Stabilization of recA Protein–ssDNA Complexes by the Single-Stranded DNA Binding Protein of *Escherichia coli*[†]

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ABSTRACT: In vitro recombination reactions promoted by the recA protein of *Escherichia coli* are enhanced by the single-stranded DNA binding protein (SSB). SSB affects the assembly of the filamentous complexes between recA protein and ssDNA that are the active form of the recA protein. Here, we present evidence that SSB plays a complex role in maintaining the stability and activity of recA–ssDNA filaments. Results of ATPase, nuclease protection, and DNA strand exchange assays suggest that the continuous presence of SSB is required to maintain the stability of recA–ssDNA complexes under reaction conditions that support their recombination activity. We also report data that indicate that there is a functional distinction between the species of SSB present at 10 mM magnesium chloride, which enhances recA–ssDNA binding, and a species present at 1 mM magnesium chloride, which displaces recA protein from ssDNA. These results are discussed in the context of current models of SSB conformation and of SSB action in recombination activities of the recA protein.

The recA protein of *Escherichia coli* is required for homologous recombination and DNA repair functions in vivo. In vitro, the recA protein promotes DNA strand exchange reactions between homologous molecules of single-stranded and duplex DNA. These reactions serve as models for homologous recombination since they proceed through synapsis and branch migration steps similar to those that are thought to occur during recombination in vivo (Radding, 1982; Cox & Lehman, 1987).

The active species in DNA strand exchange reactions is a filamentous complex of recA protein and ssDNA[1] (Cox & Lehman, 1982; Flory et al., 1984; Kahn & Radding, 1984). Presynapsis, the formation of this recA–ssDNA complex, is the first step in the DNA strand exchange pathway. Other activities of the recA protein, including ATP hydrolysis and repressor cleavage, are also activated by the binding of recA protein to ssDNA (Roberts et al., 1978; Weinstock et al., 1979).

Genetic evidence indicates that the single-stranded DNA binding protein (SSB) participates in recA-associated recombination and repair functions in *E. coli* (Glassberg et al., 1979; Vales et al., 1980; Whittier & Chase, 1981). This is supported by biochemical data that indicate that SSB plays an accessory role in these processes. SSB is an effector of the in vitro activities of the recA protein. During the presynapsis phase of DNA strand exchange reactions, SSB exerts a positive effect on the binding of recA protein to ssDNA (Flory & Radding, 1982; Cox et al., 1983a; Griffith et al., 1984). As a result, SSB increases the rate, final extent, and energetic efficiency of heteroduplex formation in these reactions (Cox & Lehman, 1981, 1982; Cox et al., 1983a,b).

Detailed studies of the presynapsis reaction have led to a preliminary understanding of the mechanism of SSB action in recombination, but many aspects of SSB action are con-

troversial or have yet to be clarified. It is clear that secondary structure in ssDNA limits the binding of recA protein to ssDNA (Muniyappa et al., 1984; Tsang et al., 1985). Under conditions required for recombination in vitro, SSB helps recA protein overcome secondary structure and bind to ssDNA. Under the same conditions, SSB interacts continuously with recA–ssDNA complexes (Morrical et al., 1986). The significance of this interaction is unclear, since recombination may be stimulated without SSB by choosing conditions that minimize ssDNA secondary structure (Muniyappa et al., 1984; Tsang et al., 1985; Kowalczykowski et al., 1987).

Other questions remain about the relationship between recA–ssDNA and SSB–ssDNA interactions during recombination. The binding of these two proteins to ssDNA is mutually exclusive under many conditions in vitro (Kowalczykowski & Krupp, 1987), suggesting that recA protein must displace SSB from ssDNA during the course of presynapsis. Conversely, other experiments have indicated that stoichiometric amounts of both proteins may remain bound to ssDNA in intact presynaptic complexes (Morrical et al., 1986). A final issue concerns the conformation of SSB involved in recombination-related reactions. SSB may assume one of at least four stable or metastable conformations at different salt concentrations, each with different ssDNA binding properties (Griffith et al., 1984; Lohman & Overman, 1985; Lohman et al., 1986a; Bujalowski & Lohman, 1986). These forms of SSB may have varying capacities to interact with recA–ssDNA complexes.

We designed experiments to address two questions about the role of SSB in recombination: What is the functional significance of SSB in reactions with recA–ssDNA? Also, how do different SSB conformations affect recA–ssDNA interactions? Our results indicate that the continuous presence of

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, *E. coli* single-stranded DNA binding protein; ϕX174(+), circular single-stranded genome of bacteriophage ϕX174; M13*mp*8(+), circular single-stranded genome of bacteriophage M13*mp*8; RFIII, linear dsDNA (substrate for DNA strand exchange reactions); RFII, nicked circular dsDNA (heteroduplex product of DNA strand exchange reactions); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TCA, trichloroacetic acid.

SSB is essential for maintaining the stability and activity of recA–ssDNA complexes. The data also indicate that a specific high-salt form of SSB may be important in recombination functions.

MATERIALS AND METHODS

E. coli recA protein (37.8 kDa/monomer) was purified as previously described (Cox et al., 1981) and was stored frozen at −70 °C in 20 mM Tris, 80% cation (pH 7.5), 1 mM DTT, 0.1 mM EDTA, and 10% glycerol. recA protein concentration was determined by the absorbance at 280 nm on the basis of an extinction coefficient of ϵ_{280} = 0.59 A_{280} mL mg^{-1} (Craig & Roberts, 1981). *E. coli* SSB protein (18.9 kDa/monomer) was purified by the method of Lohman et al. (1986b) and was stored frozen at −70 °C in either of two buffers: Buffer A contained 50 mM imidazole, 72% cation (pH 6.5), 0.15 mM NaCl, 1 mM EDTA, 1 mM DTT, and 20% glycerol. Buffer B contained 20 mM Tris, 40% cation (pH 8.4), 0.15 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 50% glycerol. SSB concentration was determined by the absorbance at 280 nm on the basis of an extinction coefficient of ϵ_{280} = 1.5 A_{280} mL mg^{-1} (Lohman et al., 1986b). M13*mp*8(+) and φX174(+) ssDNA species were prepared according to Messing (1983) and Cox and Lehman (1981), respectively. Supercoiled M13*mp*8 dsDNA was prepared according to Maniatis et al. (1982). All DNA solutions were stored at 0 °C in 10 mM Tris, 80% cation (pH 7.5), and 1 mM EDTA. Concentrations of ssDNA were determined by the absorbance at 260 nm with 36 μg mL^{-1} A_{260}^{-1} as a conversion factor. For dsDNA, the conversion factor was 50 μg mL^{-1} A_{260}^{-1}. All DNA concentrations are expressed in nucleotides. All other biomolecules and enzymes were purchased from Sigma.

Instrumentation. Absorbance measurements were obtained on a Perkin-Elmer Lambda 7 double-beam recording spectrophotometer. The two six-place cuvette holders were thermojacketed and attached to a constant-temperature water circulator. Cell path length and band-pass were equal to 1 cm and 2 nm, respectively, in all experiments.

Fluorescence measurements were obtained on an SLM Instruments 8000 series fluorometer equipped with a thermojacketed cuvette holder and constant-temperature water circulator and with a magnetic stirring motor for the sample chamber. The lens to sample focal length was 2 in., and the mean beam path lengths were 128 (source to sample) and 105 cm (sample to photomultiplier). Signal acquisition time was fixed at 5 s in all experiments. Cell path length and band-pass were equal to 1 cm and 2 nm, respectively.

ATPase Assays. ATP hydrolysis by the recA protein was monitored by a coupled spectrophotometric assay employing pyruvate kinase and lactate dehydrogenase (Morrical et al., 1986). The coupling system contained 2.24 units/mL pyruvate kinase and lactate dehydrogenase (Sigma unit definitions), 2.31 mM phosphoenol pyruvate, 1.0 mM NADH, and 0.44 mM KCl, final concentrations, in reaction buffer. Initial velocities of ATP hydrolysis were calculated from the change in NADH absorbance at 370 nm with an extinction coefficient of ϵ_{370} = 2.64 mM^{-1} cm^{-1} for NADH. All reactions were carried out at 37 °C in 1-mL cuvettes. The final reaction volume was equal to 0.91 mL in all experiments.

Reactions were started by the addition of recA protein to solutions containing 1.0 mM ATP, a selected concentration of ssDNA, and coupling system. Unless otherwise noted, standard reaction buffer contained 20 mM Tris, 80% cation (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, and 5% glycerol. In some experiments, the reaction buffer contained 1 mM MgCl$_2$ initially, and the magnesium concentration was later raised

to 10 mM with aliquots of 1.0 M MgCl$_2$. Additions of SSB were made approximately 1 min after the addition of recA protein or 1 min after the addition of MgCl$_2$ if the magnesium concentration was shifted during the reaction period.

Nuclease Protection Experiments. S1 nuclease filter binding assays for the formation of heteroduplex DNA in DNA strand exchange reactions were carried out by the procedure of Cox and Lehman (1982). All reaction mixtures contained 2.0 mM ATP, an ATP regenerating system containing 2.24 units/mL PK, 2.31 mM PEP, and 0.44 mM KCl (final concentrations), 4.0 μM [^{3}H]M13*mp*8(+) ssDNA (2.2 × 10^7 cpm/μmol, uniformly labeled), and 6.8 μM M13*mp*8 RFIII DNA (supercoiled M13*mp*8 DNA linearized by digestion with *Pst*I restriction endonuclease) in standard reaction buffer or in the same buffer containing 1 mM rather than 10 mM MgCl$_2$. The concentration of recA protein was 4.0 or 1.0 μM. All reactions were started by the addition of dsDNA. The order of addition of components was generally as follows: ATP, ssDNA, recA protein, SSB, and dsDNA. In certain experiments, the MgCl$_2$ concentration was shifted from 1 to 10 mM during the reaction as described under ATPase Assays. In these experiments, the additional MgCl$_2$ was added 8 min after the addition of recA protein and either prior to or simultaneous with the addition of dsDNA. Treatment of data was as follows: the total filter-bound counts from S1 nuclease treated control solutions containing no recA protein or SSB were subtracted from all data. One hundred percent protection was defined as the total filter-bound counts obtained from reaction mixtures treated with blank solutions containing no S1 nuclease. The extent of heteroduplex formation in individual reaction mixtures was determined relative to this value, after multiplying by a factor of 1.17 to correct for excess [^{3}H]ssDNA.

Protection by recA protein and SSB of ssDNA from DNase I degradation was monitored by a modification of the procedure of Pugh and Cox (1987). Reactions were carried out as described under ATPase Assays, uniformly tritium labeled M13*mp*8(+) ssDNA (1.6 × 10^7 cpm/μmol) being used as the binding lattice. At certain times, 25-μL aliquots were removed from the reaction mixtures and incubated with DNase I (10 Sigma units in 5 μL of reaction buffer) for 1 min at 37 °C. DNase I digestions were stopped by the addition of 40 μL of a solution containing 0.25 mg/mL calf thymus DNA and 0.375 M EDTA. Cold 10% trichloroacetic acid (0.9 mL) was then added to each digestion mixture, and the solutions were kept on ice for 30 min. Samples were applied to Whatman GF/C glass filters on a vacuum filtration apparatus. The filters were subsequently washed with 3 × 0.9 mL of cold 10% TCA and 2 × 0.9 mL of cold 95% ethanol. The filters were removed and dried under a heat lamp and then placed in glass scintillation vials and counted for tritium. This procedure resulted in 96% degradation of ssDNA in the absence of recA protein and SSB in buffers containing either 1 or 10 mM MgCl$_2$. Treatment of data was as follows: 100% protection was defined at a given concentration of ssDNA as the total tritium counts obtained from TCA precipitation and filtration of M13*mp*8(+) [^{3}H]ssDNA treated with a blank solution containing no DNase I. All data are plotted relative to this value. Background due to nonspecific filter retention of tritium was estimated by determining the total filter-bound counts from control reaction mixtures containing M13*mp*8(+) [^{3}H]ssDNA treated with DNase I in the absence of recA protein and SSB. The background was subtracted from all data and from the 100% protection control before the observed extent of protection in a given sample was calculated. This

background was <10% (typically 5%) of the 100% protection control in all experiments.

Fluorescence Titrations. Reverse titrations of SSB with ssDNA were monitored by quenching of tryptophan fluorescence in the manner previously described (Morrical et al., 1986). The excitation and emission wavelengths were 295 and 355 nm, respectively. All titrations were carried out at 37 °C in standard reaction buffer or in the same buffer containing 1 mM rather than 10 mM $MgCl_2$. Solutions contained 0.45 μM SSB, 1.0 mM ATP, and 0.44 mM KCl. Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, and NADH were omitted from the mixtures to avoid interference with fluorescence measurements. Titration curves were corrected for dilution caused by the addition of ssDNA to the solution. The initial volume was 2.0 mL in all experiments, and the correction for dilution was approximately 1.5% at the highest ssDNA concentration used. The fluorescence signal from a blank solution containing buffer, ATP, KCl, and SSB storage buffer (volume of buffer A or B equivalent to that added for a 0.45 μM SSB solution) was subtracted from all data. Under the conditions of these experiments, primary absorption by ssDNA and photobleaching of SSB tryptophan fluorescence are negligible (Morrical et al., 1986); therefore, our data do not include corrections for these effects. As a general precaution against photobleaching, exposure of a sample to the excitation beam was limited to 15–20 s while data were being acquired.

The corrected fluorescence signal of 0.45 μM SSB was normalized to a value of 1.0, and all data are plotted relative to this value. The apparent binding site size for SSB on ssDNA (n_{app}) was calculated as the ratio [ssDNA]/[SSB] at the end point of titration. Titration end points were estimated from the intersection of asymptotic lines drawn through the "plateau" region of a titration curve (SSB at low binding density) and through the linear descending portion of the curve (SSB at saturating levels).

RESULTS

Magnesium Shift Experiments. The homologous pairing, DNA strand exchange, and intrinsic ATPase activities of the recA protein are optimal in the presence of SSB and 10–13 mM Mg^{2+} ions (Cox et al., 1983a; Flory et al., 1984; Tsang et al., 1985; Morrical et al., 1986). Low Mg^{2+} concentrations do not support pairing or strand exchange. However, it is possible to stimulate strand exchange in the absence of SSB by preincubating recA protein and ssDNA at low magnesium (conditions in which ssDNA secondary structure is minimized) before running strand exchange reactions at a high magnesium concentration (Tsang et al., 1985). If the complexes resulting from such a magnesium shift are equivalent to those formed in the presence of SSB, an association of SSB with these complexes would clearly have no functional significance. We therefore wished to determine whether preincubation of recA protein with ssDNA at low [$MgCl_2$] would substitute completely for the effect of SSB in a reaction at high [$MgCl_2$].

The protocol followed is essentially that of Tsang et al. (1985). recA-ssDNA complexes were formed at a low magnesium concentration (1 mM $MgCl_2$). These complexes were incubated for 8–12 min at reaction temperature. This preincubation time is at least twice that required for the system to reach a reproducible steady state under these conditions.[2]

Table I: Data from Magnesium Shift ATPase Experiments[a]

expt	[MgCl$_2$]$_i$ (mM)	[MgCl$_2$]$_f$ (mM)	[SSB] (μM)	V_0 (μM min^{-1})[b]	no. of determinations
1	1	1	0	46.4 ± 1.3[e]	4
2	1	1	0.33	33.1 ± 0.6[c]	4
3	1	10	0	18.7 ± 0.3[d]	8
4	1	10	0.33	52.6 ± 0.6[e]	4
5	10	10	0	19.1 ± 0.2[e]	4
6	10	10	0.33	53.2 ± 1.8[e]	4

[a] ATP assays were carried out as described under Materials and Methods. All reaction mixtures contained 5.0 μM recA protein, 5.0 μM ssDNA, 1.0 mM ATP, and coupling system in reaction buffer (20 mM Tris, pH 7.5, 1 mM DTT, and 5% glycerol, plus the indicated concentration of $MgCl_2$). In experiments 3 and 4, [$MgCl_2$] was increased to 10 mM after an 8-min preincubation of recA protein and ssDNA with all other reaction components except SSB in reaction buffer containing 1 mM $MgCl_2$. In experiment 4, SSB was added 2 min after the increase in $MgCl_2$. In experiments 2 and 6, SSB was added after an 8-min preincubation of other components. i and f refer to initial and final, respectively. [b] Steady-state velocity of ATP hydrolysis measured by coupled assay. [c] Initial velocity after addition of SSB. Velocity decreases slowly with time from this value (data not shown). [d] Final velocity attained 20 min after addition of $MgCl_2$. The reaction has reached a steady state at this time, which is then maintained for at least 30 min. See text for details. [e] Steady-state velocities observed for 60 min or longer.

Longer preincubations did not affect the results. $MgCl_2$ was then added to a final concentration of 10 mM. The resulting complexes were compared over extended periods of time with complexes formed and maintained in either 1 or 10 mM $MgCl_2$ and in either the presence or absence of SSB. The comparison included detailed measurements of DNA strand exchange, DNase protection, and ATP hydrolytic activities. The use of ATP hydrolysis as an indirect measure of DNA binding is justified elsewhere (Morrical et al., 1986; Pugh & Cox, 1987; Brenner et al., 1987).

ATP Hydrolysis and ssDNA Binding. The effects of these treatments on rates of ATP hydrolysis are summarized in Table I. Experiments were conducted with a 4-fold excess of recA protein (assuming a binding stoichiometry of one recA monomer per four ssDNA nucleotide residues), so that changes in ATPase velocity primarily reflect changes in the number of binding sites available to recA protein. Control experiments conducted in 1 and 10 mM $MgCl_2$ (Table I, experiments 1 and 5) without SSB demonstrate the restriction in recA binding sites due to secondary structure at the higher magnesium concentration. When the $MgCl_2$ concentration was shifted from 1 to 10 mM, the rate of ATP hydrolysis promoted by the recA-ssDNA complex was initially as great as that observed prior to the magnesium shift (Figure 1). The advantage did not persist, however. The rate of ATP hydrolysis decreased (Figure 1), and after approximately 20 min, a final velocity was reached that was equal to the velocity in the control reaction at 10 mM $MgCl_2$ (Table I, experiments 3 and 5).

These results suggested that complexes formed by the magnesium shift protocol are unstable. This hypothesis is supported directly by the nuclease protection data presented in Figure 2. Experimental conditions were identical with those used in ATPase experiments. After $MgCl_2$ concentration was shifted from 1 to 10 mM, the level of protection of ssDNA from DNase I degradation afforded by the recA protein decreased from approximately 90% protection (the control value observed for complexes formed in 1 mM $MgCl_2$) to approximately 70% protection (the control value observed for complexes formed in 10 mM $MgCl_2$). The transition occurs over the same time interval required to reach the final ATPase

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[2] Steady state, at 1 mM $MgCl_2$, is represented by a linear time course for ATP hydrolysis achieved 4–6 min after addition of recA protein. This steady state was maintained for at least the duration of the control experiments, typically 30–60 min.
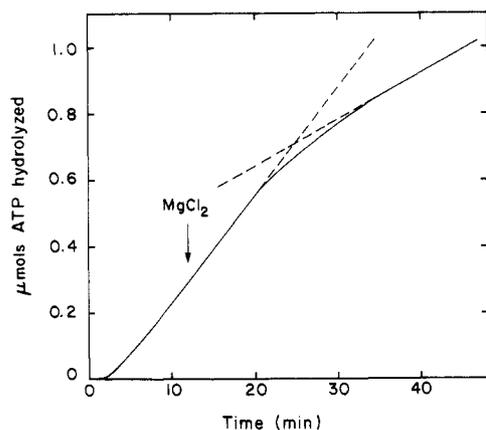
FIGURE 1: Time course of ATP hydrolysis during magnesium shift experiment. ATP hydrolysis was measured as described under Materials and Methods. The reaction mixture contained 5.0 μM recA protein, 5.0 μM ssDNA, 1.0 mM ATP, and coupling system in reaction buffer (20 mM Tris, pH 7.5, 1 mM MgCl$_2$, 1 mM DTT, and 5% glycerol). An aliquot of 1.0 M MgCl$_2$ was added to the reaction mixture at the indicated time to yield a final concentration of 10 mM MgCl$_2$.
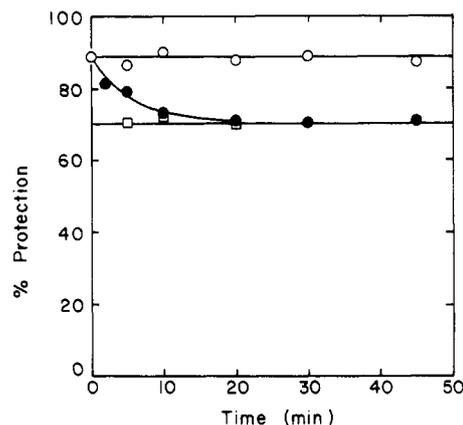

FIGURE 2: DNase I protection of ssDNA after increase in [MgCl$_2$]. The concentration of MgCl$_2$ in preincubated mixtures of recA–ssDNA complexes was shifted from 1 to 10 mM at time zero, and timed aliquots were removed and assayed for protection of ssDNA from DNase I degradation as described under Materials and Methods. Reaction mixtures contained 5.0 μM recA protein, 5.0 μM [^{3}H]-ssDNA, 1.0 mM ATP, and the coupling system for ATPase assays in reaction buffer (20 mM Tris, pH 7.5, 1 mM DTT, 5% glycerol, and the indicated concentration of MgCl$_2$). (●) indicates magnesium shift reaction (8-min preincubation of all components in reaction buffer containing 1 mM MgCl$_2$ followed by addition of MgCl$_2$ to 10 mM final concentration). (O) and (□) denote control reactions at constant MgCl$_2$ concentrations of 1 and 10 mM, respectively.

velocity following the addition of MgCl$_2$. These data are consistent with a net dissociation of recA protein from ssDNA following an increase in [MgCl$_2$] in the absence of SSB.

The effect of SSB on the velocity of recA protein catalyzed ATP hydrolysis is strongly dependent on the concentration of MgCl$_2$ as shown in Table I. Addition of SSB to recA–ssDNA complexes in 1 mM MgCl$_2$ (Table I, experiment 2) inhibits ATP hydrolysis, causing a decrease in velocity. This phenomenon is discussed further below. The highest rates of ATP hydrolysis were observed in the presence of SSB in 10 mM MgCl$_2$ (Table I, experiment 6). As reported previously (Morrical et al., 1986), SSB strongly stimulates the ATPase activity of recA–ssDNA under these conditions. When SSB is added to recA–ssDNA complexes following a 1–10 mM shift in MgCl$_2$ concentration (Table I, experiment 4), ATP hydrolysis is stimulated to the same extent. The final steady-state rate of ATP hydrolysis obtained is the same whether the ad-

Table II: Data from DNA Strand Exchange S1 Nuclease Assays[a]

expt	[recA] (μM)	[SSB] (μM)	[MgCl$_2$]$_i$ (mM)	[MgCl$_2$]$_f$ (mM)	rel extent[b]
1	4.0	0.4	10	10	1.00
2	4.0	0	10	10	0.30
3[c]	4.0	0	1	10	0.64
4[d]	4.0	0	1	10	0.35
5	4.0	0	1	1	0.03
6	1.0	0.4	10	10	0.77
7	1.0	0	10	10	0.08
8[c]	1.0	0	1	10	0.23
9[d]	1.0	0	1	10	0.10
10	1.0	0	1	1	0.01

[a]S1 nuclease assays were carried out as described under Materials and Methods. All reaction mixtures contained 4.0 μM m13*mp*8(+) [^{3}H]ssDNA, 6.8 μM M13*mp*8 RFIII ds DNA, 2.0 mM ATP, a pyruvate kinase/phosphoenolpyruvate ATP-regenerating system, and the indicated concentrations of recA protein and SSB. Reaction buffer contained 20 mM Tris, pH 7.5, 1 mM DTT, 5% glycerol, and the indicated concentration of MgCl$_2$. All reactions were started by the addition of dsDNA. Data from all experiments were normalized with respect to the percent of heteroduplex formed at t = 60 min in experiment 1. [b]Relative extent of heteroduplex formation observed after a 60-min reaction period, as measured by protection from S1 nuclease. [c]Reaction started by addition of dsDNA simultaneous with the increase in [MgCl$_2$]. [d]Reaction started by addition of dsDNA 20 min after the increase in [MgCl$_2$].

dition of SSB takes place 1 or 20 min after the shift in MgCl$_2$ concentration (data not shown), indicating that the stimulatory effect of SSB is independent of the initial binding density of recA protein under these conditions. Furthermore, the rates of ATP hydrolysis are constant over a period of at least 1 h under these conditions (data not shown), indicating that the dissociation of recA protein described above in magnesium shift experiments does not occur when SSB is present.

We conclude that the enhanced reactivity of recA–ssDNA complexes after a Mg^{2+} shift is a transient phenomenon. SSB has a significant positive effect on the stability of these complexes, maintaining a higher recA protein binding density on the ssDNA.

DNA Strand Exchange Experiments. Does the stability of recA complexes afforded by SSB affect recombination activity? To address this question, we carried out Mg-shift DNA strand exchange experiments. recA–ssDNA complexes were formed in 1 mM MgCl$_2$, and reactions were started either at 0 or at 20 min after the magnesium concentration was increased to 10 mM. Preincubation conditions were identical with those described for ATPase and nuclease protection assays. These reactions were compared to experiments in which the recA–ssDNA complexes were formed de novo at 10 mM MgCl$_2$ in the presence or absence of SSB. Results of experiments employing M13*mp*8 DNA substrates are presented in Table II. Identical results were obtained with ϕX174 substrates. The highest rate of DNA strand exchange was obtained in 10 mM MgCl$_2$ and in the presence of SSB with either excess (Table II, experiment 1) or stoichiometric (experiment 6) amounts of recA protein with respect to the concentration of ssDNA. When reaction mixtures contained 4.0 μM recA protein (a 4-fold excess), preincubation of recA–ssDNA complexes in 1 mM MgCl$_2$ led to a significant improvement in the rate and extent of DNA strand exchange (compared to experiment 2, a control reaction in 10 mM MgCl$_2$ without SSB) when the strand exchange reaction was started by the simultaneous addition of dsDNA and MgCl$_2$ (Table II, experiment 3). The yield of heteroduplex DNA observed in the magnesium shift experiment was less than that observed in the presence of SSB, however. When strand exchange was started 20 min after the magnesium shift (Table

II, experiment 4), a rate essentially identical with the control reaction without SSB (experiment 2) was observed. Since 20 min is the time required for the full extent of recA protein dissociation that we observe after a magnesium shift, it is evident that destabilization of the recA-ssDNA complex by increases in [MgCl₂] severely impairs the ability of the recA protein to carry out DNA strand exchange, even when recA protein is present in large excess. The effect of SSB is even more apparent in experiments employing stoichiometric amounts of recA protein (Table II, experiments 6-10). The rate of strand exchange obtained when dsDNA and MgCl₂ are added simultaneously (Table II, experiment 8) is much lower than that observed with excess recA protein (experiment 3) and only 30% of that observed in the presence of SSB (experiment 6). Again, the results obtained when strand exchange is initiated 20 min after the magnesium shift (Table II, experiment 9) are essentially identical with those obtained with recA-ssDNA complexes formed de novo at 10 mM MgCl₂ in the absence of SSB (experiment 7). At both recA protein concentrations, no significant heteroduplex DNA is formed during a 1-h reaction period in 1 mM MgCl₂ (Table II, experiments 5 and 10).

These results indicate that preincubation of recA-ssDNA complexes at low magnesium concentrations does not substitute for SSB in stimulating DNA strand exchange reactions of the recA protein. The enhanced stability of recA-ssDNA complexes formed in the presence of SSB translates directly into an improvement in the DNA strand exchange reaction. In the absence of SSB, the best strand exchange reactions are obtained when recA protein is present in excess and recA-ssDNA complexes are formed by the magnesium shift protocol. SSB appears to be nearly essential for reactions carried out at stoichiometric concentrations of recA protein.

Single-Stranded DNA Challenge Experiments. Fluorescence experiments described previously (Morrical et al., 1986) suggested that SSB migrates rapidly from recA-ssDNA complexes to free ssDNA when that ssDNA is added as a challenge. On the basis of the results described above, we expected that a challenge with ssDNA would reduce recA-ssDNA complex stability and therefore ATP hydrolysis catalyzed by the recA protein. Data presented in Table III show that this is the case. In these experiments, the initial ssDNA concentration is in excess relative to recA protein (one recA monomer per eight nucleotides), so that recA protein is saturated with potential ssDNA binding sites both before and after the challenge. The concentration of SSB is sufficient to saturate the initial concentration of ssDNA (1 SSB monomer per 15 nucleotides (Lohman & Overman, 1985; Lohman et al., 1986; Bujalowski & Lohman, 1986). Therefore, the SSB will bind to all the free ssDNA in the initial reaction mixture (minus half of the total ssDNA) with sufficient SSB remaining to stabilize the recA-ssDNA complexes that form on the rest of the ssDNA. Increasing the [ssDNA] by 2-fold, however, will provide enough free ssDNA to sequester all of the SSB, rendering it unavailable to the recA-ssDNA complex. Controls (Table III, experiments 1 and 2) established the ATPase velocities for complexes formed de novo at the initial and final ssDNA concentrations and with the indicated concentrations of recA protein and SSB. The lower rates of ATP hydrolysis observed at the higher ssDNA concentration reflect the expected unavailability of SSB to the recA-ssDNA complexes. In separate experiments (Table III, experiments 3 and 4), the complexes were formed and preincubated for 8-12 min at the lower ssDNA concentration. (Steady state is reached 5 min after recA protein addition under these conditions.)

Table III: Effects of ssDNA Challenge on recA Protein Binding to ssDNA As Measured by ATP Hydrolysis[a]

expt	[ssDNA]ᵢ (μM)	[ssDNA]f (μM)	V_0 (μM min⁻¹)[b]	no. of determina- tions
1	10	10	37.2 ± 0.6	6
2	20	20	22.2 ± 0.5	3
3	10	20	19.8 ± 1.5[c]	6
4	10	20	37.5 ± 1.0[d]	3

[a] ATPase assays were carried out as described under Materials and Methods. All reaction mixtures contained 1.25 μM recA protein, 0.67 μM SSB, 1.0 mM ATP, coupling system, and the indicated amount of ssDNA in standard reaction buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DTT, and 5% glycerol). In experiments 3 and 4, ssDNA concentration was increased to 20 μM after an 8-min preincubation of recA protein, SSB, and 10 μM ssDNA with all other reaction components. [b] Steady-state velocity of ATP hydrolysis measured by coupled assay. [c] Final velocity attained 10-12 min after addition of excess ssDNA. [d] Additional SSB added after addition of excess ssDNA. (Final SSB concentration equals 1.33 μM.)
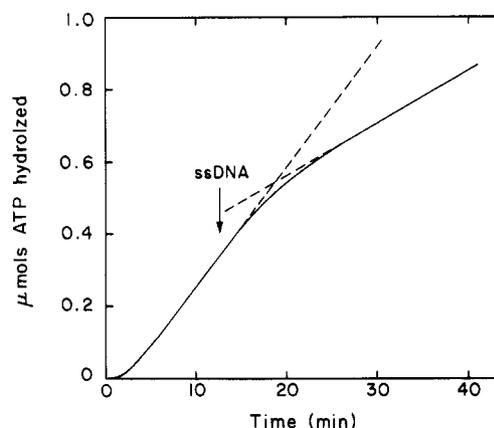

FIGURE 3: Time course of ATP hydrolysis during ssDNA challenge experiment. ATP hydrolysis was measured as described under Materials and Methods. The initial reaction mixture contained 1.25 μM recA protein, 0.67 μM SSB, 1.0 mM ATP, 10 μM ssDNA, and coupling system in standard reaction buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM DTT, and 5% glycerol). At the indicated time, the concentration of ssDNA was increased to 20 μM.

When ssDNA was added to these preformed complexes (increasing the ssDNA concentration 2-fold), the velocity of ATP hydrolysis decreased over 10-12 min before reaching a final value approximately equal to the control value at high [ssDNA] (Figure 3 and Table III, experiment 3). Subsequent doubling of the SSB concentration to saturate all ssDNA in the reaction mixture restored ATPase activity to the original level (Table III, experiment 4). These results indicate that sequestering of SSB by excess ssDNA has a similar effect on recA protein catalyzed ATP hydrolysis as incubating recA-ssDNA complexes at high magnesium concentration after a Mg shift in the absence of SSB.

SSB Binding Conformation. Bujalowski and Lohman (1986) reported that SSB can assume at least four different binding conformations in its interaction with ssDNA, depending on the salt concentration of the reaction mixture. As reported in Table I, SSB inhibits ATP hydrolysis when added to recA-ssDNA complexes in 1 mM MgCl₂ but stimulates it in 10 mM MgCl₂. We wished to determine whether this difference in the effect of SSB reflected a difference in the binding mode of SSB under the specific conditions used in our experiments.

Fluorescence-monitored titrations of SSB with ssDNA were carried out in both the low-magnesium buffer system and in standard reaction buffer containing 10 mM MgCl₂. Titration
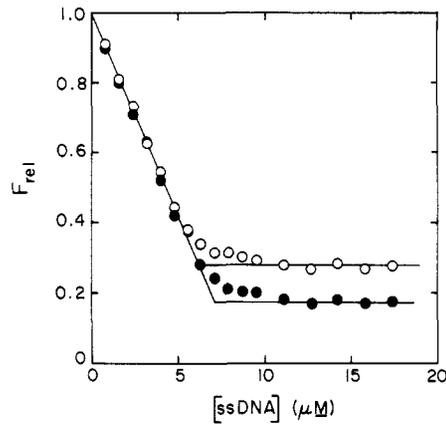
FIGURE 4: Fluorescence titrations of SSB with ssDNA. Fluorescence-monitored titrations of SSB with ssDNA were carried out as described under Materials and Methods. (●) denotes titrations carried out in buffer contained 10 mM $MgCl_2$. (O) denotes titrations carried out in buffer containing 1 mM $MgCl_2$.

curves are shown in Figure 4. The magnitude of fluorescence quenching at saturation was 82 ± 1% in 10 mM $MgCl_2$. In contrast, only 73 ± 1% quenching was seen in 1 mM $MgCl_2$. The apparent binding site size (n_{app}) of SSB was shifted to a lower value in 1 mM $MgCl_2$ as well: 55 ± 1 nucleotide residues per SSB tetramer in 1 mM $MgCl_2$ vs 64 ± 1 nucleotide residues per tetramer in 10 mM $MgCl_2$. These values may overestimate the actual site sizes because of exclusion of protein from regions of secondary structure present in native ssDNA (J. Kim and M. Cox, unpublished results). The data are consistent with a mixture of low-salt and high-salt conformations of SSB existing simultaneously in 1 mM $MgCl_2$ under the conditions used in the ATPase assays described above. Lohman and Overman (1985) and Bujalowski and Lohman (1986) previously reported that mixtures of SSB conformations may exist at intermediate concentrations of NaCl or $MgCl_2$. The data indicate that the inhibition of recA ATPase activity at low Mg^{2+} is correlated with a partial conversion of SSB to a low-salt ssDNA binding conformation.

DISCUSSION

Our results reveal several features of the mechanism by which SSB stimulates the in vitro recombination activity of recA protein: (1) SSB exerts a direct positive effect on the stability of recA–ssDNA complexes. This enhanced stability leads to improved recombination activity as measured by DNA strand exchange assays. (2) The enhancement of complex stability correlates with the continuous presence of SSB in a high-salt ssDNA binding conformation.

SSB's stabilizing effect on recA–ssDNA complexes is evident when compared with complexes formed by a magnesium shift procedure. Although preincubation in low magnesium increases the recA protein binding density, the resulting complexes are clearly not equivalent to those formed in the presence of SSB. The enhancement in ATP hydrolysis, DNase protection, and strand exchange activity is completely lost over a 20-min period due to the dissociation of recA protein. This observation is not in conflict with previous studies (Muniyappa et al., 1984; Tsang et al., 1985; Kowalczykowski et al., 1987), since the earlier work was limited to observations obtained within a few minutes of a magnesium shift. The deterioration of complexes is not observed when SSB is present and can be reversed if SSB is added after dissociation has occurred. When SSB is sequestered by addition of excess ssDNA, the deterioration of recA–ssDNA complexes is again observed. Thus, SSB blocks the dissociation of recA–ssDNA complexes under

conditions that favor the formation of secondary structure in ssDNA. The effect is functionally significant, at least in vitro, since the stabilization of complexes correlates completely with an improvement in DNA strand exchange activity.

Our conclusion that SSB stabilizes recA–ssDNA interactions is supported by protein exchange data (Neuendorf & Cox, 1986). The exchange of recA protein between recA–ssDNA complexes and a free pool of recA protein is almost completely inhibited in the presence of SSB. Our conclusion contrasts with that of Kowalczykowski et al. (1987), who used ssDNA challenge experiments to measure the migration of recA protein between polynucleotides. Their results indicated that SSB has no apparent effect on the ssDNA binding or dissociation properties of recA protein. However, we find that the addition of excess ssDNA in challenge experiments defeats the stabilizing effect of SSB on recA–ssDNA complexes. This is because SSB binds rapidly and preferentially to naked ssDNA (Morrical et al., 1986) and is effectively sequestered. Therefore, it is *probable* that the experiments of Kowalczykowski et al. (1987) did not detect the stabilization because of this sequestering effect.

Our observation that the continuous presence of SSB is required to maintain recA–ssDNA complex stability has two possible explanations: (a) The dissociation of recA protein from the nucleoprotein filament may require repeated action of SSB to remove DNA secondary structure and permit rebinding of recA protein. The result would be a cycle of SSB and recA protein binding and dissociation in a mutually exclusive interaction with ssDNA (Kowalczykowski & Krupp, 1987). (b) SSB might stabilize the recA nucleoprotein filament via a direct interaction between the two proteins. A direct interaction is suggested by the recA exchange study mentioned above (Neuendorf & Cox, 1986) and by studies of changes in SSB tryptophan fluorescence observed in the presence of recA protein and ssDNA (Morrical et al., 1986). The fluorescence data indicated that SSB associates with recA–ssDNA complexes throughout their steady-state lifetimes. Direct visualization of recA nucleoprotein complexes, however, has provided little or no evidence for a direct association of SSB with the recA protein (Thresher et al., 1988; Williams & Spengler, 1986). Either the association is weak (as suggested by the ssDNA challenge experiments presented here), or model a is correct.

Several studies have characterized the effect of salt on SSB–ssDNA interactions and have shown that different binding conformations of SSB exist in different ranges of salt concentration (Griffith et al., 1984; Lohman & Overman, 1985; Lohman et al., 1986a; Bujalowski & Lohman, 1986). Our data indicate that different SSB conformations may have quantitatively different effects on the activities of recA protein. The stabilization of recA–ssDNA complexes by SSB appears to require a specific high-salt conformation of SSB. It is evident that the relationship between SSB and recA protein is altered fundamentally by changes in salt and temperature parameters and by the nature of the lattice employed in binding studies. The implications of these effects for the in vivo function of recA protein and SSB are unclear; however, opportunities may exist for multiple pathways of complex formation in vivo. Through subtle changes in conditions, an *E. coli* cell could conceivably alter the relationship between recA protein and SSB, and thereby repress or activate recombination or repair functions.

The results of this study suggest that SSB has at least two important effects on recA protein's interactions with ssDNA. The first of these is the elimination of ssDNA secondary

structure as a barrier to recA binding, an activity that is not unique to SSB. Gene 32 protein, the helix-destabilizing protein of bacteriophage T4, is able to substitute for SSB in stimulating recombination activities of the recA protein in vitro (Muniyappa et al., 1984). Also, procedures such as the magnesium shift experiments discussed in this paper allow recA protein to overcome ssDNA secondary structure transiently. Second, SSB has a continuing effect on recA–ssDNA interactions, effectively stabilizing recA–ssDNA complexes after their formation in high magnesium. Therefore, it is possible that one important effect of SSB on recombination in vivo is to increase the active lifetime of recA–ssDNA complexes in a relatively high ionic strength environment. It is not known whether the T4 gene 32 protein has a similar activity. Further studies of these proteins and of their interactions with ssDNA are required before the detailed mechanism of homologous recombination can be understood.

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