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Joint Interactions of SSB with RecA Protein on Single-Stranded DNA

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Abstract Single-stranded DNA binding protein (SSB) is well-characterized as having a helix-destabilizing activity. The helix-destabilizing capability of SSB has been re-examined in this study. The results of restriction endonuclease protection assays and titration experiments suggest that the stimulatory effect of SSB on strand exchange acts by melting out the secondary structure which is inaccessible to RecA protein binding; however, SSB is excluded from regions of secondary structure present in native single-stranded DNA. Complexes of SSB and RecA protein are required for eliminating the secondary structure barriers under optimal conditions for strand exchange.

Key words: SSB, RecA protein, secondary structures, helix-destabilizing activity, cooperative binding, restriction endonuclease digestion

The RecA protein promotes homologous recombination in *E. coli*. *In vitro*, this protein carries out DNA strand exchange reactions between regions of single-stranded DNA and homologous duplex DNA derived from bacteriophages (M13 and ϕX 174), which provides a model for RecA protein-mediated events in homologous recombination. The first step in this strand exchange is the cooperative binding of RecA protein to single-stranded DNA with a stoichiometry of one protein monomer per 3-4 nucleotides. This RecA-ssDNA complex hydrolyzes ATP with a k_{cat} of 25-30 min^{-1}. The next step involves the pairing of the homologous regions of substrate molecules and the strand exchange itself.

Genetic evidence indicates that the single-stranded DNA binding protein (SSB) participates in the RecA protein-mediated recombination and repair functions in *E. coli* [12]. SSB stimulates RecA protein-promoted DNA strand exchange, enhancing both the rate and final extent of the heteroduplex formation [9, 10]. This stimulation reflects a positive effect of SSB on the formation and stability of filamentous RecA protein complexes on single-stranded DNA [8, 21].

SSB has been well-characterized as having a helix-destabilizing activity and as a DNA binding protein which binds single strands specifically and cooperatively [18] (Fig. 1). The stimulation of RecA protein-promoted DNA strand exchange *in vitro* may be a model for the role of SSB in general recombination. It has been demonstrated by Radding and colleagues that the major barrier to the formation of a continuous filament of RecA protein on ssDNA is the secondary structure which the DNA may possess under some conditions [10, 13, 21]. The single-stranded DNA of M13mp18 in solution is in a highly folded conformation with secondary structures resulting from intrastrand base pairing of short runs of complementary sequences. This barrier is clearly removed in the RecA system when SSB is present [13, 20, 21]. This led to a suggestion that the secondary structure is removed by means of the helix destabilizing activity of SSB, and that the SSB is then displaced by the RecA protein [16, 21]. A transient helix-destabilizing role for SSB in this system has recently been called into question by the demonstration that SSB is continuously associated with the RecA-ssDNA complex [20] and that the interaction between the two proteins is not competitive under conditions optimal for strand exchange [20]. These results, however, could also reflect the properties of a joint complex of the two proteins.

The helix-destabilizing capability of SSB has itself been called into question under some conditions. At least one major cruciform in both the bacteriophage G4 and M13 ssDNA resists denaturation by SSB [3, 12]. Lohman and colleagues have recently found that SSB exists in multiple binding modes which are influenced by ionic strength [6,

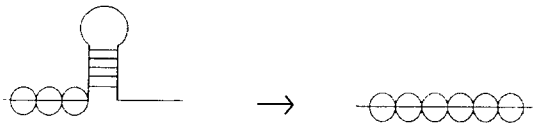

Fig. 1. Helix-destabilizing activity of SSB.

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18]. The binding mode present at a low ionic strength at 1 mM Mg^{++} in particular, is highly cooperative [18]. SSB rapidly displaces RecA protein from ssDNA in 1 mM $MgCl_2$ and strand exchange does not occur [17, 20]. SSB stimulation of strand exchange occurs only with higher Mg^{++} concentrations and is optimal at 10-13 mM [8, 10]. The SSB binding mode prevalent under these conditions has been designated as SSB_{56} by Lohman, reflecting the tetramer binding site size on poly(dT) in nucleotides [6]. SSB in this binding mode exhibits a much lower cooperativity in DNA binding [6, 19]. Evidence based on changes in the apparent binding site size on poly(dT) versus native ssDNA in high concentration of NaCl led Lohman and colleagues to suggest that it may be excluded from some regions of DNA secondary structure [18].

It has also been determined that major regions of the secondary structure in ssDNA are inaccessible to SSB binding under conditions in which SSB stimulates the strand exchange reactions. Since these regions of secondary structure are bound only when both proteins are present, the binding must reflect a joint complex of SSB and RecA protein. The formation of this joint complex is ATP-dependent.

MATERIALS AND METHODS

RecA protein of *E. coli* was purified from strain GE645 as previously described [7], and was stored frozen at -70°C in 20 mM Tris-HCl, (pH 7.5), 1 mM DTT, 0.1 mM EDTA, and 10% glycerol. The concentration of RecA protein in the stock solution was determined by absorbance at 280 nm, using an extinction coefficient ε_{280}=0.59 A_{280} $mg^{-1}ml$ [11] and a monomer molecular weight of 37,852. *E. coli* SSB protein was purified and its concentration was determined as described by Bujalowski and Lohman [6]. The stock solution of SSB contained 20 mM Tris-HCl, (pH 8.4), 0.15 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 50% glycerol. M13mp18 ssDNA was prepared using the method of Messing [23]. Poly(dT) was purchased from Sigma and had an average length of 2,000 nucleotides. An extinction coefficient of ε_{259}=8,540 $M^{-1}cm^{-1}$ was used for determining the concentration of poly(dT). All DNA solutions were stored at 0°C in 10 mM Tris-HCl, (pH 7.5) and 1 mM EDTA. M13mp18 ssDNA concentration was determined by the absorbance using 36 μg ml^{-1} (A_{260}) as the conversion factor and confirmed by direct determination of the total phosphate [22]. Restriction enzymes including *Hin*fI and *Dde*I were purchased from New England Biolab. All other biomolecules were purchased from Sigma.

Instrumentation

Fluorescence measurements were performed on an SLM Instruments 8,000 series fluorometer equipped with a thermojacketed cuvette holder, constant temperature water circulator, and magnetic stirring motor for the sample chamber.

Nuclease Digestion

Restriction enzyme digestions were carried out to determine the capability of SSB protein to melt out the secondary structures which exist in M13mp18 ssDNA. Unless otherwise indicated, all reaction mixtures contained 16 μM M13mp8 ssDNA, 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 2 mM ATP, an ATP regenerating system, and the indicated concentration of $Mg(acetate)_2$ in a standard reaction buffer. SSB protein was added after preincubation of all other components at 37°C for 5 min. After 5-10 min of incubation, 10 units of restriction enzyme were added to the reaction mixture. Digestion was stopped by addition of gel loading buffer (5% SDS, 5 mM EDTA, 0.005% bromophenol blue, and 25% glycerol). The digested DNA products were monitored by electrophoresis after applying the reaction mixture to a 1.4% agarose gel.

Fluorescence Titration

The reverse titration of SSB with M13mp18 ssDNA or poly(dT) was monitored by quenching of tryptophan fluorescence in the manner previously described [20]. The excitation and emmision wavelengths were 295 and 355 nm, respectively. All titrations were carried out at 37°C in a standard reaction buffer containing 10 mM $Mg(acetate)_2$. Pyruvate kinase, lactic dehydrogenase, phosphoenolpyruvate, and NADH were omitted to avoid interference with fluorescence measurements. The titration curves were corrected for any dilution due to the addition of DNA to the solution. The fluorescence signal coming from a blank solution containing the buffer, ATP, and SSB storage buffer was subtracted from all assay values. The corrected fluorescence signal of 0.55 μM SSB was normalized to a value of 1.0, and all data were plotted relative to this value.

RESULTS

The secondary structure destabilization or transient model for SSB action in the RecA system implicitly assume that SSB could bind to and rapidly denature all regions of secondary structure in ssDNA (Fig. 1). Two techniques were used to determine if SSB had this capability under optimal conditions for RecA protein-promoted DNA strand exchange. At first, the specific cleavage of the regions of secondary structure in ssDNA by certain restriction endonucleases was employed. The second technique employed titrations of SSB with ssDNA which either contained or did not contain regions of secondary structure (M13mp18 ssDNA and poly(dT), respectively), using the quenching of the intrinsic fluorescence of SSB as a probe

of DNA binding [6, 18, 20]. The effect of the RecA protein was also examined.

Restriction Enzyme Digestion of ssDNA

Several restriction enzymes which cleave M13mp18 ssDNA were employed in these studies and results with *Hinf*I and *Dde*I are presented in Figs. 2, 3, and 4. The specificity of *Hinf*I for duplex DNA (regions of secondary structure in the ssDNA) has been confirmed [15]. In Fig. 2, the effect of SSB on the cleavage by these enzymes was examined at two different Mg^{++} concentrations, 1 and 10 mM. In the absence of SSB, the ssDNA was cleaved into shorter ssDNA fragments by restriction enzyme (lane 2). At low Mg^{++} concentration, the secondary structure in ssDNA was minimized, and SSB was in a low salt binding mode with high cooperativity. More secondary structure existed in ssDNA at the higher Mg^{++} concentration [13, 21]. The highest concentration of SSB employed in these studies should be sufficient to saturate the DNA under either conditions [6]. Degradation of the ssDNA by both enzymes in the presence of SSB was much greater at 10 mM Mg^{++} than at 1 mM Mg^{++}. Similar results were obtained with the enzyme *Hha*I (data not shown). No degradation was observed in the control with only SSB or the recA protein, therefore, this was not a function of the nuclease contamination of the SSB (Fig. 2, lane 3) nor recA preparation (Fig. 3, lane 5). Whereas limited protection of ssDNA was observed even at 10 mM Mg^{++}, the results indicated that significant regions of DNA secondary structure existed at this Mg^{++} concentration which were not destabilized by SSB. The restriction digests of ssDNA in the presence of RecA protein were also analyzed (Fig. 3).

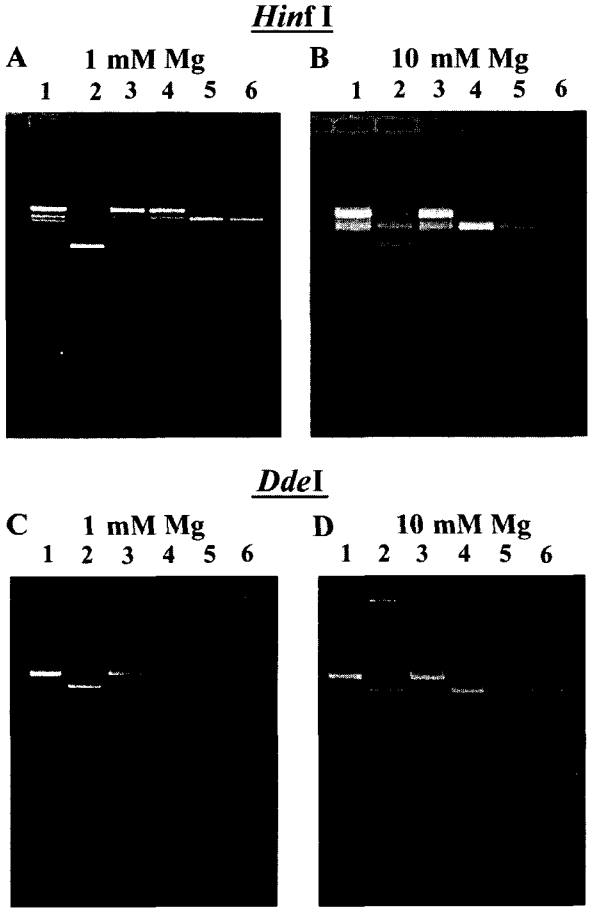

Fig. 2. Digestion of M13mp18 ssDNA by restriction endonucleases in the presence of SSB.

Reaction mixture contained in total 30 µl: 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM dithiothreitol, 16 µM (nucleotide) M13mp18 ssDNA, and the indicated concentration of $Mg(acetate)_2$. After preincubation at 37°C for 5 min, an amount of SSB indicated was added and the incubations continued for 5 min. SSB binding to ssDNA reached an endpoint within 1 min under all conditions employed [20]. Restriction enzyme, 10 units of either *Hinf*I (panels A and B) or *Dde*I (panels C and D), was then added to the reaction mixture and incubation continued for either 20 or 30 min in 10 mM or 1 mM $Mg(acetate)_2$, respectively. Digestion was halted by adding 5 µl of 10% SDS and the samples were subjected to electrophoresis on 1.4% agarose gels. Lane identification for all panels: 1) ssDNA (no SSB or restriction enzymes); 2) ssDNA, and restriction enzyme, but no SSB; 3) ssDNA 1.75 µM SSB, but no restriction enzyme; 4) ssDNA, restriction enzyme, and 0.9 µM SSB; 5) ssDNA, restriction enzyme, and 1.2 µM SSB; 6) ssDNA, restriction enzyme, and 0.9 µM SSB. SSB concentrations are reported in terms of total monomers. The monomer of SSB covers the regions of 16 nucleotides in ssDNA. When less than 1 µM SSB was used, the ssDNA could not be coated fully with SSB. The upper band in lane 1 in each panel is circular ssDNA, and the lower band is linear ssDNA (unpublished results).

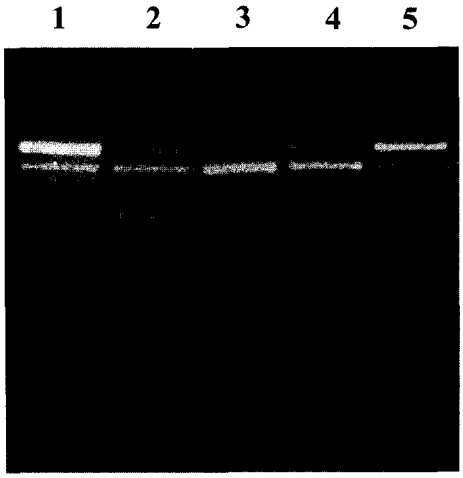

Fig. 3. Digestion of M13mp18 ssDNA by restriction endonucleases in the presence of RecA protein.

Reaction mixture contained in 30 µl total volume: 25 mM Tris-HCl (pH .5), 5% glycerol, 1 mM dithiothreitol, 16 µM (nucleotide) M13mp18 ssDNA, and 10 mM $Mg(acetate)_2$. After preincubation of these components at 37°C for 5 min, RecA protein was added as indicated below and the incubations continued for 5 min. Restriction enzyme, 10 units of either *Hinf*I or *Dde*I, was then added to the reaction mixture, and incubation continued for another 20 min. Digestion was stopped by adding 5 µl of 10% SDS and the samples were subjected to electrophoresis on 1.4% agarose gels. Lane identification for all panels: 1) ssDNA (no recA or restriction enzymes); 2) ssDNA, and *Hinf*I, but no RecA; 3) ssDNA, 6 M RecA and *Dde*I, 4) ssDNA, 6 µM RecA, ATP, and *Dde*I; 5) ssDNA, 6 µM RecA, but no restriction enzyme.

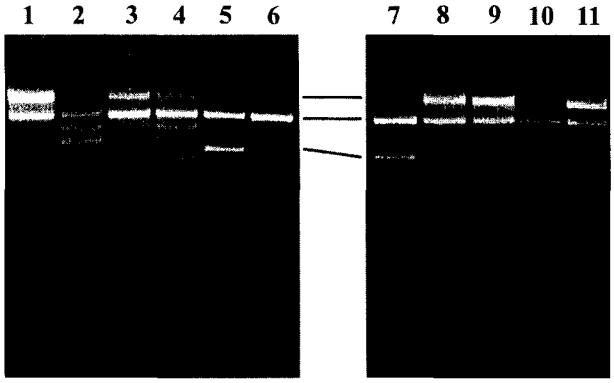

Fig. 4. Destabilization of ssDNA secondary structure by the recA protein and SSB.
Reaction mixture contained in 30 µl total volume: 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM Mg(acetate)$_2$, 16 µM (nucleotide) M13mp18 ssDNA. Except where indicated, 1 mM ATP was included along with the ATP regenerating system. After preincubation of these components at 37°C for 5 min, the indicated amount of recA protein was added and incubation continued for 5 min. SSB was then added as indicated. After an additional 5 min, 10 units of *Hin*fI were added (except where indicated). The incubation was continued for 30 min, reactions were stopped, and the samples were subjected to electrophoresis as described in Fig. 2. Lane identification: 1) DNA alone, no protein, *Hin*fI, or ATP added; 2) DNA, *Hin*fI but no recA or SSB; 3) DNA, *Hin*fI, 6 µM RecA protein, but no SSB or ATP; 4) DNA, *Hin*fI, 6 µM RecA, and ATP, but no SSB; 5) DNA, *Hin*fI, 1.8 µM SSB, but no RecA protein or ATP; 6) DNA, *Hin*fI, 1.8 µM SSB and ATP, but no RecA protein; 7) DNA, *Hin*fI, 6 µM RecA and 1.8 M SSB, but no ATP; 8) same as lane 7, but with ATP; 9) same as lane 8, but without *Hin*fI; 10) same as lane 2; 11) same as lane 1.

Significant DNA fragments of ssDNA were produced both with and without ATP, which indicated that RecA protein could not bind to the secondary structures, and that the protection from nuclease digestion by RecA protein was not complete. This observation suggests that regions of secondary structures remain in ssDNA under these conditions. This is examined further in Fig. 4. In experiments with *Hin*fI restriction enzyme, significant degradation of ssDNA was observed at 10 mM Mg^{++} in the presence of either SSB or RecA protein, each at concentrations sufficient to saturate the DNA present. When both proteins were present in the same reaction mixture, however, the ssDNA was completely protected from endonuclease digestion. This protection was not observed when the concentration of either protein alone was doubled (not shown). This indicated that the secondary structure which was inaccessible to either protein alone was eliminated when both were present. The results in Fig. 4 also demonstrated that this complete protection was ATP-dependent. Identical results were obtained in experiments carried out with *Dde*I restriction endonuclease (data not shown).

Reverse Titration of SSB Binding to ssDNA

These results were strengthened by the titration experiments presented in Fig. 5. A constant concentration of SSB was titrated with either poly(dT) or M13mp18 ssDNA.

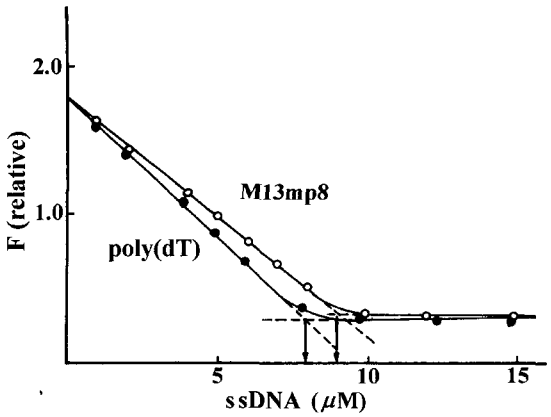

Fig. 5. Reverse titration of SSB with poly (dT) or M13mp18 ssDNA.
SSB (0.55 µM) fluorescence was monitored (λex=295 nm, λem=355 nm). In addition to SSB, the reaction mixtures contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, and 10 mM Mg(acetate)$_2$ in a 2-ml total volume. Total dilution of the reaction mixture resulting from the addition of all DNA was less than 1%.

The binding was monitored by the decrease in the intrinsic fluorescence of SSB, and the apparent binding site size was calculated by the intersection of asymptotic lines drawn on a plot as described previously [20]. The binding site size for SSB tetramer on poly(dT) was approximately 57 nucleotides which was in good agreement with results obtained by Bujalowski and Lohman [6]. A higher binding site size of 65 nucleotides was observed on M13mp18 ssDNA. Under conditions in which secondary structures existed, more M13 DNA was required for the saturation of SSB binding than poly(dT) where secondary structures could not be formed. Again, this is consistent with previous results [20]. A parallel comparison under identical conditions again indicated that SSB was excluded from at least some regions of secondary structure present in M13mp18 DNA, but absent in poly(dT).

DISCUSSION

The results presented in this study are highly relevant to the mechanism of SSB action in the RecA system. A possible explanation for the stimulatory effect of SSB on strand exchange indicates that SSB acted by melting out the secondary structures that are inaccessible to recA protein binding, and was subsequently displaced by recA protein [21], or SSB acted as an assembly factor promoting the formation of a stabilized RecA-ssDNA complex through the interaction of SSB and RecA protein [10]. From this study, it was demonstrated that under conditions (10 mM Mg^{++}) optimal for the SSB stimulation of strand exchange, SSB was excluded from significant regions of secondary structure in ssDNA. Therefore, a model for SSB action

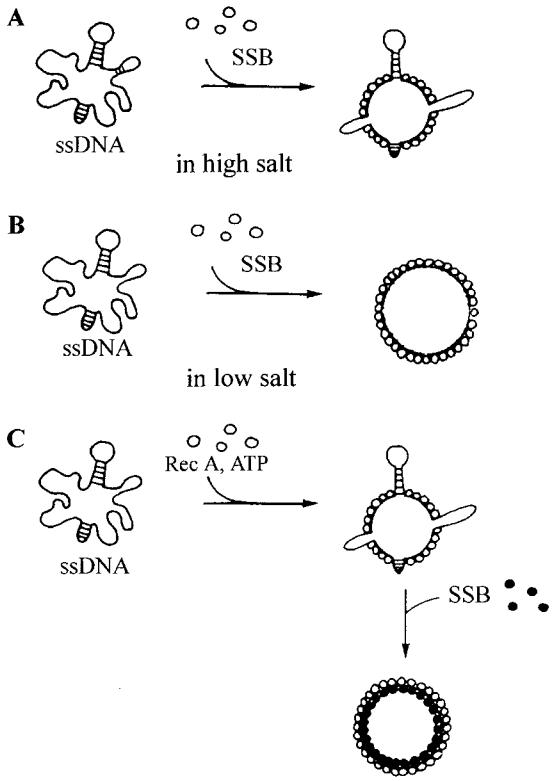

Fig. 6. Cooperative binding of SSB to ssDNA (A, B) and joint complex formation in the RecA system (C).

which depended exclusively on the helix destabilizing capability, cannot be correct. At a low salt concentration (1 mM Mg^{++}), the highly cooperative binding of SSB leads to the complete destabilization of secondary structures in ssDNA (Fig. 6B). However, the strand exchange by recA protein could not occur under this condition. At a high salt concentration (10 mM Mg^{++}), more secondary structures were formed and SSB alone could not melt out the secondary structures (Fig. 6A). All detectable secondary structures were eliminated only when both recA protein and SSB were present, indicating that a joint complex of the two proteins was responsible (Fig. 6C). The formation of this complex was ATP-dependent and may involve a specific interaction between the two proteins, as suggested elsewhere [10, 20]: SSB must be present continuously to maintain the stability of the RecA-ssDNA complex [10].

Further work is required to define the structure of the joint RecA-SSB complex implied by this and other studies, and also to determine a mechanism of action of the SSB proteins in this system.

Acknowledgments

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