Continuous Association of *Escherichia coli* Single-Stranded DNA Binding Protein with Stable Complexes of *recA* Protein and Single-Stranded DNA†

Scott W. Morrical, Jongwon Lee, and Michael M. Cox*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin—Madison, Madison, Wisconsin 53706

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ABSTRACT: The single-stranded DNA binding protein of *Escherichia coli* (SSB) stimulates *recA* protein promoted DNA strand exchange reactions by promoting and stabilizing the interaction between *recA* protein and single-stranded DNA (ssDNA). Utilizing the intrinsic tryptophan fluorescence of SSB, an ATP-dependent interaction has been detected between SSB and *recA*-ssDNA complexes. This interaction is continuous for periods exceeding 1 h under conditions that are optimal for DNA strand exchange. Our data suggest that this interaction does not involve significant displacement of *recA* protein in the complex by SSB when ATP is present. The properties of this interaction are consistent with the properties of SSB-stabilized *recA*-ssDNA complexes determined by other methods. The data are incompatible with models in which SSB is displaced after functioning transiently in the formation of *recA*-ssDNA complexes. A continuous association of SSB with *recA*-ssDNA complexes may therefore be an important feature of the mechanism by which SSB stimulates *recA* protein promoted reactions.

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INTERACTION OF SSB WITH recA-ssDNA COMPLEXES

ATP (Cox & Lehman, 1982; Radding, et al., 1983); (2) synapsis or D-loop formation, the alignment of homologous sequences and formation of a nascent heteroduplex joint between the reactant molecules (McEntee, et al., 1979; Shibata, et al., 1979; Cox & Lehman, 1981a; DasGupta & Radding, 1982); and (3) polar branch migration, the unidirectional extension of the heteroduplex region, coupled to the hydrolysis of ATP (Cox & Lehman, 1981a; Kahn, et al., 1981; West, et al., 1981). This system provides an in vitro model for the homologous pairing (synapsis) and heteroduplex extension (branch migration) phases of homologous recombination events that occur in the cell.

The single-stranded DNA binding protein (SSB) of Escherichia coli enhances both the rate and final extent of heteroduplex formation in the strand exchange reaction (Cox & Lehman, 1981a; Cox & Lehman, 1982; Wu, et al., 1982). This effect is intriguing in light of the demonstrated importance of SSB in homologous recombination (Johnson, 1977; Glassberg, et al., 1979; Vales, et al., 1980) and in DNA replication and repair processes in vivo (Glassberg, et al., 1979; Whittier & Chase, 1981). Previous studies have shown that SSB enhances strand exchange by stabilizing the presynaptic recA-ssDNA complex (Cox, et al., 1983a; Radding, et al., 1983). The resulting complex has several properties that distinguish it from complexes formed in the absence of SSB. recA-ssDNA complexes formed in the presence of SSB have apparent dissociation half-times of greater than 90 min (Cox, et al., 1983a), a stability 20-30 times that observed for these complexes in the presence of ATP alone (McEntee, et al., 1981a; Bryant, et al., 1985). With the SSB-stabilized recA-ssDNA complexes as intermediates, branch migration replaces synapsis as the rate-limiting step of strand exchange, resulting in a higher rate and efficiency of heteroduplex formation. While SSB has a modest effect on the rate of the branch migration step promoted by recA protein, the efficiency of this step with respect to the stoichiometry of ATP hydrolysis is greatly enhanced by SSB (Cox, et al., 1983b). In the absence of SSB, several thousand molecules of ATP are hydrolyzed per heteroduplex base pair formed during branch migration. This ratio improves to 100-200 ATP molecules per base pair when SSB is present.

The mechanism by which SSB participates in strand exchange has been difficult to determine, primarily because intact recA-ssDNA complexes containing SSB have not been isolated or detected. Although Tsang, et al. (1985) recently isolated complexes containing both proteins, the amounts of recA protein and SSB present appeared to be inversely proportional, suggesting that partial dissociation of recA-ssDNA complexes had allowed some SSB to bind. As a result, the two models for the action of SSB proposed to date involve only a transient role for SSB in the formation of the recA-ssDNA complex. In one model, the stabilization of recA-ssDNA interactions is a result of the helix-stabilizing activity of SSB. It is proposed that SSB melts out secondary structure in ssDNA, allowing recA protein to bind, and is then displaced by the recA protein (Flory, et al., 1984; Muniyappa, et al., 1984). Therefore, in this model SSB acts passively via the ssDNA, without interacting with recA protein. A second model involves SSB as a specific assembly factor promoting the formation of stabilized recA-ssDNA complexes through interactions of the SSB and recA protein molecules prior to the displacement of SSB (Griffith, et al., 1984; Register & Griffith, 1985). Muniyappa, et al. (1984) reported that conditions that alternately promoted or inhibited the formation of secondary structure in ssDNA affected the recA protein dependent formation of D loops in reciprocal fashion. The strong correlation they observed between secondary structure and the rate of recA protein promoted synapsis in the absence of SSB supported the first (passive) model of SSB action. Muniyappa, et al. (1984) also reported that, like SSB, both the phage T4 gene 32 protein and the β protein of phage λ increase the rate and extent of D-loop formation. The ability of three different helix-stabilizing proteins to accelerate this reaction argued against specific interactions between recA protein and SSB as a means of recA-ssDNA complex stabilization. Evidence for recA-SSB interactions in the second model was provided by electron micrographic observations (Griffith, et al., 1984).

Results from a number of other studies are not easily explained by a model involving only a transient participation of SSB in complex formation. First, the order of addition of recA protein and SSB has a remarkable effect on the rate of strand exchange (Cox & Lehman, 1982; West, et al., 1982). When SSB was mixed with ssDNA prior to the addition of recA protein, a large time lag occurred in D-loop formation, indicating that the kinetically slow dissociation of the preformed SSB-ssDNA complex was necessary before SSB-primed recA-ssDNA complex formation could begin (Cox & Lehman, 1982). This order of addition effect is not unique to the strand exchange reaction of recA protein; hydrolysis of ATP and dATP in the absence of homologous duplex is strongly inhibited when SSB is added to solutions of ssDNA prior to the addition of recA protein or nucleotide (McEntee, et al., 1980; Cohen, et al., 1983). Although recA protein is capable of binding very tightly to ssDNA under conditions similar to those used for strand exchange (Morrical & Cox, 1985; Bryant & Lehman, 1985), these results indicate that displacement of cooperatively bound SSB from ssDNA by recA protein is very slow. Second, the strong enhancement of the rate of DNA strand exchange by SSB is observed even in the presence of large excesses of recA protein (Cox, et al., 1983a), and stoichiometric levels of SSB are required even when excess recA protein is utilized. The enhancement of the efficiency of branch migration in the presence of SSB suggests that this requirement may be continuous throughout strand exchange.

It is conceivable that the previous inability to detect SSB in intact recA-ssDNA complexes is a result of the techniques applied (electron microscopy and centrifugation), which insert several potentially disruptive sample handling steps between the formation of complexes and data acquisition. We therefore sought more sensitive and less disruptive techniques to study the interaction between SSB and recA-ssDNA complexes. The SSB protein contains four tryptophan residues per 18.9-kDa monomer (Horii et al., 1980; Sancar, et al., 1981) and shows strong tryptophan fluorescence in solution. Tryptophan fluorescence is strongly quenched upon binding of SSB to ssDNA; this property has been used to characterize the SSB-ssDNA interaction (Lohman & Overman, 1985; Lohman, et al., 1986). Fluorescence therefore appeared to be a potentially useful probe for studying SSB interactions with recA-ssDNA complexes. In this study, we present SSB tryptophan fluorescence data that indicate (1) that SSB is continuously associated with recA-ssDNA complexes in the presence of ATP during and following their assembly from components, (2) that the binding of SSB to these complexes...
does not involve significant displacement of recA protein when ATP is present, and (3) that therefore the effects of SSB on recA-promoted strand exchange cannot be entirely attributed to the passive helix-distabilizing activity of this protein or to a transient role of SSB in recA-ssDNA complex formation.

**EXPERIMENTAL PROCEDURES**

**Materials.** *E. coli* recA protein was purified as previously described (Cox et al., 1981) and stored frozen at −70 °C in buffer A [20 mM Tris, 80% cation (pH 7.5), plus 1 mM DTT, 0.1 mM EDTA, and 10% glycerol]. The concentration of recA protein in stock solutions was determined by the absorbance at 280 nm, using an extinction coefficient of ε_{280} = 0.59 A_{280} mg^{-1} ml^{-1} (Craig & Roberts, 1981). *E. coli* SSB protein was purified by a modification of a published procedure (Weiner et al., 1975) and stored frozen at −70 °C in buffer B [50 mM imidazole, 72% cation (pH 6.5), plus 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, and 20% glycerol]. SSB stock concentrations were determined by the absorbance at 280 nm, using an extinction coefficient of ε_{280} = 1.5 A_{280} mg^{-1} ml^{-1} (Lohman & Overman, 1985). φX174(+) circular ssDNA (5.4 kb) was prepared by a published procedure (Cox & Lehman, 1981a). M13mp8(+) circular ssDNA (7.2 kb) was prepared by a modification of the procedure of Messing (1983). All ssDNA solutions were stored at 0 °C in 10 mL Tris and 1 mM EDTA, pH 7.5. ssDNA stock concentrations were determined by the absorbance at 260 nm, using 36 μg mL^{-1} A_{260} as a conversion factor. All ssDNA concentrations are expressed in nucleotides. ATPγS was purchased from Boehringer Mannheim; all other biochemicals and enzymes were purchased from Sigma.

**Instrumentation.** All fluorescence measurements were obtained on an SLM Instruments 8000 series scanning fluorometer equipped with a thermojacketed cuvette holder attached to a constant-temperature water circulator, and with an externally controlled magnetic stirring motor for the sample chamber. This instrument has a focal length of 2 in. (less to sample) and mean beam path lengths of 128 cm (source to sample) and 105 cm (sample to photomultiplier). Signal acquisition time was fixed at 1 s in all experiments; cell path length and band-pass were equal to 1 cm and 2 nm, respectively.

Absorbance and turbidity measurements were obtained on a Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with a thermojacketed cuvette holder 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.** Protein fluorescence spectra were obtained at an excitation wavelength of 295 nm and emission wavelengths of 320–420 nm. SSB spectra were taken at 37 ± 0.1 °C in a standard reaction buffer consisting of 20 mM Tris, 80% cation (pH 7.5), 10 mM MgCl₂, 1 mM DTT, and 5% glycerol. SSB emission was maximal at 349 nm and is greatly enhanced with respect to an equal amount (4 SSB monomer equiv) of free tryptophan. The nucleotides ATP, ADP, and ATPγS have no effect on SSB fluorescence. Fluorescence emission spectra of the recA protein were taken in 40 mM rather than 20 mM Tris to avoid light-scattering interference with fluorescence measurements due to recA filament formation, which occurs at the lower ionic strength in the absence of nucleotides (Morrical & Cox, 1985; Cotterill & Fersht, 1983a). recA emission is maximal at 345 nm in this buffer system, and at 355 nm is slightly quenched with respect to an equal amount (2 recA monomer equiv; Sancar et al., 1980) of free tryptophan; 1 mM ATP, 1 mM ADP, and 0.2 mM ATPγS each produce a small (less than 5% at 355 nm) quenching effect on recA fluorescence. Quenching effects by nucleotides are included in control values described below and under Results. The effect of ssDNA on the recA fluorescence spectrum was determined in standard reaction buffer containing either 1.0 mM ATP or 0.2 mM ATPγS.

All other fluorescence studies were carried out under the following conditions, unless specifically noted. Excitation and emission wavelengths were fixed at 295 and 355 nm, respectively. A constant temperature of 37 ± 0.1 °C was maintained. Standard reaction buffer consisted of 20 mM Tris, 80% cation (pH 7.5), 10 mM MgCl₂, 1 mM DTT, and 5% glycerol. Standard reaction mixtures contained recA protein and SSB at various concentrations, 6.75 μM ssDNA [either φX174(+) or M13mp8(+)], and 1.0 mM ATP in a total volume of 2.0 mL. In all cases, duplicate experiments involving different ssDNA species gave identical results. Additions of all components were made to temperature-equilibrated cuvettes containing rapidly stirring reaction buffer to ensure fast mixing. The order of addition of reaction components used was generally ATP, ssDNA, recA protein, and SSB. When the time course for complex formation was monitored by fluorescence, zero time was taken as the instant of addition of the last component (usually SSB). Variations in the composition of reaction mixtures and changes in the order of addition are specifically noted under Results and in figure legends.

Reverse titrations of SSB with ssDNA were carried out in standard reaction buffer at 37 ± 0.1 °C. Small aliquots of ssDNA stock solutions (concentration 0.9–1.2 mM) were added sequentially to solutions of 0.45 μM SSB, with the resulting fluorescence intensity recorded after each addition. The total change in reaction volume resulting from titration additions was less than 1%; therefore, no correction was made for dilution. The estimation of equivalence points from these titrations is described in the text.

**Treatment of Data.** Relative fluorescence intensities were obtained as fluorometer output corrected for background (the signal obtained from a blank solution of standard reaction buffer). SSB emission spectra and reverse titration curves were corrected for residual fluorescence in SSB storage buffer (buffer B). The residual level was approximately 11% of the fluorescence signal of SSB when monitored at 355 nm. This residual is included in the control values F₀ and Fₙ in all other experiments. All fluorescence data reported in the text are normalized with respect to the fluorescence of 0.45 μM SSB at standard conditions, unless specifically noted. Data points in all figures are average values from at least two separate experiments. Line- and curve-fitting procedures are described in the text. The relative fluorescence of recA protein was determined in each reaction mixture by recording fluorescence intensity prior to the addition of SSB. Where order of addition changes made this impossible, recA fluorescence was measured independently in a separate solution. According to these procedures, F_{recA} incorporates any small quenching effects by nucleotides. The fluorescence of SSB in the presence and absence of ssDNA was determined independently in all experiments. The control value F₀ was determined by adding the relative fluorescence of recA protein to that of free SSB for experiments at fixed concentrations of recA and SSB. This value is taken as the initial fluorescence intensity (at time zero) in all time courses. When recA and/or SSB concentration was varied in an experiment, F₀ was determined independently at each protein concentration as the relative fluorescence of the recA–SSB mixture in the absence of ssDNA. In all cases,
the $F_0$ calculated from the relative fluorescence intensities of separate solutions of recA and SSB was equal to the $F_0$ observed in solutions containing both SSB and recA. The control value $F_0$ was calculated by adding the relative fluorescence of recA protein to the independently determined fluorescence of SSB in the presence of ssDNA.

**Effects of Photobleaching, Light Scattering, and Absorption on Fluorescence Measurements.** Observed fluorescence intensity can be affected by photobleaching of the chromophore or by absorption or scattering of the excitation light by other molecules in the solution. The system under investigation is a complex mixture of components, so detailed controls were carried out to evaluate the effect of the above factors.

Two approaches were taken to determine what fraction, if any, of fluorescence quenching effects was attributable to photobleaching of recA protein or SSB fluorescence. (1) Continuous exposure of solutions of recA protein and SSB in the absence of ssDNA to the excitation beam for periods of time up to 60–70 s resulted in no detectable loss of fluorescence intensity. This was true at all concentrations of recA protein and SSB used in these experiments, and in no experiment was a sample continuously exposed to the excitation beam for more than 70 s. (2) NaCl was added to samples quenched as a result of protein–ssDNA complex formation to dissociate the complexes and determine if the original fluorescence intensities could be recovered. Quenched and unquenched samples (with and without SSB, respectively) were exposed to the excitation beam for 60 s and then diluted with concentrated NaCl to a final NaCl concentration of 2 M. In all cases, the final fluorescence intensities of the two samples were identical. We could detect no significant photobleaching of samples in any case under the conditions of these experiments. As a general precaution against photobleaching, exposure of all samples to the source beam was held to 10 s or less when acquiring data, except during the first minute of time courses when more rapid data acquisition was required.

The sensitivity of fluorescence signals to changes in the turbidity of reaction mixtures was tested by measuring SSB fluorescence in solutions of known turbidity. Turbid solutions of recA filaments were generated as described by Morrical and Cox (1985). recA protein (0.5–2.0 μM) was incubated with 0.45 μM SSB for 15 min at 37 ± 0.1 °C in 10 mM Tris, 80% cation (pH 7.5), and 10 mM MgCl2. The apparent turbidities of these solutions and of solutions containing recA–ssDNA complexes formed in the presence of SSB were measured as the absorbance at 355 nm. The highest turbidity observed for recA filaments produced an $A_{355}$ of 0.12 at 2.0 μM recA protein. Relative fluorescence intensities of the same recA protein solutions containing SSB and of control solutions containing recA protein but lacking SSB were also measured as described above. Fluorescence due to recA protein alone was subtracted to give apparent SSB fluorescence in the turbid solutions. The observed fluorescence of SSB was independent of solution turbidity over the $A_{355}$ range of 0–0.12. The highest turbidity measured for solutions of recA protein–ssDNA formed in the presence of ATP and SSB was approximately 0.03 under standard reaction conditions. Since the turbidity of our reaction mixtures falls within the lower part of the $A_{355}$ range determined above, changes in fluorescence intensity observed in mixtures of recA, SSB, and ssDNA cannot be attributed to light scattering by the system.

The excitation wavelength, 295 nm, was chosen to minimize interference by nucleotide or nucleic acid absorption (inner filter effects). Decreases in fluorescence intensity due to inner filter effects were evaluated in the following way. Titration of N-acetyl-l-tryptophanamide solutions were carried out with the nucleic acid or nucleotide of interest (or both). No changes in fluorescence intensity were detected under standard experimental conditions with the highest concentrations of ssDNA used in these experiments. Decreases of approximately 3% per millimolar unit of nucleotides ATP, ADP, and ATPγS were observed; however, since the experimental control values utilized included the effects of nucleotides initially present on protein fluorescence, correction for primary absorption by nucleotides was necessary only when additional ATP, ADP, or ATPγS was added to a reaction mixture. Using the method of Birdsall et al. (1983), a correction of approximately 9% resulted at the highest nucleotide concentration used in these experiments.

**ATP Regeneration and ATPase Assay.** An ATP regenerating system consisting of 2.24 units/mL pyruvate kinase, 2.31 mM phosphoenolpyruvate, and 0.44 mM KCl was included in some reaction mixtures; 2.24 units/mL lactate dehydrogenase and 1.0 mM NADH were added to the regenerating system to form a coupled spectrophotometric assay system for recA ATPase activity. Decreases in NADH concentration as a function of time were monitored by the absorbance at 370 nm at a constant temperature of 37 ± 0.1 °C. NADH absorbance was measured at 370 nm rather than 340 nm (the absorbance maximum) in order to accommodate the high concentration of NADH needed for the coupled assay while remaining within the linear absorbance range of the spectrophotometer. Initial rates expressed as micromolar ADP produced per minute were calculated from $-\Delta A_{370}$ per minute data by using an extinction coefficient of $e_{370} = 2.64$ mM⁻¹ cm⁻¹ for NADH. All time courses were linear following an initial induction ($r$) time of 1–2 min. Slightly longer lag times were observed in the presence of SSB. Initial velocities were calculated from the linear portions of time courses since ATP is regenerated by the coupling system and [ATP] = [ATP], in the steady state. Doubling the concentration of pyruvate kinase and lactate dehydrogenase in the reaction mixtures had no effect on the observed rate of NADH oxidation; therefore, ATP hydrolysis by the recA protein was strictly rate limiting in the coupled assay. Reported $K_{m}$ values for ATP in recA protein promoted ATP hydrolysis range from 20 to 90 μM in the presence or absence of SSB (Weinstock et al., 1983a; Cox et al., 1988b). Doubling the ATP concentration from 1 mM to 2 mM had no significant effect (<3% increase) on the rate of ATP hydrolysis, so that ATP hydrolysis is being measured under maximum velocity conditions in all experiments reported. Reaction volumes of 0.91 mL were contained in 1-mL cuvettes. ATP hydrolytic reactions were started by the addition of recA protein to cuvettes containing buffer, ATP, ssDNA, and coupling system. SSB was added last, 1 min after the addition of recA protein. All other reaction conditions were as described for the fluorescence experiments.

**RESULTS**

**Interaction of SSB with ssDNA in the Absence or Presence of recA Protein**

**Fluorescence Properties of SSB and recA Protein.** The effects of ssDNA on the fluorescence spectra of SSB and recA protein in standard reaction buffer are presented in Figure 1. SSB fluorescence is strongly quenched by ssDNA. At low binding density (excess ssDNA), the extent of quenching exceeds 80% when monitored at 355 nm (Figure 2). With 1.0 mM ATP present, the emission spectrum of recA protein in the presence of stoichiometric amounts of ssDNA was indistinguishable from the spectrum in the absence of ssDNA.
Because of the relatively high fluorescence of SSB, and because of the additivity of SSB and recA fluorescence, changes in the SSB signal brought about by quenching can be easily detected above a high background of recA fluorescence.

MgCl₂ Induces the High-Salt Binding Mode of SSB. Lohman and Overman (1985) observed that SSB may assume one of at least two distinct ssDNA binding modes, depending on the NaCl concentration of the solution. The “high-salt” binding mode is characterized by a ssDNA binding site size of 65 ± 5 nucleotide residues per SSB tetramer (16 ± 1 residues per monomer) and 83 ± 3% quenching of tryptophan fluorescence in the ssDNA-bound protein. This binding mode exclusively is observed at NaCl concentrations greater than 0.1–0.2 M. The “low-salt” binding mode is characterized by a ssDNA binding site size of 33 ± 3 nucleotide residues per SSB tetramer (8 ± 1 residues per monomer) and 51 ± 3% quenching of tryptophan fluorescence in the ssDNA-bound protein. This binding mode exclusively is observed at NaCl concentrations less than 10 mM. It is postulated that (SSB)₃ (the low-salt form) arises through binding of ssDNA to two SSB monomers in a tetramer, while in (SSB)₆ (the high-salt form) ssDNA is wrapped to interact with all four monomers of the tetramer, giving twice the apparent binding site size and a higher degree of fluorescence quenching. The transition between the two forms at intermediate salt concentrations appears to be correlated with a decrease in the cooperativity parameter (ω) for SSB as the salt concentration is increased (Ruyechan & Wetmur, 1976; Lohman & Overman, 1985; Lohman et al., 1986).

Under the conditions employed in this study (particularly with 10 mM MgCl₂ replacing NaCl), and in the absence of recA protein, SSB interacts with ssDNA in its high-salt binding mode. Figure 2 shows a reverse fluorescence titration performed by adding incremental amounts of M13mp8(+) ssDNA to a constant total concentration of SSB. Identical results were obtained with φX174(+) ssDNA. The calculated apparent binding site size of SSB, n_app, is equal to the SSB concentration divided by the concentration of ssDNA obtained at the intersection of a line drawn through the “plateau” region of the binding curve (low binding density, SSB saturated with ssDNA) and the best line drawn through the linear descending portion of the curve. Five separate titrations gave an average value of 17 ± 2 nucleotide residues per SSB monomer (68 ± 8 residues per tetramer) for n_app, which is very close to the value obtained by Lohman and Overman (1985) under high-salt conditions.² In addition, quenching in the saturated mixture averaged 85 ± 2%, again well within experimental error of the value obtained by Lohman and Overman (1985) at high [NaCl]. These results establish that relatively low concentrations of MgCl₂ induce a binding mode of SSB approximately equivalent to that observed at high NaCl concentrations and demonstrate that interactions between SSB and free ssDNA occur via this binding mode under conditions routinely used in the study of recA protein promoted DNA strand exchange. These conclusions are consistent with results obtained elsewhere (T. Lohman, personal communication). Control Values for Fluorescence Measurements. All protein fluorescence data reported in subsequent sections were compared to two control values, determined independently for each

² A binding site size of 45 nucleotides per SSB tetramer is observed under these conditions when a poly(dT) lattice is utilized. The difference may reflect secondary structure unavailable to SSB binding in the polynucleotide employed here. The extent of fluorescence quenching observed is consistent with the “high-salt” binding mode for both M13mp8(+) and poly(dT) (T. Lohman, personal communication).
probably attributable to increased secondary structure in
compared to the 1 recA monomer per 4 nucleotide stoichiometry
protein binds to ssDNA with high affinity but at a reduced
number of sites (1 recA per 9-10 nucleotide residues) com-
concentration (40 mM Tris and
Absence
plexes formed in the absence of ATP, fluorescence decreases
These experiments were carried out at a relatively high buffer
with time to the level of
fluorescence intensities are equivalent to
fluorescence control", or simple $F_0$. This value represents the
relative fluorescence intensity from a solution containing given
concentrations of SSB and recA protein in the presence of all
other reaction components except ssDNA. $F_0$ therefore in-
cludes any small quenching effects of nucleotides on recA
protein and is equal to the sum of the intrinsic fluorescence
of SSB (in the absence of ssDNA) and the fluorescence of
recA protein in a reaction mixture. The second control value
will be referred to as "quenched fluorescence control", or
simply $F_Q$. This value represents the calculated sum of the
fluorescence of SSB in the presence of ssDNA and the
fluorescence of recA protein, both of which are determined
independently in each experiment in the presence of all other
non-protein reaction components. In practice, $F_Q$ is the
minimum possible fluorescence intensity in a given reaction
mixture (assuming no further quenching effects on recA
protein), attained when the SSB is maximally bound to the
ssDNA under the conditions of the experiment. $F_0$, on the
other hand, is the maximum possible fluorescence intensity
in a given reaction mixture, attained when all SSB is displaced
from ssDNA.

**SSB and recA Protein Bind ssDNA Competitively in the Absence of ATP.** On addition of SSB to recA-ssDNA
complexes formed in the absence of ATP, fluorescence decreases with time to the level of $F_0$ (Figure 3), suggesting that SSB has
displaced a stoichiometric amount of recA protein from the
ssDNA and coated the single strand as (SSB)$_{65}$. Final
fluorescence intensities are equivalent to $F_0$ at both saturating
and subsaturating concentrations of SSB in these experiments.
These experiments were carried out at a relatively high buffer
concentration (40 mM Tris and 10 mM MgCl$_2$, pH 7.5) to
block recA filament formation and thereby prevent competition
for recA monomers by aggregation reactions (Morrical & Cox,
1985). Under these conditions, it has been shown that recA
protein binds to ssDNA with high affinity but at a reduced
number of sites (1 recA per 9-10 nucleotide residues) com-
pared to the 1 recA monomer per 4 nucleotide stoichiometry
measured at lower buffer or MgCl$_2$ concentrations (Morrical
& Cox, 1985). The difference in apparent stoichiometries is probably attributable to increased secondary structure in

**FIGURE 3:** Interaction of SSB with recA-ssDNA complexes formed in the absence of ATP. The indicated amounts of SSB were added to preincubated mixtures of 1.0 &muM recA protein and 3.38 &muM ssDNA in 40 mM Tris, 80% cation (pH 7.5), 10 mM MgCl$_2$, 1 mM DTT, and 5% glycerol. All other conditions were as described under Experimental Procedures. The horizontal lines drawn represent the values of $F_0$, and solid squares (■) denote $F_0$ values determined at the indicated SSB concentrations.

**FIGURE 4:** Interaction of SSB with recA-ssDNA complexes formed in the presence of ATP. SSB (0.45 &muM) was added at time zero to a solution containing 2.0 &muM recA, 6.75 &muM ssDNA, and 1.0 mM ATP. All other conditions were as described under Experimental Procedures. The control values $F_0$ and $F_Q$ are described in the text.

**ssDNA at the higher ionic strength, which appears to be in-
accessible to recA protein binding (Muniyappa et al., 1984; Flory et al., 1984). Our results indicate that (SSB)$_{65}$ has access to all of its normal binding sites under conditions where recA binding may be restricted, whether or not recA protein is present. The SSB-ssDNA interaction appears to be much stronger than the recA-ssDNA interaction in the absence of nucleotide effectors. These and other results (S. Morrical and J. Lee, unpublished results) indicate that SSB and recA protein compete for ssDNA binding sites in the absence of ATP.

**Binding of SSB to recA-ssDNA Complexes in the Presence of ATP.** When SSB is added to a preincubation mixture containing ssDNA, ATP, and excess recA protein, a time-
dependent decrease in net fluorescence intensity is observed
(Figure 4). Taking $F_0$ as the initial point, fluorescence
decreases until leveling off at a value roughly midway between
$F_0$ and $F_Q$, a quenching effect of approximately 50% the
magnitude observed in the absence of recA protein. The rate
at which the fluorescence intensity decreases is also much
slower than that observed in the absence of recA protein (see
Figure 7), requiring nearly 60 s to reach its final quenched
value. This quenching effect is dependent on ssDNA, as no
similar decrease occurs when SSB is added to recA protein
and ATP (i.e., in the total fluorescence control). Partial
quenching is also dependent on ATP since, as shown in the
previous section, SSB fluorescence is quenched to the same
extent in the presence or absence of recA protein when ATP
is deleted from the system. Controls described under Ex-
perimental Procedures demonstrate conclusively that the
decreases in signal intensity observed when SSB is added to
recA-ssDNA complexes cannot be attributed to photo-
bleaching or to interference due to absorption or scattering
of the excitation beam. Instead, they are solely the result of
quenching effects on protein fluorescence. Fluorescence assays
performed in the presence of a pyruvate kinase–phosphoen-
olpyruvate ATP regenerating system revealed tryptophan
fluorescence quenching effects identical with those observed
in reactions without a regenerating system (data not shown).
Under these conditions, the final partially quenched fluores-
cence intensity observed was stable for more than 1 h. Thus,
the ATP-dependent quenching effect on protein fluorescence
we have observed is continuously maintained while the
ATP/ADP ratio remains high.

In our reaction mixtures, decreases in fluorescence intensity
could be brought about through quenching of SSB tryptophan
fluorescence, recA tryptophan fluorescence, or both. The three simplest explanations for our observations can be stated as follows: (1) Both recA protein and SSB interact normally with ssDNA in the complexes we observe, with quenching of SSB fluorescence as observed in (SSB)$_{65}$ and with no quenching effect on recA fluorescence. Binding of the two proteins in this case is mutually exclusive, so that the decreased magnitude of fluorescence quenching seen would result from SSB binding at a reduced number of sites (those not occupied by recA). (2) The interaction of SSB with a recA-ssDNA complex involves a specific conformation of SSB unique to this interaction, producing a qualitatively different fluorescence quenching effect on SSB. In this case, SSB is not competing with recA protein for ssDNA binding sites but is binding to the intact recA-ssDNA complex in a manner that is not necessarily analogous to its normal interaction with ssDNA. (3) All or part of the quenching effect is due to quenching of recA fluorescence by some novel mechanism in the presence of SSB, ssDNA, and ATP, while SSB fluorescence remains largely unquenched (presumably SSB is almost completely displaced from the ssDNA after a transient participation in complex formation). Several observations argue against case number 3. The insensitivity of recA fluorescence to quenching by nucleotides, ssDNA, or dsDNA and the high susceptibility of SSB fluorescence to quenching by single-stranded poly-nucleotides argue against recA and for SSB as the protein species undergoing fluorescence quenching effects in stabilized recA-ssDNA complexes. In some experiments, the extent of fluorescence quenching is greater than the total intrinsic fluorescence of recA protein in the experiment. Finally, if recA protein fluorescence is quenched in the complex and most or all of the SSB is free in solution, it should be possible to obtain a net fluorescence intensity significantly lower than $F_0$ by adding excess ssDNA to completely quench the fluorescence of the free SSB in its high-salt ssDNA binding form. Excess ssDNA was added to a recA-ssDNA complex preformed with ATP and SSB and exhibiting the typical partial quenching of fluorescence shown in Figure 4. In this experiment, fluorescence decreased rapidly to a level equal to $F_0$, not less than $F_0$ (Figure 5). $F_0$ again represents the fluorescence intensity expected if (SSB)$_{65}$ is saturated with ssDNA and recA protein is unquenched. Our inability to generate fluorescence intensities lower than $F_0$ in experiments of this type, combined with the results described above, argues strongly against quenching of recA fluorescence in an SSB-free recA-ssDNA complex as a viable explanation for the observed fluorescence quenching in this system. Therefore, the fluorescence quenching we have described is the result of a continuous association of SSB with a recA-ssDNA complex. Experiments described in the next section were carried out to distinguish between cases 1 and 2. The experiment presented in Figure 5 may imply that the association of SSB with recA-ssDNA complexes is relatively loose. This possibility will be discussed below in another context.

Characterization of the ATP-Dependent Interaction between SSB and recA-ssDNA Complexes

As described previously the partial quenching of SSB fluorescence due to the continuous association of SSB with recA-ssDNA complexes in the presence of ATP could result from (1) a lower number of (SSB)$_{65}$-ssDNA interactions due to mutually exclusive binding of recA protein and SSB or (2) the interaction of SSB in an altered conformation with an intact recA-ssDNA complex. In an attempt to distinguish between these two possibilities, experiments were carried out to determine the stoichiometries of recA protein and SSB in the complex. All complexes described below were formed in the presence of ATP.

Effect of recA Protein Concentration. recA protein binds to ssDNA at a stoichiometry of 1 recA monomer per 3.5-4.0 nucleotide residues in both the absence (Menetski & Kawczynowski, 1985; Morrical & Cox, 1985; Bryant & Lehman, 1985) and presence (Tsang et al., 1985; Neuendorf & Cox, 1986) of SSB under conditions in which such binding is not inhibited by secondary structure in the ssDNA. Minor differences in the reported binding site size can be attributed entirely to differences in the extinction coefficient used to determine recA protein concentration in work carried out in different laboratories.

If binding of recA protein and SSB is mutually exclusive, the extent of fluorescence quenching of SSB due to ssDNA binding should be inversely proportional to the concentration of recA protein. The results presented in Figure 6 are not consistent with this prediction. At recA concentrations greater...
than 1.7 μM (one recA monomer per four nucleotides in these reaction mixtures), the extent of quenching of the SSB fluorescence is completely independent of recA protein concentration. This is true even when a 4-fold excess of recA protein is added. At levels of recA protein insufficient to saturate the ssDNA (recA < 1.7 μM at 6.75 μM ssDNA), the observed extent of fluorescence quenching is greater due to the direct interaction of SSB with ssDNA in regions not coated by recA protein. The inability to decrease the fluorescence quenching effect with a large excess of recA protein suggests that we are observing two distinct SSB binding modes. One corresponds to (SSB)₆₅ in the absence of recA protein and the other to a new binding mode that is observed only in the presence of recA protein.

The time course of fluorescence quenching observed in these experiments supports the same interpretation. The rate of SSB association with recA–ssDNA complexes as monitored by this fluorescence change is also independent of [recA] at saturating recA protein levels (Figure 7). When recA protein is in excess, the fall in fluorescence intensity from the initial value of F₀ to the final value of approximately (F₀ + F₀)/2 occurs with an approximate time of 15 s. In contrast, binding of (SSB)₆₅ to ssDNA in the absence of recA protein is essentially complete within 5 s after addition of SSB to the ssDNA solution (Figure 7). The methods employed in these experiments do not permit accurate data acquisition in the period 0–5 s. At subsaturating recA concentrations, time courses are obtained that are apparently biphasic within the limits of our measurements, with a fast phase consistent with the rapid binding of (SSB)₆₅ to ssDNA not coated by recA protein, and with a slow phase representing the qualitatively different association of SSB with recA–ssDNA complexes observed and described above.

The observation that both the rate and extent of fluorescence quenching due to SSB association with recA–ssDNA complexes (+ATP) exhibit saturation behavior with respect to recA concentration argues against a competition between recA protein and SSB for ssDNA binding sites. Since saturation occurs at approximately one recA monomer per four ssDNA nucleotide residues, a concentration sufficient to bind all of the ssDNA with recA protein alone, our data suggest that SSB is interacting with the recA–ssDNA complex rather than with ssDNA that is free of recA protein.

Effect of ATPγS. When SSB is added to mixtures of recA protein, ssDNA, and ATPγS, a decrease in fluorescence in-

FIGURE 7: Time course for complex formation; effect of recA protein concentration. Reactions were started by addition of 0.45 μM SSB to solutions containing 6.75 μM ssDNA, 1.0 mM ATP, and the indicated amount of recA protein. All other conditions were as described under Experimental Procedures. recA protein concentrations were (0) 0, (m) 0.5, (O) 2.0, and (D) 4.0 μM. The value of F₀ was subtracted from all data and controls. Thus, the fluorescence of recA protein (which increases as [recA] is increased) has been subtracted out, and the magnitude of the SSB-dependent fluorescence quenching effect in each case is plotted relative to the [recA]-independent control value (F₀ - F₀).

FIGURE 8: SSB fluorescence quenching effects in ATPγS-treated recA–ssDNA complexes. SSB, recA protein, and ssDNA concentrations were 0.45, 2.0, and 6.75 μM, respectively. ATPγS (0.2 mM) either replaced ATP in the initial reaction mixture (0) or was added to a complex preformed in 1.0 mM ATP (O). (m) denotes the value of F₀ in this experiment. All other conditions were as described under Experimental Procedures.

The maximum extent of quenching will simply be attained at a density similar to that seen in the presence of ATP is observed (Figure 8). The final magnitude of fluorescence quenching is reduced, however, with only 20–25% of the quenching effect produced by free ssDNA observed. Addition of ATPγS to a complex preformed in the presence of ATP causes fluorescence intensity to increase to an equivalent level (Figure 8). The difference in the magnitudes of quenching observed with ATPγS vs. ATP could reflect a loss of SSB binding sites in the presence of ATPγS due to the binding of additional recA protein or could be due to a reduced affinity of ATPγS for the complex in the presence of ATPγS brought about by a conformational change in the recA protein. The stoichiometry of recA protein binding to ssDNA at saturation in the presence of ATPγS and SSB is one recA monomer per four nucleotides (Neuendorf & Cox, 1986). This value is identical with the apparent stoichiometry of recA protein binding in the complexes studied in this work. It is therefore unlikely that significant amounts of additional recA protein are bound upon the addition of ATPγS. Instead, this observation represents a second piece of evidence suggesting a direct interaction between recA–ssDNA complexes and SSB. Evidence that ATPγS produces a conformational change in recA protein has been presented elsewhere (Menetski & Kowalczykowski, 1985).

ATPγS induces irreversible binding of recA protein to ssDNA (McEntee et al., 1981b; Weinstock et al., 1981b). This leads to the formation of long filaments containing ssDNA uniformly coated with recA protein that are easily visualized by electron microscopy (DiCapua et al., 1982; Flory & Radding, 1982). The observation of smooth, continuous filaments of recA protein and ssDNA indicates that binding of recA protein to ssDNA in the presence of ATPγS is complete and independent of secondary structure. Thus, it is important that a reduced but significant SSB–dependent fluorescence quenching effect is observed even in the presence of ATPγS. This result again suggests that SSB interacts continuously with a complex saturated with recA protein and supports the argument that this interaction cannot be a normal binding interaction between SSB and regions of ssDNA inaccessible to recA protein binding.

Effect of SSB Concentration. If SSB binds to a reduced number of sites on ssDNA when recA protein and ATP are present, normal levels of fluorescence quenching should be observed at lower, subsaturating concentrations of SSB. The maximum extent of quenching will simply be attained at a
complexes. Neuendorf and Cox (1986) have shown that a nucleotide residues is required for stable recA DNA complex stoichiometric ratio of approximately 1 SSB monomer per 20 binding of SSB to ssDNA are rare or nonexistent in these quenching in these reactions is approximately half that seen formation. This is approximately equivalent to the SSB concentration above the equivalence point. Since the mixture. The final fluorescence intensities from reactions with conditions of SSB rapidly increase the net fluorescence of the again argue against competitive binding of recA protein and extents of fluorescence quenching expected for SSB binding in the absence of recA protein, a value that is independent of ssDNA is completely coated by (SSB)₆S, and subsequent ad-
sion of SSB rapidly increase the net fluorescence of the mixture. The final fluorescence intensities from reactions with all components (including recA) are significantly greater than the control values at all concentrations of SSB used in the reactions (Figure 9). At high [SSB], the magnitude of quenching in these reactions is approximately half that seen in the absence of recA protein, a value that is independent of SSB concentration above the equivalence point. Since the magnitude of fluorescence quenching is independent of SSB concentration at high concentrations of SSB, these results again argue against competitive binding of recA protein and SSB and argue for case number 2 as described above. Normal extents of fluorescence quenching expected for SSB binding to ssDNA are not observed even at very low SSB concentrations. This in turn suggests that sites large enough for normal binding of SSB to ssDNA are rare or nonexistent in these complexes. Neuendorf and Cox (1986) have shown that a stoichiometric ratio of approximately 1 SSB monomer per 20 nucleotide residues is required for stable recA–ssDNA complex formation. This is approximately equivalent to the SSB concentration required to obtain the maximum extent of fluorescence quenching observed in the presence of recA protein and ATP.

Time courses for fluorescence quenching obtained at various SSB concentrations resemble that in Figure 4, except at very high [SSB], where an interesting phenomenon occurs. The initial rate of fluorescence decrease was essentially independent of SSB concentration throughout the range examined (data not shown). This suggests that a rearrangement of recA protein on ssDNA or a conformational change in recA protein is the rate-limiting step in complex formation. At high [SSB], fluorescence intensity goes through a minimum before the final magnitude of approximately (F₀ + F₀)/2 is attained (data not shown). The effect was most noticeable at 1.35 μM SSB, the highest SSB concentration examined, and was easily detectable at 0.675 μM SSB. Occasionally, a slight minimum was observed when working at 0.45 μM SSB, though the effect was irregular, varying with reaction conditions and protein preparations. We speculate that at high concentrations of SSB, some direct binding of SSB to ssDNA can occur during the relatively slow assembly of the stable recA–ssDNA complex, producing a transiently higher magnitude of fluorescence quenching due to the interaction of (SSB)₆S with ssDNA. This directly bound SSB is slowly displaced by a complex containing recA protein, ssDNA, and SSB in continuous association, restoring the lower level of fluorescence quenching we attribute to this complex. The transient phase we observe in some experiments may reflect the SSB interaction during complex assembly observed by Register and Griffith (1985) by electron microscopy.

recA Protein Promoted ATP Hydrolysis in the Presence and Absence of SSB. ATP hydrolysis by the recA protein is dependent on the interaction of recA protein and single-stranded DNA under the conditions employed in these studies; therefore, factors that prevent or inhibit binding of ssDNA by recA protein should simultaneously prevent or inhibit ATP hydrolysis. If SSB and recA protein compete for binding sites on ssDNA, SSB should then be expected to inhibit the ATPase activity of recA protein by decreasing the number of sites available for recA protein binding. This type of inhibition was in fact observed by Cohen et al. (1983) when the substrate dATP was the last reaction component added to the reaction mixture. Cohen and co-workers preincubated recA protein and SSB with ssDNA in the absence of dATP before starting the reaction with dATP. As described above, SSB competitively displaces recA protein from ssDNA in the absence of nucleotide substrate; therefore, the SSB concentration dependent decreases in the rate of dATP hydrolysis observed by Cohen et al. (1983) reflect the loss of ssDNA-bound recA protein due to this competition. This order of addition also strongly inhibits the strand exchange reaction of recA protein (Cox & Lehman, 1982).

We have measured the effect of SSB on recA-catalyzed ATP hydrolysis when SSB is the last component added (the order of addition in which DNA strand exchange is optimal). In both the presence and absence of SSB, the initial velocity is dependent on recA protein concentration at constant [ssDNA]; however, as shown in Figure 10, SSB has a strong stimulatory effect, rather than an inhibitory effect, on ATP hydrolysis at high recA protein concentrations when SSB is added last. A stimulation of 2.5–3-fold is observed at saturating concentrations of recA protein. The partial quenching of SSB fluorescence described above is observed for more than 1 h in the presence of an ATP regenerating system (see above). Therefore, ATP hydrolysis catalyzed by recA protein is stim-
ulated by SSB under conditions in which SSB is known to interact continuously with the recA-ssDNA complex.

With ATP saturating, the apparent value of \( k_{\text{cat}} \) at various concentrations of recA protein and ssDNA is given by the ratio \( v_0 / [\text{recA}] \) (in units of min\(^{-1}\)). The true (optimal) value of \( k_{\text{cat}} \) should occur at a recA protein concentration just sufficient to saturate the single-stranded DNA, since further increases in [recA] do not lead to corresponding increases in \( v_0 \) when recA protein is in excess. Therefore, by measuring \( k_{\text{cat}} \) as a function of [recA], we could estimate the apparent binding stoichiometry of recA protein to ssDNA in the presence and absence of SSB. These results are shown in Figure 11. In the absence of SSB, \( k_{\text{cat}} \) is optimal at a recA/ssDNA ratio of 1 monomer per 10 nucleotide residues, with a maximum \( k_{\text{cat}} \) of approximately 19 min\(^{-1}\). This 1/10 apparent stoichiometry of binding is similar to that observed previously in the absence of ATP at 10 mM MgCl\(_2\) and relatively high buffer concentration (Morrical & Cox, 1985) and may reflect a decrease in the total amount of ssDNA available for recA binding due to secondary structure (Muniyappa et al., 1984; Flory et al., 1984). In the presence of SSB (added last), \( k_{\text{cat}} \) is optimal at a recA/ssDNA ratio of approximately 1/4, with a maximum \( k_{\text{cat}} \) of approximately 29 min\(^{-1}\). Identical results were obtained at a 2-fold higher concentration of SSB (Figure 11). Two observations are important here: First, at constant [ssDNA], \( k_{\text{cat}} \) is optimal at a higher recA concentration in the presence of SSB than in its absence. The optimal \( k_{\text{cat}} \) is observed at a recA/ssDNA ratio of 1/4 in the presence of SSB, a value in good agreement with reported stoichiometries of recA protein to ssDNA at saturation. This again suggests that the associated SSB is not displacing recA protein but rather is present in addition to the normal saturating complement of recA protein. The fact that the optimal recA/ssDNA ratio does not change when SSB concentration is doubled is another strong indication that recA protein and SSB do not compete for binding sites in these complexes. Second, the maximum \( k_{\text{cat}} \) value measured was more than 50% higher in the presence of SSB than in its absence. While less ssDNA is available to recA protein binding in the absence of SSB, the same maximum \( k_{\text{cat}} \) would be expected when the available ssDNA was saturated. This indicates that the observed stimulation of recA ATPase activity by SSB is not entirely due to an increase in the amount of recA protein bound to ssDNA. There is an additional stimulatory effect, which suggests a direct interaction between recA protein and SSB. These results again support the notion that SSB is associated with recA-ssDNA complexes (+ATP) in a novel binding mode that does not involve displacement of significant amounts of recA protein.

**ADP and Order of Addition Effects.** Previous studies have revealed two important characteristics of the recA-ssDNA complex active in strand exchange that should be shared by our SSB-containing complexes if they are chemically competent for the DNA strand exchange reaction. First, Cox et al. (1983b) showed that levels of ADP on the order of 50-60% of the total nucleotide pool caused SSB-stabilized recA-ssDNA complexes to dissociate, halting the strand exchange reaction. Second, preincubation of SSB with ssDNA prior to the addition of recA protein (an order of addition inverse to that used in the experiments described above) strongly inhibited both the initial rate and final extent of D-loop formation by blocking the formation of the active recA-ssDNA complex (Cox & Lehman, 1982; West et al., 1982). The results of these studies allowed us to make several predictions concerning the fluorescence behavior of our system in response to inhibitory levels of ADP and to a reversed order of protein addition.

If the complex observed by fluorescence is the same complex active in strand exchange, we would expect to see dissociation of this complex in a mixture of 50% ADP and 50% ATP. In our fluorescence-monitored system, dissociation would be detectable as a decrease in fluorescence intensity, since dissociation of recA protein would open up regions of free ssDNA for direct binding by (SSB)\(_{65}\), producing the normal fluorescence quenching effect. The complex was preformed in 100% ATP, and then an equivalent amount of ADP was added to quickly raise the ADP content of the solution to 50%. Figure 12 shows that the predicted decrease in fluorescence intensity was actually observed. The decrease was relatively slow, but a final intensity equal to \( F_0 \) (the value expected if all recA
is dissociated) was reached within 6–8 min after addition of ADP. Thereafter, the signal was stable at $F_0$. When the ADP concentration was rapidly increased from 0% to 75% in a similar experiment, a much faster drop ensued in which $F_0$ was reached within 40 s (data not shown). Thus, the complex we have studied is dissociated by levels of ADP known to disrupt the active, SSB-stabilized complex of recA protein and ssDNA in a manner consistent with published results.

We also predicted that a reversed order of addition of recA protein and SSB would inhibit complex formation. Here, complex formation would be detected as an increase in fluorescence intensity, since we are starting with ssDNA fully coated by (SSB)$_{65}$, which must be displaced in the reaction. When SSB was preincubated with ssDNA and the reaction started by addition of recA protein, we saw no significant increase in fluorescence intensity from $F_0$ (the level expected for ssDNA fully coated by (SSB)$_{65}$) during a 5-min monitoring period (Figure 13). A slow increase in fluorescence intensity was subsequently observed (data not shown); however, the level of fluorescence typically seen with complexes described in this work was never attained within a 30-min monitoring period. This observation is consistent with the long time lags and decreased extents of DNA strand exchange reactions observed when SSB is added prior to recA protein (Cox & Lehman, 1982). When the same experiment was repeated with ATP$_2$S replacing ATP in the mixture, a somewhat faster increase in fluorescence intensity was observed (Figure 13), but at a rate that was still much slower than the rate of SSB association measured for the normal order of addition. [Note that this rate of increase is also considerably slower than the rate observed when ATP$_2$S is added to a complex preformed in ATP (Figure 8).] Obviously, the addition of SSB to ssDNA prior to recA protein strongly inhibits the formation of this complex, even in the presence of ATP$_2$S, which causes recA protein to bind rapidly and irreversibly to ssDNA. These results are consistent with a scheme in which (SSB)$_{65}$-ssDNA must dissociate before an active complex of recA protein, ssDNA, and SSB can form, and further identifies the complex studied in these experiments with the stable complex of recA protein and ssDNA active in DNA strand exchange.

**DISCUSSION**

Our principal conclusion is that SSB is associated with recA-ssDNA complexes in a form that is detectable by tryptophan fluorescence quenching effects. The observed quenching effects are a property of SSB in the system and cannot be attributed to quenching of recA fluorescence in a complex that lacks SSB. The association occurs under conditions that are optimal for recA protein promoted DNA strand exchange. The interaction of SSB with recA-ssDNA complexes revealed in this study has several important characteristics: (1) It is continuous, with no detectable displacement of SSB for up to 60 min at any concentration of recA protein. (2) It is ATP dependent. In the absence of ATP, our data are consistent with an interaction in which SSB and recA protein compete for ssDNA binding sites and SSB always exhibits properties consistent with the (SSB)$_{65}$ binding conformation. When ATP is present, the interaction is demonstrably altered to one that is noncompetitive; i.e., stoichiometric amounts of both recA protein and SSB now appear to be associated with the complex. (3) It appears to be distinct from the normal binding interactions of SSB with ssDNA as determined by kinetic properties.

The interaction of SSB with recA-ssDNA complexes in the presence of ATP does not involve significant displacement or exclusion of recA protein from ssDNA binding sites brought about by the normal binding of (SSB)$_{65}$ to those sites. The evidence that the interaction is instead a novel one takes several forms. First, both the rate and extent of the observed quenching of SSB fluorescence are lower than those observed when (SSB)$_{65}$ binds directly to ssDNA. Second, both the rate and extent of this fluorescence quenching effect are independent of either recA protein or SSB concentration above saturating levels. If recA protein and SSB competed for ssDNA binding sites, the amount of SSB bound as monitored by fluorescence quenching should be dependent upon the relative concentration of the two proteins. Third, this effect exhibits saturation at recA protein levels equivalent to those required to saturate ssDNA with recA protein, i.e., one recA monomer per four nucleotide residues. The $k_{sat}$ or turnover number for recA protein promoted ATP hydrolysis also reaches a maximum at the same stoichiometry in the presence of SSB; doubling the SSB concentration does not alter this observed stoichiometry. These results indicate that there is not significant displacement of recA protein by SSB in these complexes. Fourth, this fluorescence quenching effect exhibits saturation with respect to SSB concentration at levels similar to those required to saturate ssDNA alone with SSB. This suggests that stoichiometric levels of both SSB and recA protein are continuously present in this complex. Even at very low SSB concentrations, we find no evidence for a normal association of SSB with ssDNA when saturating levels of recA
protein and ATP are present. Fifth, some quenching of SSB fluorescence is observed even when ATP$_y$S is present and recA protein irreversibly forms unbroken filaments on ssDNA. Combined, these data argue that SSB associates with an intact recA-ssDNA complex in a novel manner.

Several additional experimental observations suggest, but do not demonstrate, that a direct interaction occurs between recA protein and SSB. The maximum turnover number for recA protein promoted ATP hydrolysis is enhanced by greater than 50% in the presence of SSB. In addition, the extent of fluorescence quenching of SSB in these complexes is rapidly reduced when ATP$_y$S is added. Binding and hydrolysis of nucleotides can only occur with recA protein, and one simple interpretation of these results is that SSB and recA protein interact in these complexes. Lehman and Overman (1985) reported that at least two conformations of SSB with significantly different tryptophan fluorescence properties can be induced by changes in the ionic media in which ssDNA binding reactions take place. It is interesting to speculate that a direct interaction between recA protein and SSB may induce a similar conformational change in SSB, causing SSB to interact with the recA-ssDNA complex in a manner distinct from its regular high-salt interaction with ssDNA.

We have observed that the stoichiometry of recA protein binding to ssDNA is reduced under conditions that favor the formation of ssDNA secondary structure, consistent with results reported elsewhere (Muniyappa et al., 1984; Flory et al., 1984). It is also clear that this barrier is removed in the presence of SSB. The data reported here, however, suggest that this effect is not simply a result of the helix-stabilizing capability of SSB. SSB is not displaced after playing a transient role in recA-ssDNA complex formation but instead remains associated with the complex. Thus, experimental observations that seemed to reflect helix destabilization by SSB (Muniyappa et al., 1984) may instead reflect the properties of joint complexes of recA protein and SSB.

The SSB-containing complexes we have detected by fluorescence exhibit properties consistent with those expected for the presynaptic complexes characterized in recA protein promoted DNA strand exchange reactions. ADP dissociates these complexes, and a reverse order: of addition of proteins inhibits their formation. The reaction between SSB and recA-ssDNA complexes monitored by fluorescence quenching is also kinetically competent to be the first step in the strand exchange reaction. Complex formation proceeds with a half-time of approximately 15 s, while the half-times for synopsis with homologous duplex and for the subsequent extension of heteroduplex are approximately 45 s and 10 min, respectively (Cox et al., 1983b); therefore, complex formation in our reaction mixtures occurs faster than the rate-limiting step of DNA strand exchange and fast enough to account for the rate of homologous pairing in the strand exchange pathway. These facts are consistent with a model in which the continuous interaction of SSB with recA-ssDNA is an integral part of the presynaptic phase of DNA strand exchange, and not an unproductive side reaction.

Our observation that SSB stimulates ATP hydrolysis when it is the last component added is consistent with earlier reports of stimulatory effects of SSB on ATP hydrolysis in the presence or absence of homologous duplex DNA (Cox & Lehman, 1982; Cox et al., 1983b). By using a coupled assay for ATP hydrolysis employing pyruvate kinase and lactate dehydrogenase, we have demonstrated that the same stimulatory effect occurs under conditions in which ATP concentration is maintained at high levels and ADP concentration at minimal levels throughout the reaction period. Other workers have reported inhibition of recA-ssDNA-catalyzed ATP and dATP hydrolysis by SSB (McIntee et al., 1980; Cohen et al., 1983). As shown in this study, this inhibition is the result of a different order of addition of reaction components. When SSB is added to ssDNA in a reaction mixture before either ATP or recA protein, a very stable SSB-ssDNA complex forms that almost completely blocks the binding of recA in the presence or absence of ATP, thereby inhibiting ATP hydrolysis as well as recA-dependent recombination activities.

The properties of the joint SSB-recA-ssDNA complex revealed in this study also suggest explanations for the inability to detect this interaction in previous studies. SSB appears to be transferred rapidly to ssDNA added as a challenge after complex formation. This suggests that the interaction of SSB with the complex is relatively weak and might be disrupted in the steps required to fix recA-ssDNA complexes for electron microscopy or centrifugation (Griffith et al., 1984; Solis & Lehman, 1983). ATP$_y$S, which is often used to stabilize recA-ssDNA complexes in these experiments, may have an adverse effect on SSB interactions with recA-ssDNA. SSB represents 10% or less of the total protein mass in the complexes defined in these experiments and may not be observed in electron micrographs if, as we suspect, it is bound in a form distinct from its normal mode of binding to ssDNA. Direct observation of a complex containing both SSB and recA protein may be elusive for one or more of these reasons. Utilization of fluorescence quenching for these studies is convenient and nondisruptive. The fluorescence quenching results we observe clearly reflect a previously undetected effect of SSB.

These results do not provide information about the structural details of the joint SSB-recA-ssDNA complex. We cannot rigorously eliminate models in which recA protein and SSB are interspersed on the ssDNA in a specific pattern that may change somewhat under different ionic conditions. On the basis of available data on the stoichiometries of the proteins in the complex, we prefer a model in which SSB interacts directly with recA filaments complexed to ssDNA, or to very short regions of ssDNA exposed because of some structural feature of the recA filaments bound to them. It is tempting to speculate that the continuous association of SSB reflects a continuous role for SSB in recA protein promoted DNA strand exchange, but this also remains to be rigorously demonstrated. Virtually all of the many effects of SSB on recA protein promoted DNA strand exchange could reflect properties of recA complexes acquired in the presynapsis or complex formation phase of the reaction.

While the detailed mechanism remains to be elucidated, SSB does appear to affect all stages of recA protein promoted DNA strand exchange (Cox & Lehman, 1981a, 1982; Radding et al., 1982; Wu et al., 1982; Cox et al., 1983b). SSB also affects recA protein promoted proteolysis in a manner suggesting that the complexes described in this report are also the active species in the proteolytic reaction (Resnick & Sussman, 1982). It has previously been suggested, on the basis of the stoichiometries of recA protein and SSB required for optimal DNA strand exchange, that both proteins are bound to ssDNA in the active complex in a novel structure (Cox & Lehman, 1982; Julin et al., 1986). The experiments reported herein support this view.

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Registry No. ATP, 56-65-5; ATP\textsubscript{γ}S, 35094-46-3; ADP, 58-64-0.

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