur (data not shown).

[2] S. C. Kowalczykowski, manuscript in preparation.

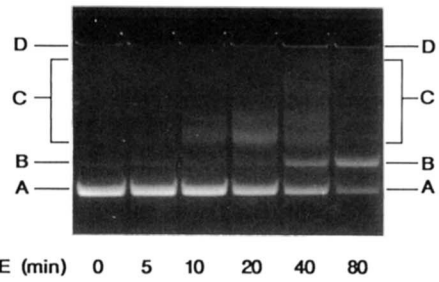

FIG. 1. **RecA protein-promoted DNA strand exchange at 1 mM magnesium acetate in the presence of PVA.** Reactions were performed as described under "Materials and Methods" at 1 mM magnesium acetate and 7.5% PVA. *Band A* indicates linear M13 dsDNA substrate molecules; *band B* indicates gapped circular heteroduplex DNA product molecules (form II DNA); *region C* indicates joint molecule intermediate DNA species; *band D* indicates homology-dependent DNA networks. *Lanes 1–6* are 0-, 5-, 10-, 20-, 40-, and 80-min time points, respectively.

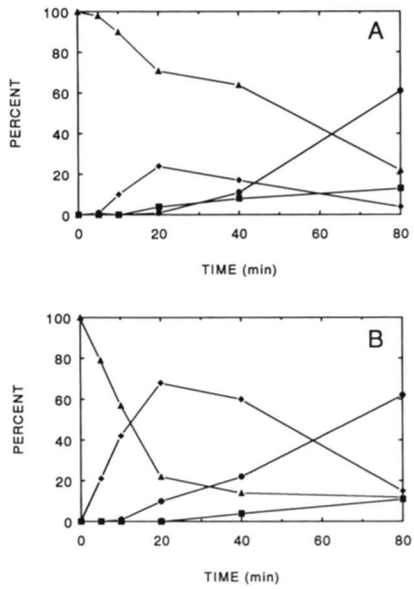

FIG. 2. **Time courses for recA protein-promoted DNA strand exchange.** Reactions were performed as described under "Materials and Methods." *Triangles* indicate linear M13 dsDNA substrate molecules; *diamonds* indicate joint molecule intermediate DNA species; *circles* indicate gapped circular heteroduplex DNA product molecules; *squares* indicate homology-dependent DNA networks. *Panel A* indicates reactions performed at 1 mM magnesium acetate and 7.5% PVA; *panel B* indicates reactions performed at 6 mM magnesium acetate and no macromolecule.

We examined DNA strand exchange at 1 mM magnesium acetate in the presence of varying amounts of PVA. The results in Table I show that PVA in excess of 2.5% is required for joint molecule formation. At 5% PVA, both joint molecule formation (only 19% of the linear dsDNA substrate is utilized in 80 min) and form II DNA formation (only 7% produced in 80 min) are slow. At 7.5% PVA, joint molecule formation is much more effective, and branch migration converts most of the joint molecule intermediate species to form II DNA product molecules (see Fig. 2*A*). However, some homology-dependent DNA networks are formed via presumed reinitiation of pairing by the ssDNA that is partially displaced from the joint molecules (see Chow *et al.*, 1988; Lavery and Kowalczykowski, 1990). At 10% PVA, joint molecule formation by recA protein is quite rapid. Although many of the joint molecules are converted to form II DNA product molecules, a large (and experimentally variable) amount of homology-dependent DNA networks is formed (see Fig. 3*A*). Thus, even though the rate of substrate utilization is rapid, joint molecules do not accumulate, and instead they become involved in the reinitiation reactions that lead to network formation.

PEG Also Enhances recA Protein-promoted DNA Strand Exchange—To demonstrate that the effect of PVA on recA protein-promoted DNA strand exchange is not unique to this macromolecule we examined the reaction in the presence of PEG. As with PVA, PEG in excess of 2.5% is required for joint molecules to appear at 1 mM magnesium acetate (Table 1). Although the PEG and PVA concentration dependences for joint molecule formation are similar, the DNA strand exchange reaction is not identical in the presence of these macromolecules. Linear dsDNA substrate molecules are taken up into joint molecules at similar rates in PEG and PVA; however, at low polymer concentrations, form II DNA product molecule formation is less effective in the presence of PEG, suggesting that extension of heteroduplex DNA (*i.e.* branch migration) is not as favorable in the presence of this polymer. At 10% PEG, both the rate of substrate utilization and the rate of form II DNA product molecule formation are comparable to those at 10% PVA. However, in the presence of PEG, homology-dependent DNA networks are formed more slowly, and joint molecule intermediate species accumulate (see Fig. 3).

In the Absence of SSB Protein, Homology-dependent DNA Network Formation Is Favored over Form II DNA Product Molecule Formation—At elevated magnesium ion concentration, recA protein-promoted DNA strand exchange is greatly stimulated by SSB protein (Cox *et al.*, 1983). To determine if the reaction is stimulated similarly by SSB protein at 1 mM magnesium acetate in volume-occupied solution, we examined DNA strand exchange in the absence of SSB protein under these conditions. When DNA strand exchange is performed at 1 mM magnesium acetate and 10% PVA, joint molecules are formed effectively in the absence of SSB protein (Fig. 4*A*); linear dsDNA substrate molecules are taken up into joint molecules almost as quickly as in the presence of SSB protein (compare Fig. 4*A* and Fig. 3*A*). However, no gapped circular product molecules are formed in the absence of SSB protein. Instead, a large number of homology-dependent DNA networks appears. Presumably, in the absence of SSB protein, recA protein easily binds the ssDNA displaced from joint molecules and promotes the reinitiation reactions that form extensive DNA networks. Thus, SSB protein appears to be needed either to extend the heteroduplex DNA or to block the reinitiation reactions that are inhibitory to the resolution of form II DNA product molecules (see Chow *et al.*, 1988). Similar results are seen at 1 mM magnesium acetate and 7.5% PVA (data not shown); however, joint molecule formation is slowed somewhat more by the omission of SSB protein under these conditions (see Lavery and Kowalczykowski, 1992).

When DNA strand exchange is performed at 10% PEG in the absence of SSB protein, joint molecule formation also occurs (Fig. 4*B*). As was true at 10% PVA, when SSB protein is omitted from the reaction form II DNA product molecule formation is dramatically inhibited, and a large number of homology-dependent DNA networks appears (compare Fig. 4*B* and Fig. 3*B*). However, in the presence of PEG these networks are formed more slowly, and there is increased accumulation of intermediate species.

RecA Protein ssDNA-dependent ATPase Activity in Volume-occupied Solution—At elevated magnesium ion concentration, SSB protein stimulates joint molecule formation by removing secondary structure from ssDNA and allowing formation of saturated recA protein-ssDNA presynaptic com-

TABLE I

Effects of PVA and PEG on DNA strand exchange

Reactions were performed in TMD buffer containing 5 μM M13 ssDNA, 10 μM M13 dsDNA, 3 μM recA protein, 0.45 μM SSB protein, and PVA or PEG as indicated.

[Polymer]	Maximum rate of substrate utilization[a]		Maximum rate of appearance[a]					
			Joint molecules		Product molecules		DNA networks	
	PVA	PEG	PVA	PEG	PVA	PEG	PVA	PEG
%	%/min		%/min		%/min		%/min	
0	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
2.5	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
5	0.25	0.14	0.20	0.13	0.13	0.05	<0.05	<0.05
7.5	1.9	1.8	1.8	1.8	1.3	0.68	0.20	<0.05
10	6.0	6.8	2.8	6.8	1.0	0.78	3.4	0.33

[a] The maximum rate (%/min) of both substrate utilization and species appearance was determined from a reaction time course (similar to that shown in Fig. 2).

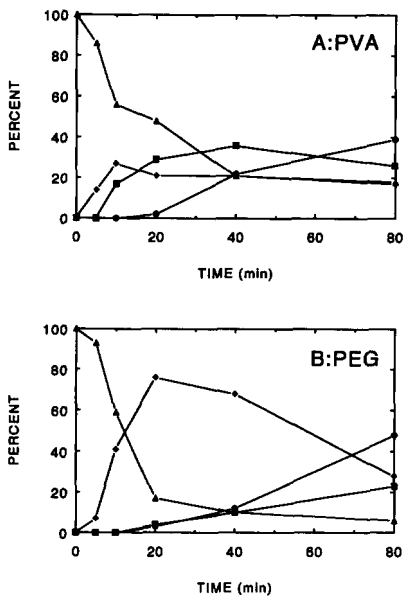

FIG. 3. **Comparison of DNA strand exchange at 10% PVA and 10% PEG.** Reactions were performed as described under "Materials and Methods." *Triangles* indicate linear M13 dsDNA substrate molecules; *diamonds* indicate joint molecule intermediate DNA species; *circles* indicate gapped circular heteroduplex DNA product molecules; *squares* indicate homology-dependent DNA networks. *Panel A* indicates reactions performed at 1 mM magnesium acetate and 10% PVA; *panel B* indicates reactions performed at 1 mM magnesium acetate and 10% PEG.

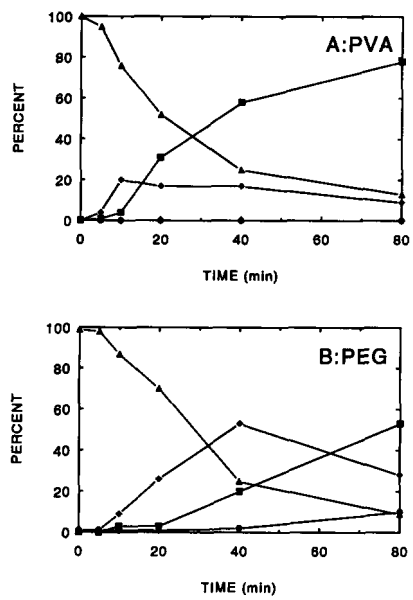

FIG. 4. **DNA strand exchange in the absence of SSB protein.** Reactions were performed as described under "Materials and Methods" with SSB protein omitted. *Triangles* indicate linear M13 dsDNA substrate molecules; *diamonds* indicate joint molecule intermediate DNA species; *circles* indicate gapped circular heteroduplex DNA product molecules; *squares* indicate homology-dependent DNA networks. *Panel A* indicates reactions performed at 1 mM magnesium acetate and 10% PVA; *panel B* indicates reactions performed at 1 mM magnesium acetate and 10% PEG.

plexes (Muniyappa *et al.*, 1984). Consistent with this, examination of ssDNA-dependent ATPase activity under these conditions shows that more recA protein is involved in ATP hydrolysis when SSB protein is present (Kowalczykowski and Krupp, 1987; Menetski and Kowalczykowski, 1989). Because joint molecule formation at 1 mM magnesium acetate in volume-occupied solution occurs almost as quickly in the absence of SSB protein as it does in its presence, we investigated the effect of PVA and PEG on ssDNA-dependent ATPase activity. At 1 mM magnesium acetate in the presence of excess recA protein, PVA results in a concentration-dependent increase in the observed rate of ATP hydrolysis (Table II; see Lavery and Kowalczykowski (1992) for recA protein concentration dependence). The rate of ATP hydrolysis increases with increasing PVA concentration up to 7.5% PVA; beyond this concentration there is no further increase in the observed rate of hydrolysis. When PEG is added to the ATPase reaction in place of PVA, similar results are obtained

TABLE II

Effect of volume-occupying agents on ssDNA-dependent ATPase activity

Reactions were performed in TMD buffer containing 5 μM M13 ssDNA, 3 μM recA proteins, and PVA or PEG as indicated.

[Polymer]	Steady-state rate of ATP hydrolysis	
	PVA	PEG
%	μM/min	
0	23.0	23.0
2.5	29.6	25.9
5	33.9	26.9
7.5	41.0	28.3
10	41.0	31.9

(Table II). However, the rate of ATP hydrolysis increases throughout the range of concentrations tested (*i.e.* up to 10%), and the presence of PEG has less of a stimulatory effect on

the observed rate of hydrolysis.

At 1 mM magnesium acetate, SSB protein displaces recA protein from ssDNA (Kowalczykowski *et al.*, 1987), and therefore ssDNA-dependent ATPase activity is inhibited (Kowalczykowski and Krupp, 1987). This displacement of recA protein by SSB protein contributes to the lack of recA protein-promoted DNA strand exchange activity under these conditions (Roman and Kowalczykowski, 1986); therefore, we were curious about the effect of SSB protein on ATPase activity at 1 mM magnesium acetate in volume-occupied solution. The effect of SSB protein on the observed rate of ATP hydrolysis is shown in Fig. 5. In the absence of PVA or PEG the addition of SSB protein results in a rapid and complete inhibition of ATP hydrolysis, as recA protein is displaced from the ssDNA. At 2.5% PVA or PEG, ATP hydrolysis is also inhibited by the addition of SSB protein; however, the kinetics of inhibition are less rapid. At 5% PVA or PEG, inhibition occurs even more slowly and is not complete. At polymer concentrations equal to or greater than 7.5%, the addition of SSB protein no longer affects the observed rate of ATP hydrolysis. Interestingly, these are the polymer concentrations at which we observe effective joint molecule formation by recA protein.

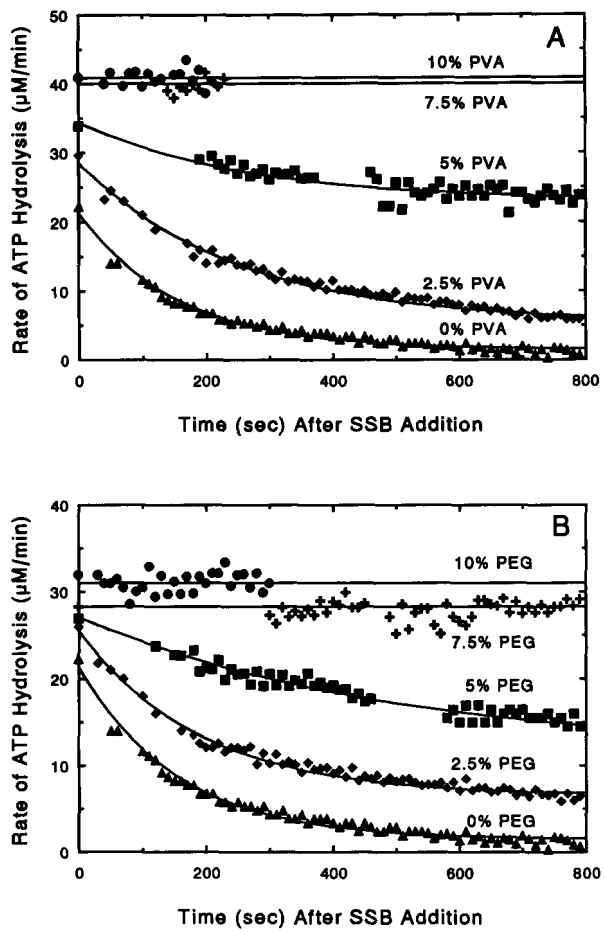

FIG. 5. **Effect of SSB protein on ssDNA-dependent ATP hydrolysis by recA protein.** The hydrolysis of ATP was measured as described under "Materials and Methods" at 1 mM magnesium acetate, and PVA or PEG as indicated. After recA protein had attained a steady-state rate of ATP hydrolysis, 0.45 μM SSB protein was added ($t = 0$). *Triangles* indicate reactions in which no PVA or PEG was present; *diamonds* indicate reaction performed at 2.5% PVA or PEG; *squares* indicate reactions performed at 5% PVA or PEG; *crosses* indicate reactions performed at 7.5% PVA or PEG; *circles* indicate reactions performed at 10% PVA or PEG. *Panel A* indicates reactions performed in PVA; *panel B* indicates reactions performed in PEG.

TABLE III

Binding of RecA protein to etheno M13 DNA

Reactions were performed at 25 °C in TMD buffer containing 3 μM etheno M13 DNA, 1 μM recA protein, and 10% PEG as indicated.

	Salt titration midpoint[a]		Relative fluorescence increase[b]	
	−PEG	+PEG	−PEG	+PEG
	mM			
No cofactor	280	530	1.6	1.8
0.5 mM ADP	145	350	1.8	1.9
0.5 mM ATP	610	1,000	2.1	2.7

[a] The salt titration midpoint is the NaCl concentration required to disrupt one-half of the recA protein-etheno M13 DNA complex present.

[b] The relative fluorescence increase is the increase in etheno M13 DNA fluorescence caused by recA protein binding; it is determined by dividing the fluorescence of the protein-DNA complex by the fluorescence of the free DNA and protein at the end of a salt titration.

The Stability of recA Protein-Etheno M13 DNA Complexes Is Affected by the Presence of PEG—In the presence of PVA or PEG, recA protein shows enhanced activity in the competition with SSB protein for ssDNA sites. To determine if this difference can be attributed to an increased affinity for ssDNA, we measured the salt sensitivity of recA protein binding to etheno M13 DNA, a modified ssDNA whose fluorescence increases upon protein binding. The salt titration midpoint (defined as the concentration of NaCl required to dissociate one-half of the recA protein-DNA complex present) is related to the affinity of the protein for ssDNA (Menetski and Kowalczykowski, 1985). Table III shows salt titration midpoint values in the absence and presence of 10% PEG. Under all conditions examined, the presence of 10% PEG results in a dramatic increase in the observed salt titration midpoint. Additionally, the relative fluorescence increase of etheno M13 DNA caused by recA protein binding in the presence of ATP is considerably higher in the presence of PEG (Table III). Thus, the addition of PEG to reaction buffer does increase the apparent affinity with which recA protein binds ssDNA.

The Association of recA Protein with Etheno M13 DNA—Examination of mutant recA proteins has indicated that the ability to compete with SSB protein for binding sites is related to the association rate of recA protein with ssDNA; a decreased rate of association has been correlated with a decreased ability to compete (Menetski and Kowalczykowski, 1990) whereas an increased rate of association has been correlated with an increased ability to compete[3] (Lavery and Kowalczykowski, 1988; see Kowalczykowski, 1991b). To determine if the increased ability observed in volume-occupied solution can be attributed to an increased rate of recA protein association, we examined the association of recA protein with etheno M13 DNA in the absence and presence of 10% PEG. The results in Fig. 6 show that recA protein association with ssDNA is affected by the presence of 10% PEG. In the absence of PEG, the increase in fluorescence resulting from the binding of recA protein to etheno M13 DNA can be fit by a single exponential equation. In the presence of 10% PEG, the fluorescence increase is not fit by a single exponential; however, the data are described well by a double-exponential equation, suggesting that some recA protein is rapidly binding ssDNA, while a fraction is associating with the DNA more slowly.

[3] M. V. V. S. Madiraju, P. E. Lavery, S. C. Kowalczykowski, and A. J. Clark, submitted for publication.

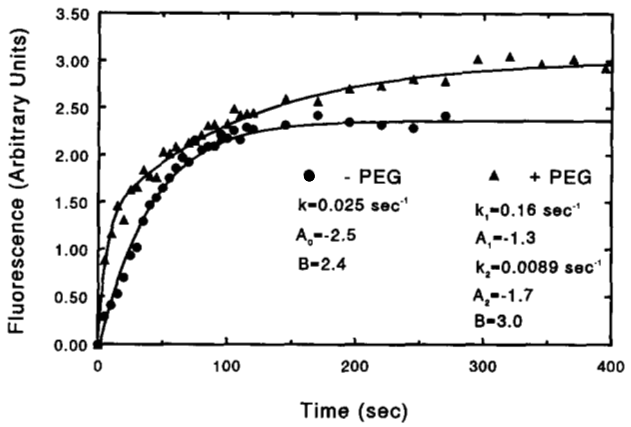

FIG. 6. **The association of recA protein with etheno M13 DNA.** Reactions were performed as described under "Materials and Methods," and the increase in etheno M13 DNA fluorescence caused by recA protein binding was monitored over time. *Circles* indicate reactions performed at 1 mM magnesium acetate in the absence of PEG; *triangles* indicate reactions performed at 1 mM magnesium acetate and 10% PEG. For the single exponential fit in the absence of PEG, k is the apparent first-order rate constant (s^{-1}), A_0 is the amplitude of the fluorescence change caused by recA protein binding (arbitrary units), and B is the maximum fluoresence of the recA protein-etheno M13 DNA complex. For the double-exponential fit in the presence of PEG, k_1 is the apparent first-order rate constant of the rapidly binding component of recA protein (s^{-1}), A_1 is the amplitude of the fluorescence change caused by the binding of this component (arbitrary units), k_2 is the apparent first-order rate constant of the slower binding portion component (s^{-1}), A_2 is the amplitude of fluorescence change caused by the binding of this component (arbitrary units), and B is the maximum fluorescence of the recA protein-etheno M13 DNA complex.

A comparison of best fit parameters indicates that the fraction of recA protein which binds rapidly in the presence of PEG associates with the DNA about six times more quickly than recA protein binding in the absence of PEG; the fraction of recA protein which binds slowly in the presence of PEG associates with the DNA more slowly than recA protein in the absence of PEG. The amplitude of the fluorescence change caused by recA protein binding is greater in the presence of PEG than in its absence; this is consistent with the higher relative fluorescence increase values obtained from salt titrations. Thus, in the presence of PEG the more rapid association of some recA protein with ssDNA could contribute to the increased ability of the recA protein to compete with SSB protein for ssDNA binding.

DISCUSSION

To simulate the *in vivo* effects of macromolecular crowding we examined *in vitro* activities of recA protein in solutions containing the volume-occupying agents PVA or PEG. Although recA protein-promoted DNA strand exchange in "dilute" solution requires elevated magnesium ion concentration, we find that the presence of PVA or PEG allows the reaction to proceed effectively at 1 mM magnesium acetate. In the presence of PEG, both the rate of recA protein association with ssDNA and the steady-state affinity of recA protein for ssDNA are increased; consequently, the ability of recA protein both to compete with SSB protein for ssDNA and to bind to ssDNA-containing secondary structures is enhanced. Hence, at 1 mM magnesium acetate, the addition of PVA or PEG to the strand exchange reaction both eliminates the inhibitory effect of SSB protein on presynaptic complex formation and enables recA protein to form more fully saturated presynaptic complexes in the absence of SSB protein. Moreover, in the presence of these macromolecules, subsequent joint molecule formation is no longer dependent on elevated magnesium ion concentration.

The binding of recA protein to ssDNA and ATP results in the formation of presynaptic complexes that are capable of participating in DNA strand exchange. Because a consequence of this binding is the hydrolysis of ATP, presynaptic complex formation can be monitored by measuring ATP hydrolysis. At 1 mM magnesium acetate, ssDNA-dependent ATP hydrolysis is inhibited rapidly by the addition of SSB protein; under these conditions, recA protein is displaced from ssDNA by SSB protein (Kowalczykowski *et al.*, 1987) and is therefore unable to form presynaptic complexes (Roman and Kowalczykowski, 1986). At 1 mM magnesium acetate, the addition of PVA or PEG to the ATPase reaction results in a concentration-dependent reduction in the inhibitory effect of SSB protein, and ultimately, the effect of SSB protein addition becomes negligible. Thus, at 1 mM magnesium acetate, recA protein is no longer displaced from ssDNA by SSB protein in the presence of PVA or PEG, *i.e.* presynaptic complex formation can occur.

The resistance of recA protein to displacement by SSB protein in the presence of PVA or PEG signifies a heightened ability in the competition with SSB protein for DNA binding sites. An examination of association rates indicates that a fraction of the recA protein present associates with ssDNA more rapidly in solution containing PEG. Because an increased rate of recA protein association is correlated with an increased ability in the competition with SSB protein (see Kowalczykowski, 1991b), this likely contributes to the enhanced activity observed in volume-occupied solution. Additionally, an examination of the salt concentration required to disrupt recA protein-ssDNA complexes indicates that the steady-state affinity of recA protein for ssDNA is increased in solution containing PEG. This possibly also contributes to the resistance of recA protein to displacement by SSB protein.

The inability of recA protein to compete with SSB protein during the formation of presynaptic complexes is not the only obstacle to DNA strand exchange at 1 mM magnesium acetate. The lack of DNA strand exchange by mutant recA proteins that can compete with SSB protein under these conditions indicates that steps subsequent to presynapsis require elevated magnesium ion concentration (see Lavery and Kowalczykowski, 1990). Magnesium ion concentration affects the aggregation state of recA protein (Brenner *et al.*, 1988; Cotterill and Fersht, 1983; Roman and Kowalczykowski, 1986), and the formation of a specific recA protein aggregate is thought to be necessary for the synapsis of presynaptic complexes with dsDNA (Radding, 1988). Consistent with this, the addition of spermidine, which is known to precipitate recA protein at high concentrations (Griffith and Shores, 1985), reduces the magnesium ion concentration required for DNA strand exchange (Lavery and Kowalczykowski, 1990). Along these same lines, Minton (1981, 1983) predicted that the aggregation state of self-associating proteins increases, and filament-filament interactions are favored in volume-occupied solution. Thus, like spermidine, the addition of PVA or PEG at 1 mM magnesium acetate must induce the formation of the recA protein aggregate required for synapsis and subsequent DNA strand exchange; *i.e.* the presence of these macromolecules must promote sufficient self-association (and therefore filament-filament interactions) for synapsis so as not to require elevated magnesium ion concentration.

Although spermidine and volume-occupying agents reduce the magnesium ion concentration required for DNA strand exchange, they do not eliminate the need for the presence of

magnesium. Thus, magnesium ion must function at two levels in the DNA strand exchange process. Although the binding of recA protein to DNA does not require magnesium, ATP hydrolysis is dependent on magnesium ion (Ogawa *et al.*, 1978). Presumably, it is an ATP-magnesium ion complex that binds to recA protein and induces the high affinity ssDNA binding state necessary for joint molecule formation (Menetski *et al.*, 1988). Thus, there is an absolute requirement for magnesium ion to complex with ATP and recA protein during DNA strand exchange. Although this complex is necessary for DNA strand exchange, it is not sufficient. Additional magnesium ion is needed to induce the formation of a specific recA protein aggregate that is necessary for synapsis; however, in contrast to the absolute requirement for magnesium ion during presynaptic complex formation, spermidine or volume-occupying agents can substitute for magnesium in the latter synaptic function. Thus, we would propose that there are two distinct functions for magnesium ion in the recA protein-promoted DNA strand exchange reaction; the first is presynaptic, with magnesium ion playing an essential role in formation of the high affinity state complex; the second is synaptic, with elevated magnesium ion concentrations playing an essential role in the formation of the aggregate needed for homologous pairing.

It has been demonstrated that recA protein-promoted DNA strand exchange is significantly reduced when SSB protein is omitted from the reaction (Cassuto *et al.*, 1980; McEntee *et al.*, 1980; Shibata *et al.*, 1980; Cox *et al.*, 1983). At 1 mM magnesium acetate in volume-occupied solution, the omission of SSB protein does not affect joint molecule formation in the dramatic fashion that it does at elevated magnesium ion concentration. Under these conditions, the most marked effects of SSB protein omission are an inhibition of form II DNA product molecule formation and an increased appearance of homology-dependent DNA networks. Because the observed rate of ATP hydrolysis by recA protein is quite high in the absence of SSB protein and is not increased by its presence under these conditions, SSB protein does not appear to be aiding recA protein in the formation of saturated presynaptic complexes, as has been proposed to occur at elevated magnesium ion concentration (Munniyappa *et al.*, 1984; Kowalczykowski and Krupp, 1987). Instead SSB protein appears to be needed either to extend the region of heteroduplex DNA, producing the form II DNA molecules of complete strand exchange, or to suppress the reinitiation reactions that lead to the formation of homology-dependent DNA networks and are inhibitory to the resolution of form II DNA (see Chow *et al.*, 1988). These ideas are addressed more fully in the accompanying paper (Lavery and Kowalczykowski, 1992).

At 1 mM magnesium acetate, buffer containing either PVA or PEG can support DNA strand exchange by recA protein. However, probably because of the differences in the sizes of these polymers, the reaction is not identical in the presence of PVA and PEG. Although joint molecule intermediate species appear at comparable rates in PVA and PEG, their conversion to form II DNA product molecules tends to be more effective in buffer containing PVA; more intermediate species accumulate in PEG. This is best explained by comparing ssDNA-dependent ATPase activity in the presence of these two macromolecules. The observed rate of ATP hydrolysis is less in the presence of PEG; however, the k_{cat} for ATP hydrolysis by recA protein is the same in the presence of these two polymers (see Lavery and Kowalczykowski, 1992). This indicates that less recA protein is bound to the ssDNA in the presence of PEG, *i.e.* presynaptic complexes are less saturated with recA protein. These observations suggest that regions devoid of recA protein in the presynaptic complex are not inhibitory to joint molecule formation; however, these discontinuities do impede the branch migration that converts the joint molecules to product molecules.

Another difference observed when PEG is added to the DNA strand exchange reaction in place of PVA is that homology-dependent DNA networks are formed more slowly in the presence of PEG. These networks are formed when recA protein reinitiates successive rounds of pairing between the ssDNA displaced from one joint molecule and the duplex region of another; SSB protein suppresses this network formation, presumably by coating the displaced DNA strand and inhibiting reinitiation reactions (Chow *et al.*, 1988). Under conditions in which recA protein can rapidly displace SSB protein from ssDNA, network formation occurs even when SSB protein is present in the strand exchange reaction (see Lavery and Kowalczykowski, 1990). Along these lines, recA protein displaces SSB protein from ssDNA more slowly in the presence of PEG than in the presence of PVA (data not shown), which accounts for the significantly slower network formation observed in reactions containing PEG. In volume-occupied solution, the most extensive homology-dependent DNA network formation is, as expected, observed in reactions that do not contain SSB protein, in which recA protein can bind easily to the ssDNA displaced from joint molecules.

Although the specifics of the reaction are not identical in the presence of PVA and PEG, the addition of either of these macromolecules enables recA protein to promote DNA strand exchange at the otherwise nonpermissive magnesium ion concentration of 1 mM. The inhibitory effect of SSB protein on the formation of recA protein-ssDNA presynaptic complexes is alleviated, and subsequent synapsis and strand exchange are no longer dependent on elevated magnesium ion concentration. Additionally, SSB protein does not appear to be aiding recA protein in the formation of saturated presynaptic complexes, and as a result, joint molecule formation is not greatly reduced by the omission of SSB protein under these conditions. Because biochemical studies conducted in volume-occupied solution may represent the *in vivo* situation more accurately, they should advance our understanding of biological processes. Hence, our examination of DNA strand exchange in volume-occupied solution suggests that a low *in vivo* magnesium ion concentration would not compromise the ability of recA protein to promote recombination reactions.

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