Reprinted from *J. Mol. Biol.* (1987) **193**, 97–113

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(Received 24 March 1986, and in revised form 28 August 1986)

The effect that *Escherichia coli* single-stranded DNA binding (SSB) protein has on the single-stranded DNA-dependent ATPase activity of RecA protein is shown to depend upon a number of variables such as order of addition, magnesium concentration, temperature and the type of single-stranded DNA substrate used. When SSB protein is added to the DNA solution prior to the addition of RecA protein, a significant inhibition of ATPase activity is observed. Also, when SSB protein is added after the formation of a RecA protein–single-stranded DNA complex using either etheno M13 DNA, poly(dA) or poly(dT), or using single-stranded phage M13 DNA at lower temperature (25°C) and magnesium chloride concentrations of 1 mM or 4 mM, a time-dependent inhibition of activity is observed. These results are consistent with the conclusion that SSB protein displaces the RecA protein from these DNA substrates, as described in the accompanying paper. However, if SSB protein is added last to complexes of RecA protein and single-stranded M13 DNA at elevated temperature (37°C) and magnesium chloride concentrations of 4 mM or 10 mM, or to poly(dA) and poly(dT) that was renatured in the presence of RecA protein, no inhibition of ATPase activity is observed; in fact, a marked stimulation is observed for single-stranded M13 DNA. A similar effect is observed if the bacteriophage T4-coded gene *32* protein is substituted for SSB protein. The apparent stoichiometry of DNA (nucleotides) to RecA protein at the optimal ATPase activity for etheno M13 DNA, poly(dA) and poly(dT) is $6(\pm 1)$ nucleotides per RecA protein monomer at 4 mM-$MgCl_2$ and 37°C. Under the same conditions, the apparent stoichiometry obtained using single-stranded M13 DNA is 12 nucleotides per RecA protein monomer; however, the stoichiometry changes to 4·5 nucleotides per RecA protein monomer when SSB protein is added last. In addition, a stoichiometry of four nucleotides per RecA protein can be obtained with single-stranded M13 DNA in the absence of SSB protein if the reactions are carried out in 1 mM-$MgCl_2$. These data are consistent with the interpretation that secondary structure within the natural DNA substrate limits the accessibility of RecA protein to these regions. The role of SSB protein is to eliminate this secondary structure and allow RecA protein to bind to these previously inaccessible regions of the DNA. In addition, our results have disclosed an additional property of the RecA protein–single-stranded DNA complex: namely, in the presence of complementary base-pairing and at elevated temperatures and magnesium concentrations, a unique RecA protein–DNA complex forms that is resistant to inhibition by SSB protein.

1. Introduction

In the accompanying paper, we demonstrated through direct DNA binding studies that *Escherichia coli* single-stranded DNA binding (SSB†) protein had essentially no direct effect on

† Abbreviations used: SSB protein, *E. coli* single-stranded DNA binding protein; ATP-γ-S, adenosine-5′-*O*′-(3-thiotriphosphate); etheno M13 DNA, single-stranded M13 DNA containing 1,N^6-etheno-adenosine and 3,N^4-etheno-cytidine residues.

0022-2836/87/010097-17 $03.00/0

either the equilibrium stability or the rate of dissociation of a RecA protein–single-stranded DNA complex (Kowalczykowski *et al.*, 1987). The binding of these two proteins was found to be competitive, with SSB protein always displacing RecA protein unless ATP-γ-S or ATP (under limited conditions) was present. Although those studies did not demonstrate the existence of any direct interaction between RecA and SSB proteins, it is clear that SSB protein has a dramatic effect on the rates of D-loop formation and strand assimilation (McEntee *et al.*, 1980; Shibata *et al.*, 1980; Cassuto *et al.*, 1980; Cox & Lenham, 1982; West *et al.*, 1982; Cox *et al.*, 1983*a*,*b*). Therefore, SSB protein must play some role in these reactions, and our DNA binding results would imply that its effect is primarily at the level of the association of RecA protein and single-stranded DNA. Muniyappa *et al.* (1984) and Kahn & Radding (1984) proposed that the function of SSB protein (and phage T4-coded gene *32* protein) is to remove secondary structure from single-stranded DNA, which impedes the presynaptic association of RecA protein and DNA, and which also inhibits the subsequent steps in strand assimilation. Their model would imply that RecA protein is able to displace SSB protein, and this is consistent with our DNA binding studies, which demonstrated that RecA protein is capable of displacing DNA-bound SSB protein in the presence ATP-γ-S or ATP (at elevated temperatures and magnesium concentrations).

To gain further insight into the role of SSB protein in RecA protein-catalyzed strand assimilation, we investigated the effects of SSB protein on the single-stranded DNA-dependent ATPase activity of RecA protein. Since efficient catalysis of the strand assimilation reaction requires a stoichiometric amount of RecA protein relative to the amount of single-stranded DNA present, it is likely that a study of the single-stranded DNA-dependent ATPase activity of RecA protein would provide insight into the strand assimilation reaction itself. In addition, the reported effects of SSB protein on RecA protein-catalyzed single-stranded DNA-dependent reactions have been confusing, and although it is generally observed that SSB protein inhibits the single-stranded DNA-dependent ATPase activity of RecA protein (McEntee *et al.*, 1980; Cassuto *et al.*, 1980; Cohen *et al.*, 1983), SSB protein has also been reported to stimulate this activity (Cox *et al.*, 1983*c*; Tsang *et al.*, 1985). In contrast, the effect of SSB protein on RecA protein single-stranded DNA-dependent protease activity is generally stimulatory (Resnick & Sussman, 1982; Moreau & Roberts, 1984) or no effect is observed (Phizicky & Roberts, 1981). Thus, SSB protein is capable of affecting the activity of RecA protein in widely divergent ways, though the molecular mechanism of these effects is not understood.

In this paper, we have characterized extensively the effect of SSB protein on the ATPase activity of RecA protein and have observed that SSB protein can, in fact, either inhibit or stimulate this activity. The effect observed depends on a variety of experimental conditions including order of addition, temperature, magnesium concentration and DNA substrate used. Our data, and our interpretation of those data, provide a unifying framework within which to understand all of the disparate results reported for the effect of SSB protein on RecA protein ATPase and protease activities. The results obtained are consistent with the competitive effects observed in the previously described DNA-binding studies and together they clearly show that the inhibition of RecA protein ATPase activity results from the displacement of RecA protein from the DNA by SSB protein. Also, and perhaps most importantly, these ATPase activity studies demonstrate clearly that SSB protein is not required for maximum activity on DNA substrates lacking secondary structure (in fact, SSB protein inhibits ATPase activity) but that SSB protein is required for maximum activity on native single-stranded phage M13 DNA under most conditions. We have established that this requirement for SSB protein is due to the presence of secondary structure within single-stranded M13 DNA, which limits the availability of this DNA to RecA protein, and that the role of SSB protein is to eliminate this inhibitory secondary structure. Finally, it is also demonstrated that the presence of secondary structure or homologous pairing is important in the formation of a unique RecA protein–DNA complex that is resistant to inhibition (i.e. displacement) by SSB protein.

2. Materials and Methods

(a) *Chemicals and buffers*

All chemicals used were reagent grade and all solutions were made in glass-distilled water. ATP was purchased from PL Biochemicals and phosphoenol pyruvate and NADH were purchased from Sigma; all except the NADH were dissolved as concentrated stock solutions at pH 7. The buffer used in all experiments consisted of 20 mM-Tris·HCl (pH 7·5), 0·1 mM-dithiothreitol, 0·5 mM-ATP, 2 mM-phosphoenol pyruvate and 4 mM-$MgCl_2$ at 37°C unless otherwise indicated.

(b) *DNA*

Single-stranded M13 phage DNA and etheno M13 DNA were prepared as described (Menetski & Kowalczykowski, 1985). Poly(dA) and poly(dT) were purchased from PL Biochemicals and their concentrations were determined using extinction coefficients of 9400 and 8520/M nucleotide per cm at 260 nm and 264 nm, respectively.

(c) *Proteins*

RecA protein was purified from *E. coli* strain KM1842 using the procedure described by Cox *et al.* (1981) or from strain JC12772 (Uhlin & Clark, 1981) (kindly provided by Dr A. J. Clark of University of California at Berkeley), with no difference in the results obtained. Protein concentration was determined using an extinction coefficient of $2{\cdot}7 \times 10^4$/M per cm at 280 nm.

SSB protein was isolated from strain RLM727 using a preparative protocol provided by Dr Roger McMackin of Johns Hopkins University. Protein concentration was determined by using an extinction coefficient of $3{\cdot}0 \times 10^4$/M per cm at 280 nm (Ruyechan & Wetmur, 1975).

Gene *32* protein was prepared from T4 phage (33^-, 55^-, 61^-) as described (Kowalczykowski *et al.*, 1981*b*). Protein concentration was determined using an extinction coefficient of $3{\cdot}7 \times 10^4$/M per cm at 280 nm.

Lactate dehydrogenase and pyruvate kinase were purchased from Sigma. Both are provided as ammonium sulfate suspensions and working solutions of these enzymes were made by centrifuging a homogeneous sample of the suspension, drawing off the supernatant, and then dissolving the protein pellet in buffer at a concentration of 5 units of each/μl.

(d) *ATPase assay*

The ATPase assay used was described by Kreuzer & Jongeneel (1983) and is based on the fact that upon hydrolysis of ATP to ADP by RecA protein, one equivalent of phosphoenol pyruvate is converted to pyruvate by pyruvate kinase when the ADP is regenerated to ATP. The pyruvate is subsequently converted to lactate upon the oxidation of 1 equivalent of NADH to NAD^+ by lactate dehydrogenase. This oxidation of NADH results in a decrease in the absorbance at 340 nm that can be readily followed. Thus, the rate of change of the absorbance at 340 nm is proportional to the rate of steady-state ATP hydrolysis. For the 0·5 ml volume employed in the ATPase assays described here, the rate of ATP hydrolysis in μmol/min is equal to the absolute value of the slope of the absorbance change time-course (dA/dt) multiplied by 4·94.

The ATPase assays were conducted by first dissolving 1 mg of NADH in 10 ml (or, in some cases, 5 ml) of reaction buffer. To 0·5 ml of this buffer, 12·5 units each of pyruvate kinase and lactate dehydrogenase were added and allowed to incubate at the appropriate temperature for 1 to 2 min; DNA was then added, followed by RecA protein. When SSB protein is present, the order of addition is indicated. The absorbance data were collected as a function of time using a Hewlett–Packard 8450 spectrophotometer equipped with the 89100A temperature controller. The slope (i.e. absorbance change per unit time) was obtained by using the dA/dt-software key provided on the HP8450, averaged over the time interval during which the hydrolysis rate was linear. In standard reaction buffer, when the concentration of single-stranded M13 DNA exceeds the RecA protein concentration (e.g. 0·2 μM-RecA protein and 3 μM-DNA), this ATPase assay yields a steady-state rate of ATP hydrolysis of 28·3 mol ATP/mol RecA protein per min. This value is in good agreement with previously obtained values using a different assay method (Weinstock *et al.*, 1981; Kowalczykowski, 1986).

3. Results

(a) *Effect of SSB protein on RecA protein ATPase activity stimulated by single-stranded M13 DNA*

The addition of RecA protein to a cuvette containing single-stranded M13 DNA, ATP and the components of the ATP-regenerating/NADH-oxidizing system yields the type of data shown in Figure 1 (left-most trace). The steady-state hydrolysis of ATP to ADP by RecA protein results in an equimolar conversion of NADH to NAD by the coupled ATP-regenerating and NADH-oxidizing enzymes. The optical density decreases in a linear manner due to the oxidation of NADH until all of the NADH is exhausted. The total absorbance change for this concentration of NADH is approximately $-0{\cdot}7\ A_{340}$ unit. The slope of the linear portion of such data is proportional to the rate of ATP hydrolysis and, for the left-most trace in Figure 1, the slope is $-8{\cdot}37 \times 10^{-4}\ A_{340}$/s; this slope corresponds to 4·13 nmol of ATP hydrolyzed per minute under these experimental conditions.

If SSB protein is added to a cuvette containing the single-stranded M13 DNA *prior* to the addition

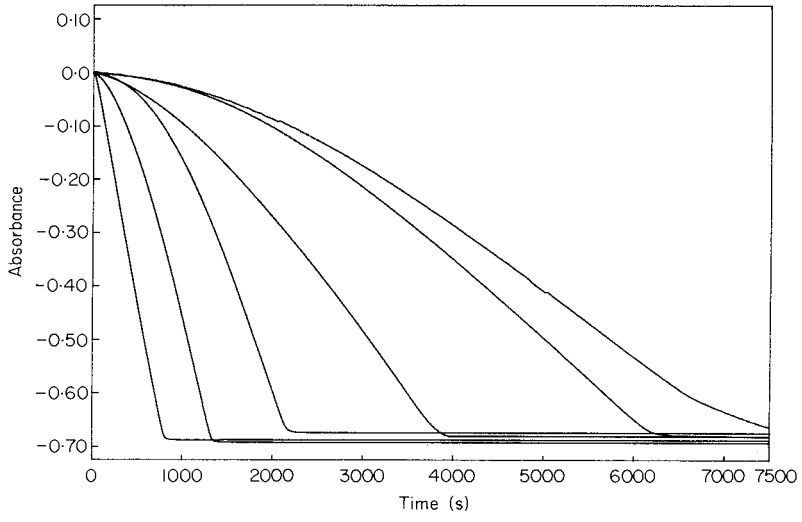

Figure 1. Effect of SSB protein on RecA protein ATPase activity when SSB protein is added to single-stranded M13 DNA prior to the addition of RecA protein. SSB protein was added to 3 μM-single-stranded M13 DNA in standard reaction buffer; after 1 to 2 min, 0·8 μM-RecA protein was added at time zero. The concentrations of SSB protein (in μM) were (from left to right): 0, 0·1, 0·2, 0·3, 0·4 and 0·5.

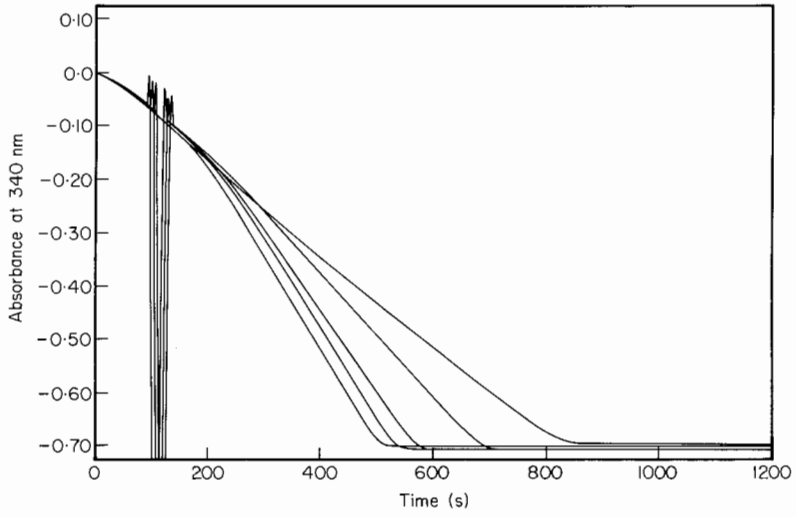

Figure 2. Effect of SSB protein on RecA protein ATPase activity when SSB protein is added last. RecA protein (0·8 μM) was added to 3 μM-single-stranded M13 DNA in standard reaction buffer at time zero. After 100 s (discontinuity in each trace), various amounts of SSB protein (in μM) were added (from right to left): 0, 0·1, 0·2, 0·4 and 0·5.

of RecA protein, a dramatic inhibition of the ATPase activity is observed (Fig. 1). With increasing concentrations of SSB protein, a pronounced lag in ATP hydrolysis is observed, with little hydrolysis occurring for several minutes at SSB protein concentrations of 0·4 and 0·5 μM. On the basis of the binding experiments described in the accompanying paper (Kowalczykowski *et al.*, 1987), saturation of the DNA by SSB protein occurs at approximately 0·3 μM-SSB and, therefore, a competition for the available DNA-binding sites is expected above this SSB protein concentration. The appearance of a lag in the ATP hydrolysis data is consistent with the interpretation that the two proteins compete for the same DNA binding sites. Following the lag, the rate of ATP hydrolysis increases with time, though at the higher SSB protein concentrations it does not achieve the rate that is observed in the absence of SSB protein. The results suggest that RecA protein is capable of displacing only a limited amount of the SSB protein.

In contrast to this inhibitory effect of SSB protein on RecA protein ATPase activity when it is added *prior* to the RecA protein, the effect of SSB protein on the ATPase activity when added *after* the addition of RecA protein to the single-stranded DNA is shown in Figure 2. Note that these experimental conditions are identical with those in Figure 1, except that the order of RecA and SSB protein addition is reversed. The data in Figure 2 demonstrate that the addition of SSB protein 100 seconds after the addition of RecA protein to a cuvette containing single-stranded DNA and ATP results in a stimulation, rather than inhibition, of the ATPase activity. This stimulatory effect is dependent upon the SSB protein concentration and appears to saturate at concentrations of SSB protein greater than 0·2 μM. Thus, it is clear that under identical conditions, SSB protein can exert completely opposite effects on the ATPase activity of RecA protein depending upon the order of addition of the RecA and SSB proteins. These effects are summarized in Figure 3, where the terminal rate of ATP hydrolysis (i.e. determined from the final linear range of experimental time points) is plotted as a function of SSB protein concentration for both sets of experiments. It is clear that when SSB protein is added first (filled circles), a greater than fourfold inhibition of ATP hydrolysis rate occurs; but when SSB protein is added last (filled squares), a greater than twofold stimulation in the rate is observed. The apparent DNA-binding stoichiometry for SSB protein obtained from the inhibition data is approximately 9(±1) nucleotides per SSB protein monomer and is approximately 15(±2) nucleotides per SSB protein monomer for the SSB protein stimulation data.

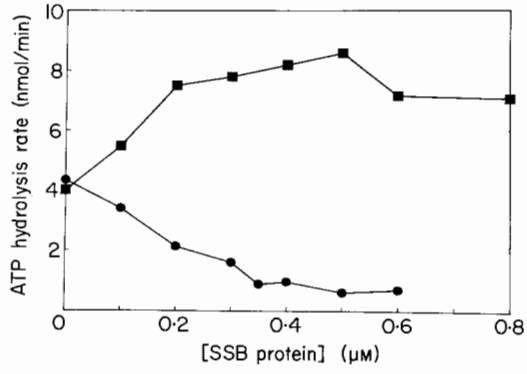

Figure 3. Observed ATP hydrolysis rates plotted against SSB protein concentration. The terminal ATP hydrolysis rate obtained from the data in Figs 1 and 2 and other data, not shown, is plotted as a function of the SSB protein concentration. The filled squares represent the rate obtained when SSB protein is added last; the filled circles represent the rate obtained when SSB protein is added prior to the RecA protein.

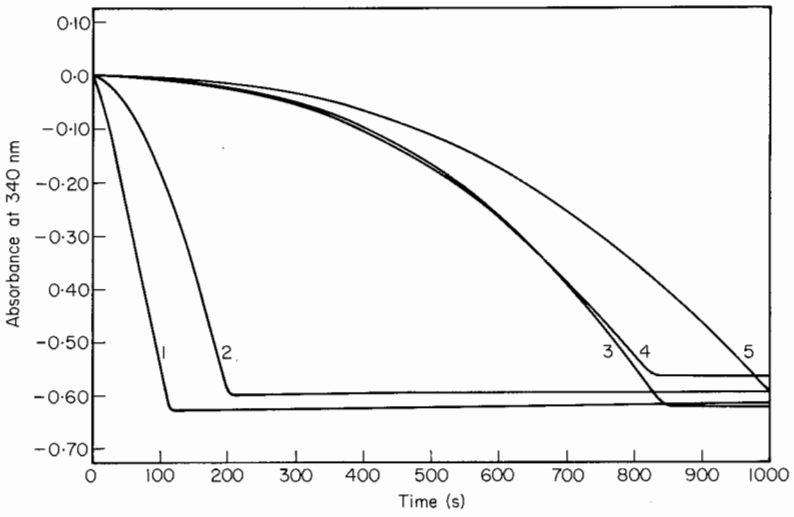

Figure 4. Effect of the order of component addition on the observed ATP hydrolysis rate. Components were added in the order indicated below. The final concentration of each ingredient was: RecA protein (2 μM), SSB protein (3·5 μM), and single-stranded M13 DNA (35 μM). Reactions were carried out in standard reaction buffer. Time zero represents the time of addition of the last component: curve 1, RecA protein and DNA were pre-incubated for 2 min in the absence of ATP and then followed by the addition of a mixture containing SSB protein and ATP; curve 2, RecA and SSB proteins were added simultaneously to a solution containing ATP and DNA; curve 3, SSB protein, DNA and ATP (added in that order) were pre-incubated for 1 min and then followed by the addition of RecA protein; curve 4, RecA protein and DNA were pre-incubated in the absence of ATP for 2 min, followed by the addition of SSB protein and incubation for an additional 5 min, and then followed by the addition of ATP; curve 5, SSB protein and DNA were pre-incubated for 1 min in the absence of ATP, followed by the addition of RecA protein and incubation for an additional 30 min, and then followed by the addition of ATP.

Although these opposite effects of SSB protein may appear contradictory, the order of SSB protein addition has been shown to have a significant effect on the RecA protein-catalyzed strand assimilation reaction (Cox & Lehman, 1982; Cox *et al.*, 1983*b*; Radding *et al.*, 1983), and those reported effects parallel the results observed here. To determine which components were important to prevent inhibition of ATPase activity by SSB protein, the order of addition of the components was varied and the results are shown in Figure 4. If RecA protein is pre-incubated with single-stranded DNA for two minutes, and SSB protein and ATP are added as a mixture (curve 1), then no inhibition by SSB protein is observed compared to a control experiment carried out in the absence of SSB protein (not shown). Also, if RecA and SSB proteins are added simultaneously to a solution containing DNA and ATP, only a slight lag is observed (curve 2). However, if SSB protein is pre-incubated with the single-stranded DNA and then followed by adding either RecA and then ATP (curve 5), or by adding ATP and then RecA protein (curve 3), a significant inhibition is obtained. Finally, if RecA protein is pre-incubated with single-stranded DNA in the absence of ATP for two minutes, followed by incubation with SSB protein for five minutes, and then ATP is added, a significant inhibition is again observed (curve 4). Thus, in order to prevent inhibition of RecA protein single-stranded DNA-dependent ATPase activity by SSB protein, it is necessary to incubate RecA protein, ATP and DNA together either prior to, or simultaneously with, the addition of SSB protein. If SSB protein is allowed to bind to the single-stranded DNA first, either directly or by displacement of RecA protein in the absence of ATP, then a significant inhibition of ATPase activity is observed.

(b) *Effect of SSB protein on RecA protein ATPase activity stimulated by etheno M13 DNA*

In the accompanying paper (Kowalczykowski *et al.*, 1987), it was demonstrated that the fluorescent, chloroacetaldehyde-modified single-stranded M13 DNA referred to as etheno M13 DNA (Menetski & Kowalczykowski, 1985) was incapable of forming complexes with RecA protein that were resistant to displacement by SSB protein. This result is in contrast to those obtained with unmodified single-stranded M13 DNA. To determine whether these observations are paralleled in the effects of SSB protein on ATPase activity, the effect of SSB protein on RecA protein ATPase activity stimulated by etheno M13 DNA was investigated. ATPase assays were conducted exactly as those described for Figure 2, where the SSB protein was added last; however, etheno M13 DNA was employed as a substrate rather than the native DNA. In contrast to the results obtained using the unmodified DNA substrate, the addition of SSB protein to the RecA protein–etheno M13 DNA assay mixture resulted in an *inhibition* of ATPase activity (raw data not shown). This inhibitory effect of SSB protein on the ATP hydrolysis rate using etheno M13 DNA as a

substrate is shown in Figure 5, where the terminal ATP hydrolysis rate (triangles) is plotted as a function of the SSB protein concentration. Maximal inhibition by SSB protein occurs at a stoichiometry of approximately 17(±2) nucleotides per SSB protein monomer, which is consistent with the stoichiometry of SSB protein binding to etheno M13 DNA obtained from direct DNA-binding studies (Kowalczykowski *et al.*, 1987). When etheno M13 DNA is used as the DNA substrate, the inhibition of RecA protein DNA-dependent ATPase activity by SSB protein observed is not instantaneous and the measured ATP hydrolysis rate decays with a half-time of approximately two to three minutes. These results suggest that a time-dependent displacement of the RecA protein from the DNA by SSB protein occurs, resulting in an inhibition of ATPase activity. This inhibitory effect is also consistent with the direct DNA binding experiments that show a time-dependent displacement of RecA protein from etheno M13 DNA by SSB protein, occurring with a half-time of 2·4 minutes (Kowalczykowski *et al.*, 1987).

At this point, the question can be raised as to why SSB protein has an inhibitory effect on RecA protein ATPase activity when using etheno M13 DNA as a substrate. Although the chemical modification may have some "unknown" effect on RecA protein ATPase activity, it is known that chloroacetaldehyde modifies both the adenine and cytosine bases so that complementary base-pairing is prevented (Barrio *et al.*, 1972; Tolman *et al.*, 1974). Thus, etheno M13 DNA is completely devoid of any secondary structure and, as will be shown below, the stimulatory effect of SSB protein is dependent on the presence of secondary structure within the DNA.

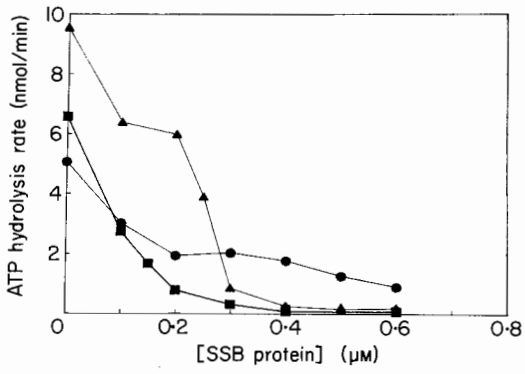

Figure 5. Effect of SSB protein on RecA protein ATPase activity stimulated by etheno M13 DNA, poly(dT) and poly(dA). RecA protein (0·8 μM) was mixed with the DNA in standard reaction buffer, followed by the addition of SSB protein. The terminal ATP hydrolysis rate was determined and plotted *versus* the SSB protein concentration: (▲) etheno M13 DNA (5·1 μM); (■) poly(dT) (3 μM); (●) poly(dA) (3 μM).

(c) *Dependence of ATPase activity on RecA protein concentration*

To clarify the relationship between the binding of SSB and RecA proteins to single-stranded DNA further, the dependence of the ATP hydrolysis rate on RecA protein concentration, at a fixed DNA concentration, in both the absence and the presence of SSB protein was determined. Figure 6 displays the steady-state rate of ATP hydrolysis plotted as a function of the RecA protein concentration when single-stranded M13 DNA is used as the substrate. In the absence of SSB protein, the rate of ATP hydrolysis increases linearly with RecA protein concentration until a plateau is achieved, at approximately 0·25 μM-RecA protein (filled squares). This plateau value corresponds to an hydrolysis rate of 4·3 nmol of ATP hydrolyzed per minute and occurs at an apparent stoichiometry of approximately 12(±2) nucleotides per RecA protein monomer. This apparent binding stoichiometry is significantly greater than the *minimum* values obtained from DNA binding studies (Silver & Fersht, 1982, 1983; Menetski & Kowalczykowski, 1985; Morrical & Cox, 1985; Bryant *et al.*, 1985) and suggests that much of the DNA is not capable of supporting the single-stranded DNA-dependent ATPase activity of RecA protein. Consistent with this interpretation is the fact that the binding stoichiometry observed from the ATPase data is in good agreement with binding stoichiometries determined from direct DNA binding data that were obtained at the higher magnesium concentrations, which are more likely to stabilize secondary structure (Morrical & Cox, 1985; Tsang *et al.*, 1985).

To study the effect of SSB protein on the observed RecA protein DNA-binding stoichio-

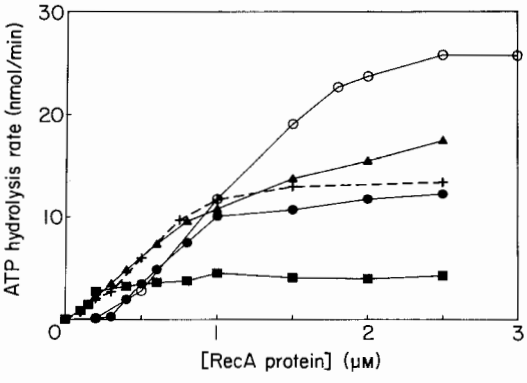

Figure 6. ATP hydrolysis rate plotted against RecA protein concentration. When SSB protein is present, it is always added last to the reaction mixture. All reactions, except + data, were carried out in standard reaction buffer and contained the following: (■) 3·0 μM-single-stranded M13 DNA; (●) 3·0 μM-single-stranded M13 DNA and 0·6 μM-SSB protein; (○) 6·0 μM-single-stranded M13 DNA and 1·2 μM-SSB protein; (▲) 5·1 μM-etheno M13 DNA; (+) 3·0 μM-single-stranded M13 DNA and 0·6 μM-SSB protein, except at 10 mM-$MgCl_2$.

metry, ATPase assays were initiated by adding increasing concentrations of RecA protein to the single-stranded M13 DNA, followed by the addition of a saturating amount of SSB protein (0·6 μM) after two minutes, which yielded raw data similar to that shown in Figure 2. The steady-state ATP hydrolysis rate obtained after addition of SSB protein is plotted as a function of RecA protein concentration in Figure 6 (filled circles). It is clear that SSB protein has two different effects: at low RecA protein concentrations it inhibits ATPase activity, but at high RecA protein concentrations it stimulates ATPase activity. At concentrations less than 0·3 μM-RecA protein, SSB protein almost completely inhibits the ATPase activity, whereas above 0·3 μM-RecA protein, a linear increase in ATP hydrolysis rate is seen with increasing RecA protein concentration. Note that an apparent saturation is again observed, but it occurs at a RecA protein concentration of 1 μM and at a plateau value of 12·3 nmol of ATP hydrolyzed per minute; these values are approximately threefold greater than those obtained in the absence of SSB protein (filled squares). The apparent stoichiometry of RecA protein binding to the DNA can be obtained from the steeply rising region of the data by determining the amount of protein required to saturate the DNA (1·0 μM), less the amount required to initiate ATP hydrolysis (0·3 μM), assuming that little or no RecA protein is bound at concentrations less than 0·3 μM (Kowalczykowski *et al.*, 1987). This yields an apparent DNA binding stoichiometry of 4·3 nucleotides per RecA protein monomer. This stoichiometry is consistent with that obtained from DNA-binding studies and suggests that, in the absence of SSB protein, only one-third of the single-stranded DNA is accessible to RecA protein, but that the addition of SSB protein allows RecA protein somehow to utilize all of the single-stranded M13 DNA as a substrate for ATPase activity. This interpretation is consistent with the approximately threefold increase in both the binding stoichiometry and the plateau ATP hydrolysis rate.

To be certain that the rate of ATP hydrolysis was, in fact, proportional to the amount of RecA protein–DNA complex present, an experiment identical with that described above was carried out, except that the amount of single-stranded M13 DNA and SSB protein was doubled. The results obtained are also shown in Figure 6 (open circles) and they demonstrate that when the DNA concentration is doubled, both the amount of RecA protein required for saturation (1·8 μM) and the plateau value for ATP hydrolysis (25·8 nmol ATP hydrolyzed/min) are doubled. Thus, the amount of ATP hydrolysis obtained for a given set of conditions is indeed proportional to the amount of RecA protein–single-stranded DNA complex present.

To ensure that the effect of SSB protein on RecA protein ATPase activity does not change under the conditions normally used for the RecA protein strand assimilation reaction, experiments identical with those described in this section were carried out in a buffer containing 10 mM-$MgCl_2$. Those data obtained in the presence of SSB protein are plotted in Figure 6 (crosses) and are very similar to the equivalent data obtained at 4 mM-$MgCl_2$, except that there is no inhibition by SSB protein at low RecA protein concentrations (i.e. the lag is absent) and that at all RecA protein concentrations the ATP hydrolysis rates are approximately 15 to 20% greater. In the absence of SSB protein (not shown), the data at 10 mM-$MgCl_2$ are similar to those at 4 mM-$MgCl_2$, except that the ATP hydrolysis rates at all RecA protein concentrations are 15 to 20% greater at the higher magnesium concentration. Thus, the effect of SSB protein is similar under these two sets of conditions.

If the inability to utilize more than half of the single-stranded M13 DNA as a substrate for ATPase activity is due to the presence of secondary structure in the M13 DNA, then it might be expected that the etheno M13 DNA substrate, which is totally devoid of secondary structure, would not show both the anomalously high stoichiometry and low plateau ATP hydrolysis rate, nor would SSB protein be required for maximum activity. When increasing concentrations of RecA protein are added to etheno M13 DNA, in the absence of SSB protein, an increase in hydrolysis rate is observed (Fig. 6, filled triangles). In contrast to the data obtained using unmodified M13 DNA as a substrate (in the absence of SSB), the results obtained with etheno M13 DNA show an apparent saturation at approximately 0·9 μM-RecA, which corresponds to an apparent stoichiometry of 6($\pm$1) nucleotides per RecA protein monomer.

A comparison of the results in Figure 6 shows that the etheno M13 DNA data are very similar to the single-stranded M13 DNA data obtained in the *presence* of SSB protein (except for the inhibition at low RecA protein concentrations at the lower $MgCl_2$ concentration); i.e. the increase in ATP hydrolysis rate per micromolar RecA protein concentration is similar; the apparent stoichiometry of DNA binding is similar; and the final plateau values for the ATP hydrolysis rate, when normalized for DNA concentration differences, are similar. Thus, it appears that the properties of etheno M13 DNA as a substrate for RecA protein ATP hydrolysis activity are very comparable to those of single-stranded M13 DNA *only* if SSB protein is present, and are quite different from those of single-stranded M13 DNA alone. Since etheno M13 DNA has no secondary structure and since SSB protein can remove secondary structure from native DNA, it is logical to conclude that etheno M13 DNA (in the absence of SSB protein) and single-stranded M13 DNA in the *presence* of SSB protein are similar because they are effectively free from secondary structure. Therefore, it is likely that one function of SSB protein is to remove secondary structure from native single-stranded DNA and therefore allow RecA protein "access" to these regions of DNA.

(d) *Properties of synthetic polynucleotides*

If the similarities, with regard to RecA protein binding stoichiometry and the plateau ATP hydrolysis rate, between etheno M13 DNA in the absence of SSB protein and single-stranded M13 DNA in the presence of SSB protein are due solely to the absence of secondary structure, then synthetic homopolynucleotides should behave similarly to the etheno M13 DNA. Therefore, the ATPase activity as a function of the RecA protein concentration was investigated using poly(dT) as a substrate. The results are displayed in Figure 7 (filled triangles) and show that the ATP hydrolysis rate increases with increasing RecA protein concentration until an apparent stoichiometry of 6(±1) nucleotides per RecA protein monomer is reached. This stoichiometry is similar to that obtained with single-stranded M13 DNA in the presence of SSB protein and is identical with the value obtained with etheno M13 DNA. Also, the poly(dT) data display the same gradual increase in ATP hydrolysis rate at the higher RecA protein concentrations that is observed with etheno M13 DNA and, when normalized for the DNA concentration difference, the ATP hydrolysis rates are nearly identical. The maximum hydrolysis rate with poly(dT) (≈8 nmol ATP hydrolyzed/min) exceeds that obtained with single-stranded M13 DNA alone and approaches that obtained with single-stranded M13 DNA in the presence of SSB protein. Thus, the behavior of poly(dT) as a substrate for RecA protein ATPase activity is more similar to that of etheno M13 DNA and that of the SSB protein–single-stranded M13 DNA mixture, than to that of single-stranded M13 DNA alone. Data for poly(dA) (squares) are also shown in Figure 7 and they demonstrate that its behavior is similar to that of poly(dT), except that the gradual increase at higher RecA protein concentrations is absent.

Etheno M13 DNA in the absence of SSB protein and single-stranded M13 DNA in the presence of SSB protein behave similarly with regard to the stoichiometry of RecA protein ATPase activity, but they differ in one important property. The addition of SSB protein to RecA protein that is bound to etheno M13 DNA results in an *inhibition* of ATPase activity (Fig. 5), whereas with single-stranded M13 DNA the opposite effect is observed (Fig. 3). Again, if this contrasting behavior is due solely to differences in secondary structure of these two DNA molecules, then both poly(dT) and poly(dA) would be expected to behave similarly to etheno M13 DNA with regard to the effect of SSB protein. As expected, when SSB protein is added two minutes after incubation of RecA protein and either polynucleotide, a time-dependent inhibition of ATPase activity is observed (not shown). The data for the terminal steady-state rate of ATP hydrolysis as a function of the SSB protein concentration for both poly(dT) and poly(dA) are plotted in Figure 5. The apparent stoichiometry obtained from this ATPase activity inhibition data is 17(±4) nucleotides per SSB protein monomer for poly(dT), and 22(±3) for poly(dA); these values are comparable to those observed for etheno M13 DNA. Thus, both poly(dT) and poly(dA) behave similarly to etheno M13 DNA with regard to their behavior as substrates for RecA protein DNA-dependent ATPase activity.

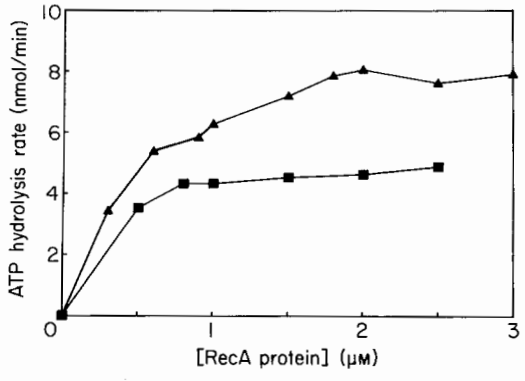

Figure 7. ATP hydrolysis rate plotted against RecA protein concentration. All reactions were carried out in standard reaction buffer and contained the following: (▲) 3·0 μM-poly(dT); (■) 3·0 μM-poly(dA).

(e) *Inhibition by SSB protein is prevented by homologous pairing*

As mentioned previously, the observation that SSB protein inhibited the ATPase activity of RecA protein when etheno M13 DNA, poly(dA) or poly(dT) is used as a substrate, but not when single-stranded M13 DNA is used, suggests that somehow secondary structure or the presence of fortuitous base-pairing within the single-stranded M13 DNA prevented inhibition by SSB protein. To test this hypothesis, the effect of SSB protein on a mixture of poly(dA) and poly(dT) was determined. To carry out this experiment, poly(dT) and poly(dA) were separately pre-incubated in the presence of ATP at 37°C with a saturating amount of RecA protein. The two polynucleotide solutions were then mixed together and allowed to incubate for ten minutes. During this time the separate strands are renatured to form duplex poly(dA)·poly(dT) as judged by an appropriate hypochromic effect (not shown). Afterward, a saturating amount of SSB protein was added and the ATP hydrolysis rate was monitored further. The results observed (not shown) demonstrate that SSB protein does *not* inhibit the RecA protein ATPase activity when using renatured duplex poly(dA)·poly(dT); this is in marked contrast to the inhibitory effect of SSB on the individual strands of poly(dA) or poly(dT) (Fig. 5). Thus, these results, as well as the contrasting effects of SSB protein obtained using etheno M13 DNA *versus* single-stranded M13 DNA, demonstrate that SSB protein cannot inhibit the ATPase activity of RecA protein if the DNA substrate used is capable of forming secondary structure (i.e. single-stranded M13 DNA) or

complementary duplex structure (i.e. poly(dA)·poly(dT)). Both of these observations can be generalized to conclude that, in the presence of regions of homologously paired complementary DNA sequences, SSB protein is unable to inhibit RecA protein ATPase activity. The significance of this result will be addressed in the Discussion.

(f) *Effects of temperature, magnesium concentration and NaCl concentration*

Since temperature, magnesium ion concentration and salt concentration affect both the physical properties of RecA protein (e.g. binding affinity, aggregation state) and the stability of DNA secondary structure, the impact of these variables on the behavior of SSB protein regarding RecA protein ATPase activity was investigated.

If an ATPase assay using single-stranded M13 DNA as a substrate in standard buffer is carried out at 25°C, the rate of ATP hydrolysis in the absence of SSB protein is observed to be about threefold slower than at 37°C (not shown). Surprisingly, at this temperature, the effect of adding SSB protein is to *inhibit* the ATPase activity when the RecA protein concentration is 0·8 μM (Table 1). This result is in distinct contrast to the stimulatory effect of SSB protein observed at 37°C (Fig. 3). Concentrations of SSB protein as low as 0·1 μM begin to inhibit the activity of RecA protein, and the apparent stoichiometry of maximum SSB protein inhibition at 25°C is approximately 10(±3) nucleotides per SSB protein monomer (not shown); this value is in agreement with the stochiometry observed previously at 37°C (Fig. 3). Thus, SSB protein inhibits the ATPase activity of RecA protein at 25°C, in contrast to the stimulatory effect observed at 37°C. These results are consistent with the observations from DNA-binding studies, which show that SSB protein will displace RecA protein completely from single-stranded M13 DNA at 25°C but not at 37°C (Kowalczykowski *et al.*, 1987).

The DNA-binding studies also suggested that the amount of RecA protein resistant to SSB protein displacement is increased when the magnesium chloride concentration is increased (Kowalczykowski *et al.*, 1987). Therefore, to determine whether the stimulation by SSB protein could be restored by increasing the magnesium concentration, the effect of SSB protein on RecA protein ATPase activity was also determined at 25°C, but at 10 mM-$MgCl_2$ (see Table 1). In contrast to the inhibition obtained in buffer containing 4 mM-$MgCl_2$, SSB protein is observed to stimulate the ATPase activity of RecA protein in buffer containing 10 mM-$MgCl_2$ in the same way as is observed with 4 mM-$MgCl_2$ at 37°C. At 25°C, the ATP hydrolysis rate in the absence of SSB protein shows relatively little dependence (≈20 to 30%) on the $MgCl_2$ concentration from 1 mM to 10 mM when excess RecA protein is present relative to the DNA site concentration (i.e. 1·5 μM-RecA protein and 3 μM-single-stranded M13 DNA). However, under identical conditions in the presence of SSB protein, an almost linear increase in the hydrolysis rate is observed ranging from zero at 1 mM-$MgCl_2$ to a value of 8·4 μM-ATP hydrolyzed per minute at 10 mM-$MgCl_2$. At 10 mM-$MgCl_2$, the ATP hydrolysis rate in the presence of SSB protein is nearly threefold greater than in its absence at these concentrations of RecA protein and single-stranded M13 DNA. Thus, both elevated temperature and high $MgCl_2$ concentrations are conditions which favor stimulation of ATPase activity by SSB protein.

To verify this relationship of temperature and magnesium concentration on the effect of SSB protein ATPase activity, experiments identical with those in Figure 2 were carried out in 1, 4 and 10 mM-$MgCl_2$ and at 25, 37 and 45°C (RecA protein is stable to at least 50°C, data not shown). The results are summarized in Table 1 and demonstrate that, at elevated temperatures and $MgCl_2$ concentrations, the effect of SSB protein is stimulatory and, at lower temperature and $MgCl_2$ concentrations, the effect is inhibitory. The data in Table 1 also show that high magnesium concentrations can compensate for low temperatures and *vice versa*.

Since the stability of secondary structure is diminished at lower magnesium concentrations, it was of interest to determine whether all of the single-stranded M13 DNA was accessible to RecA protein at lower $MgCl_2$ concentrations. To determine whether this was the case, the RecA protein concentration dependence of ATPase activity was determined at 1 mM-$MgCl_2$ in the absence of SSB protein and is shown in Figure 8. A gradual hyperbolic increase in the rate of ATP hydrolysis is observed and, although the saturation point is not as clearly defined as in other data, an apparent stoichiometry at saturation of approximately 3·5 nucleotides per RecA protein monomer is obtained from the initial and terminal slopes. Since the binding appears to be weaker under these conditions, this apparent stoichiometry will represent an underestimate of the true value. Even with this limitation, this value is in good agreement with the apparent stoichiometry values obtained

Table 1
Effect of SSB protein on RecA protein ATPase activity

	Magnesium concentration (mM)		
Temperature (°C)	1	4	10
25	−	−/+	+
37	−	+	+
45	−	+	+

Effect of 0·6 μM-SSB protein when added last to RecA protein (0·8 μM) and single-stranded M13 DNA under standard conditions: +, stimulation; −, inhibition; −/+, inhibition at 0·8 μM-RecA protein but stimulation at 1·5 μM-RecA protein.

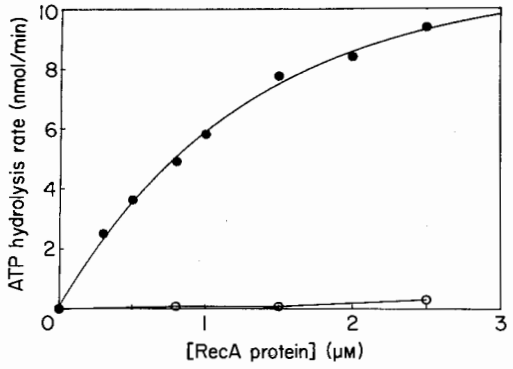

Figure 8. ATP hydrolysis rate at 1 mM-$MgCl_2$ plotted against RecA protein concentration in the presence and absence of SSB protein. Reactions were carried out in standard reaction buffer at 37°C, except that the $MgCl_2$ concentration was 1 mM. Reactions contained: (●) 3 μM-single-stranded M13 DNA; (○) 3 μM-single-stranded M13 DNA and 0·6 μM-SSB protein added last.

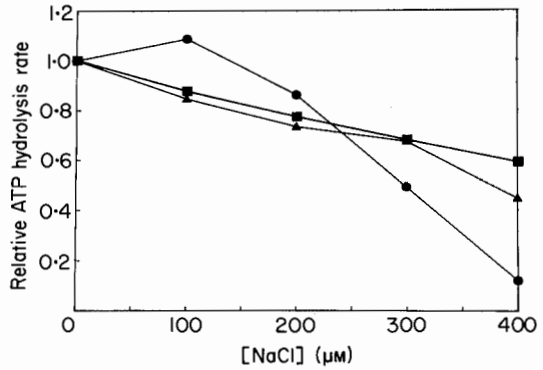

Figure 9. Salt dependence of the relative RecA protein ATP hydrolysis rate. Reactions were carried out in standard reaction buffer except that NaCl was added after the addition of all other components; all reactions contained 3·0 μM-DNA, 0·8 μM-RecA protein, and, when present, 0·6 μM-SSB protein; (●) single-stranded M13 DNA; (▲) single-stranded M13 DNA plus SSB protein added after RecA protein addition; (■) etheno M13 DNA. Data were normalized to represent 1·0 at zero NaCl by dividing each data set by the absolute ATP hydrolysis rates observed at zero NaCl.

using single-stranded M13 DNA at the higher $MgCl_2$ concentration, but only when SSB protein is present (Fig. 6). In addition, the data in Figure 8 yield a maximum hydrolysis rate of 9·4 nmol ATP hydrolyzed per minute. This value is comparable to the plateau hydrolysis rates obtained using DNA without secondary structure, i.e. etheno M13 DNA, poly(dA), and poly(dT), or single-stranded M13 DNA at 4 or 10 mM-$MgCl_2$ in the *presence* of SSB protein (see Figs 6 and 7), but is not in agreement with the values obtained with single-stranded M13 DNA at higher $MgCl_2$ concentrations in the *absence* of SSB protein. Therefore, at 1 mM-$MgCl_2$, single-stranded M13 DNA possesses little or no secondary structure with a stability sufficient to limit the binding of RecA protein. As might be expected from the results in Table 1, at 1 mM-$MgCl_2$, SSB protein has an inhibitory effect on RecA protein ATPase activity at all RecA protein concentrations tested, and this is also shown in Figure 8.

Finally, the effect of increasing NaCl concentrations on the ATPase activity of RecA protein using single-stranded M13 DNA as a substrate was investigated, both in the presence and absence of SSB protein. The results are presented in Figure 9 and show that, in the presence of SSB protein, only a 55% inhibition is observed at 400 mM-NaCl compared to a 90% inhibition in the absence of SSB protein. This finding may suggest that the RecA protein–DNA complex is more stable in the presence of SSB protein, but it may also reflect the fact that the increased salt concentration results in a more stable DNA secondary structure, and that SSB protein facilitates the elimination of this structure at the higher salt concentrations. To attempt to distinguish between these possibilities, the NaCl sensitivity of the ATPase activity was also determined using etheno M13 DNA as the substrate (see Fig. 9). The salt sensitivity using etheno M13 DNA is virtually identical with that of the unmodified M13 DNA in the *presence* of SSB, again suggesting that the similarity of these two sets of experimental conditions results from the absence of secondary structure in the DNA molecules rather than from some specific effect of SSB protein on RecA protein. Because the affinity of RecA protein is greater for etheno M13 DNA than it is for single-stranded M13 DNA, this interpretation of the data is not unequivocal. However, it is consistent with all of the other data presented in this paper that have illustrated in many ways that etheno M13 DNA alone and single-stranded M13 DNA *plus* SSB protein are equivalent due to the absence of secondary structure.

(g) *Effect of ATP concentration*

We have interpreted the stimulatory effects of SSB protein on the ATPase activity of RecA protein as being due to the elimination of secondary structure by SSB protein. For this to be the correct explanation, RecA protein must be able subsequently to displace the SSB protein from this region of the DNA (for further details of the proposed model, see Discussion). Since both ATP-γ-S and ATP induce a high-affinity single-stranded DNA binding form for RecA protein, it is likely that this form of the protein is responsible for displacement of the SSB protein. Consistent with this expectation, RecA protein is able to displace SSB protein in the presence of ATP-γ-S or ATP (under limited conditions) (Kowalczykowski *et al.*, 1987). Therefore, we expected to see an effect of ATP concentration on the stimulatory effect of SSB protein that we have reported here.

Figure 10 shows the effect of ATP concentration on the RecA protein ATPase activity in both the presence and the absence of SSB protein. The data

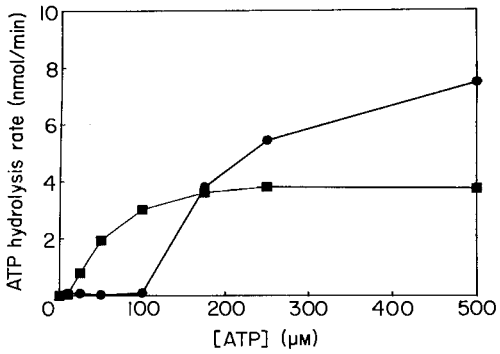

Figure 10. Effect of ATP concentration on RecA protein ATPase activity in the presence and absence of SSB protein. Reactions were carried out in standard reaction buffer except that the ATP concentration was varied as indicated. Reactions contained 3 μM-single-stranded M13 DNA, 0·8 μM-RecA protein, and when present, 0·6 μM-SSB protein was added last: (■) in the absence of SSB protein; (●) in the presence of SSB protein.

demonstrates that, at low ATP concentrations (< 100 μM), the addition of SSB protein inhibits the ATPase activity totally, whereas, at higher ATP concentrations (> 200 μM), a marked stimulation by SSB protein is observed. At ATP concentrations greater than 200 μM, a gradual increase in the hydrolysis rate is observed in the presence of SSB protein, but not in its absence. This finding suggests that, at higher ATP concentrations, RecA protein is able to compete more effectively with SSB protein. In the absence of SSB protein, the apparent K_m value for ATP is ≈50 μM-ATP, whereas in its presence, the value is ≈150 to 200 μM. Thus, the ATP concentration dependence of RecA protein ATPase activity in the presence of SSB protein reflects not only the intrinsic ATP dependence of RecA protein ATPase activity, but also superimposed is the ATP dependence of the competition of RecA and SSB proteins for the limited DNA-binding sites. Since the apparent K_m value for ATP observed in the presence of SSB protein is greater than in its absence, this implies that SSB protein acts as a competitor of RecA protein ATPase activity, which is consistent with our DNA-binding studies (Kowalczykowski *et al.*, 1987). Also consistent with our expectations is the fact that, in the presence of SSB protein, increasing ATP concentrations promote a continuously elevated steady-state level of ATP hydrolysis. Since the ATP hydrolysis rate observed in the absence of SSB protein shows no dependence on ATP concentration above 200 μM, the ATP dependence in the presence of SSB protein is presumably the result of an increase in the steady-state amount of RecA protein–DNA complex present *versus* the amount of SSB protein–DNA complex present. Thus, these data suggest that the ATP-bound species of RecA protein is responsible for the displacement of SSB protein.

(h) *Effect of bacteriophage T4-coded gene 32 protein*

To determine whether the stimulatory effects of SSB protein that we have observed are unique to the *E. coli* SSB protein, or whether they are a general property of helix-destabilizing proteins, the effect of bacteriophage T4-coded gene *32* protein was investigated. When added last, gene *32* protein was found to stimulate the ATPase activity of RecA protein if single-stranded M13 DNA is used as a substrate. This is identical with the behavior of SSB protein (see Figs 2 and 3). The maximum amount of stimulation is approximately two- to threefold and saturation of this stimulatory effect occurs at an apparent stoichiometry of 7·5(±1) nucleotides per gene *32* protein monomer at 4 mM-$MgCl_2$ and at 5·5(±1) nucleotides per gene *32* protein monomer at 10 mM-$MgCl_2$. These values are consistent with the range of site sizes for DNA binding that have been reported for gene *32* protein (see Kowalczykowski *et al.*, 1981*a*).

Since this behavior of gene *32* protein with regard to stimulation of RecA protein ATPase activity is very similar to that observed with SSB protein, it was of interest to determine what the RecA protein concentration dependence of the single-stranded M13 DNA-dependent ATPase activity is in the presence of an optimal amount of gene *32* protein (0·85 μM). As illustrated in Figure 11, the results obtained with gene *32* protein can be nearly superimposed on those obtained with SSB protein. Thus, it is very likely that both *E. coli* SSB protein and T4-coded gene *32* protein act to stimulate the ATPase activity and, by inference, the strand assimilation activity of *E. coli* RecA protein by a similar mechanism.

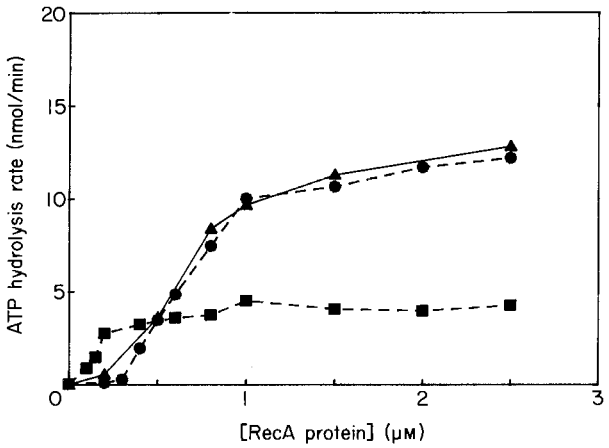

Figure 11. ATP hydrolysis rate as a function of the RecA protein concentration in the presence of gene *32* protein. Reactions were carried out in standard reaction buffer and contained RecA protein at the indicated concentration, 3·0 μM-single-stranded M13 DNA, and 0·85 μM-gene *32* protein added last (▲). For comparison, the equivalent data with SSB protein (●) and without SSB protein (■) from Fig. 6 are also plotted.

4. Discussion

(a) *Effects of SSB protein*

In this paper, we have shown that the effects of SSB protein on the single-stranded DNA-dependent ATPase activity of RecA protein are quite complex and can result in either an inhibition or a stimulation of activity, depending on temperature, magnesium concentration, type of DNA substrate and the order of addition of the components. When SSB protein is added to a solution of single-stranded M13 DNA prior to the addition of RecA protein, an inhibition of ATPase activity is observed (Fig. 1). This inhibition takes the form of both the appearance of a lag in the onset of ATP hydrolysis and a decrease in the final steady-state hydrolysis rate (Fig. 3). The maximum inhibition ($\approx$75%) by SSB occurs at an apparent stoichiometry of approximately ten nucleotides per SSB protein monomer (Fig. 3) and corresponds approximately to an SSB protein concentration required for saturation of the single-stranded DNA.

When SSB protein is added to the solution *after* the formation of a RecA protein–DNA complex, then one of two totally opposite effects is observed, depending on the type of DNA substrate, temperature and magnesium concentration used. When using either etheno M13 DNA, poly(dA), or poly(dT), the addition of excess SSB protein completely inhibits the ATPase activity with inhibition half-times of approximately 1·9 and 4·2 minutes for etheno M13 and poly(dT), respectively (not shown). In contrast, when single-stranded M13 DNA is used as a substrate at 37°C and either 4 mM or 10 mM-$MgCl_2$, a marked stimulation rather than inhibition of ATPase activity is observed at higher RecA protein concentrations (Fig. 6).

A comparison of the DNA to RecA protein stoichiometry for optimal ATPase activity provides insight into the nature of this unique stimulatory effect of SSB protein when single-stranded M13 DNA is used as a substrate. At a ratio of 6($\pm$1) nucleotides per RecA protein monomer, maximum ATPase activity is observed when etheno M13, poly(dA) and poly(dT) are used as substrates in the absence of SSB. However, for single-stranded M13 DNA in the absence of SSB protein, the apparent stoichiometry is 12($\pm$2) nucleotides per recA protein monomer. These results suggest that approximately one-half to two-thirds of the single-stranded M13 DNA is inaccessible to the RecA protein in the absence of SSB. In the presence of SSB protein (added last), an apparent stoichiometry of 4·5($\pm$1) nucleotides per recA protein monomer is obtained from the ATPase activity data at two different single-stranded M13 DNA concentrations (3 μM and 6 μM), which is in agreement with the etheno M13, poly(dA) and poly(dT) stoichiometries. Also consistent with the DNA inaccessibility interpretation is the fact that the maximum rate of ATP hydrolysis observed with single-stranded M13 DNA and saturating amounts of RecA protein is approximately one-third of the rate obtained with single-stranded M13 DNA in the presence of SSB (Fig. 6).

Finally, the data obtained at 1 mM-$MgCl_2$ (Fig. 8) suggest that, at this lower magnesium concentration, where secondary structure will be less stable, all of the single-stranded M13 DNA is available to RecA protein. This implies that at 1 mM-$MgCl_2$, RecA protein is able to remove any existing secondary structure without the aid of SSB protein. However, this RecA protein–single-stranded M13 DNA complex is quite dynamic, as evidenced by the fact that, when shifted to 10 mM-$MgCl_2$, its ATPase activity will slowly decay to the lower rate observed at the higher $MgCl_2$ concentrations (unpublished results); this suggests that some RecA protein has dissociated and DNA secondary structure has reformed.

These differences in stoichiometries and maximum ATP hydrolysis rates suggest to us that one difference between etheno M13, poly(dA) and poly(dT) *versus* single-stranded M13 DNA is that the presence of stable secondary structure prevents access of RecA protein to these regions of the DNA. The main function of SSB protein then is to remove this secondary structure and subsequently allow RecA protein to bind to these previously base-paired regions. This role for SSB protein was initially proposed by Muniyappa *et al.* (1984), and our data are consistent with such an interpretation. Thus, in this regard, the properties of etheno M13 DNA (which lacks secondary structure) are equivalent to those of single-stranded M13 DNA in the presence of SSB protein, which effectively would have no secondary structure.

However, one property of single-stranded M13 DNA that is unique and rather puzzling is that SSB protein does *not* ultimately inhibit the ATPase activity of RecA protein when this DNA is used as a substrate and when SSB protein is added *last* (at higher temperatures and magnesium concentrations). The observation that SSB protein inhibits ATPase activity when etheno M13 DNA is used as the substrate, but not when unmodified single-stranded M13 DNA is used, suggests to us that the fortuitous base complementarity present within single-stranded M13 DNA was somehow necessary (though not sufficient) for this curious effect. Consistent with this hypothesis are the data obtained using poly(dA) and poly(dT). When either of these two polynucleotides is used as a substrate for ATP hydrolysis alone, SSB protein completely inhibits ATPase activity (Fig. 5). However, if these two DNA molecules are allowed to renature in the presence of RecA protein and then followed by SSB protein addition, no inhibition of ATPase activity is observed. The ATP hydrolysis rate obtained on the poly(dA)·poly(dT) duplex DNA substrate that forms as a result of renaturation is consistent with the rate observed for duplex DNA-stimulated ATP hydrolysis (Kowalczykowski, 1985, and unpublished results). The fact that SSB protein is unable to inhibit ATPase activity in this case is consistent with the proposal that the presence of

complementary sequences within the DNA that are capable of base-pairing is responsible for the resistance to inhibition by SSB protein. This is probably the result of a unique RecA protein–DNA complex that forms with the complementary DNA sequences. However, the result that SSB protein inhibits the ATPase activity of RecA protein on single-stranded M13 DNA in 4 mM-$MgCl_2$ at 25°C but not at 37°C, suggests that base complementarity is a necessary though not sufficient requirement for this effect. Thus, some property of RecA protein that is enhanced at higher temperatures and magnesium concentrations and/or some property of SSB protein (presumably DNA binding) that is diminished at these conditions is also required. Currently, we are uncertain as to the molecular basis of the effects of temperature and magnesium concentration.

(b) *Mechanism of the SSB protein effect*

The mechanism by which SSB protein enhances the strand assimilation activity of RecA protein is of considerable interest, and a number of hypotheses have been put forward. Cox & Lehman (1982) suggested that a specific interaction between RecA and SSB protein prevented the dissociation of RecA protein from single-stranded DNA (also Cox *et al.*, 1983*a*,*b*). In the accompanying paper, we demonstrated through equilibrium and kinetic DNA-binding studies that SSB protein does not stabilize directly the RecA protein–single-stranded DNA complex (Kowalczykowski *et al.*, 1987). However, it is possible that SSB protein can act *indirectly* to form a more stable RecA protein–DNA complex by allowing RecA protein to bind to regions of DNA that previously contained secondary structure and to form a unique complex that is resistant to SSB displacement. This is the likely explanation for the observation that the lifetime of RecA protein–single-stranded M13 DNA complexes formed in the presence of SSB protein (or in 1 mM-$MgCl_2$) is tenfold greater than those formed in the absence of SSB protein (Kowalczykowski *et al.*, 1987).

It has also been suggested that SSB protein may serve as an assembly factor for RecA protein binding to single-stranded DNA (Griffith *et al.*, 1984) and, with regard to single-stranded M13 DNA, our data support this interpretation. For this particular DNA molecule, SSB protein is required for complete saturation of the DNA by RecA protein. However, since SSB protein inhibits binding of RecA protein to DNA substrates that are devoid of secondary structure, participation in assembly is not a general function of SSB protein. Hence, its role in assembly is actually a subset of SSB protein's main function, which is to remove secondary structure from single-stranded M13 DNA.

The idea that the role of SSB protein is to remove secondary structure present in single-stranded DNA was put forward by Muniyappa *et al.* (1984), and the results presented here completely substantiate this concept. In the absence of SSB protein, RecA protein is unable fully to saturate native single-stranded M13 DNA due to the presence of secondary structure in the DNA, and the role of SSB protein is to denature this structure and subsequently allow RecA protein to bind to these regions. The inability of RecA protein to denature local duplex structures is consistent with the observation that RecA protein is unable to destabilize duplex DNA (Cazenave *et al.*, 1984; Kowalczykowski, unpublished results), whereas SSB protein is capable of doing so (Segal *et al.*, 1972; Williams *et al.*, 1983).

Furthermore, our studies extend the original proposal for the role of SSB protein described by Muniyappa *et al.* (1984) in that we have demonstrated an additional feature of the RecA protein–DNA interaction in the presence of SSB protein; namely, that when DNA possessing complementary sequences capable of base-pairing is used as a substrate for ATPase activity (e.g. single-stranded M13 or poly(dA)·poly(dT)), the resultant RecA protein–DNA complex formed is refractory to inhibition by SSB protein at elevated temperatures and magnesium concentrations. Thus, there must be a unique RecA protein–DNA complex that forms within duplex DNA structures.

All of the results described in this paper are accommodated in the model presented in Figure 12,

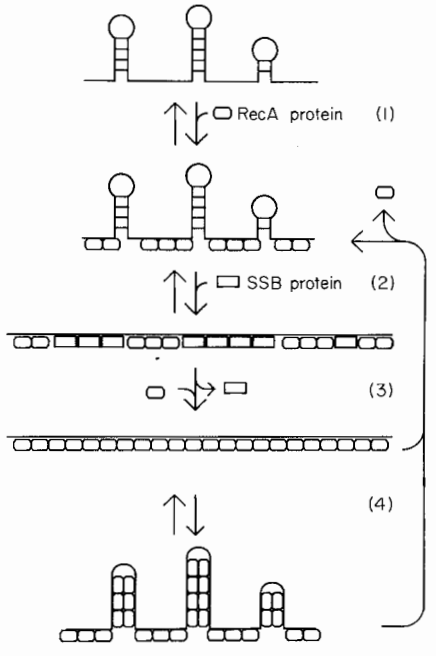

Figure 12. A model for the effect of SSB protein on the single-stranded DNA-dependent ATPase activity of RecA protein: (1) binding of RecA protein to regions of DNA devoid of secondary structure; (2) melting of secondary structure due to SSB protein binding; (3) competition of RecA and SSB proteins for DNA-binding sites showing only the situation where RecA protein can displace the SSB protein; and (4) formation of a unique RecA protein–DNA complex involving secondary structure.

which represents an extension of a model proposed by Muniyappa *et al.* (1984). In the first step, RecA protein is shown binding to the unstructured and weakly structured regions of native single-stranded DNA. In 1 mM-$MgCl_2$ these regions would represent nearly all of the DNA, but in 4 mM-$MgCl_2$ only approximately one-half to one-third of the DNA would be accessible to RecA protein. The remainder of the single-stranded DNA would be unavailable for RecA protein binding due to a kinetic limitation that prevents RecA protein from "melting-into" the stably base-paired regions of secondary structure within the DNA. Such a "kinetic block" to helix destabilization of native duplex DNA has been observed for T4-coded gene *32* protein (Jensen *et al.*, 1976). The addition of SSB protein (or gene *32* protein) results in denaturation of the secondary structure due to the helix-destabilizing property of this protein (step (2)). At this point, a transient ternary complex of RecA protein, SSB protein and single-stranded DNA is formed, and the ultimate type of protein–DNA complex finally formed in step (3) depends on the reaction conditions. At elevated temperatures ($\geqslant$37°C) and high magnesium concentrations ($\geqslant$4 mM-$MgCl_2$), RecA protein will displace the SSB protein from the DNA, resulting in a single-stranded DNA molecule that is essentially saturated with RecA protein, as shown in step (3). At the lower temperatures and $MgCl_2$ concentrations (see Table 1), and at low RecA protein concentrations, the competition for the limited DNA binding sites favors SSB protein binding, and RecA protein is displaced from the DNA, resulting in a nearly complete inhibition of ATPase activity (not illustrated). These interpretations of the effects of SSB protein on ATPase activity are completely consistent with the direct DNA-binding studies described by Kowalczykowski *et al.* (1987). Thus, there is a competition between RecA protein and SSB protein for DNA binding, and the outcome of this competition is determined by a combination of both kinetic and equilibrium properties.

The RecA protein–DNA complex formed in step (3) is kinetically labile, and dissociation of RecA protein results in the re-formation of DNA secondary structure (return arrows); thus, SSB protein is constantly required in our proposed mechanism. This continual requirement is consistent with the stoichiometric requirement of SSB protein (see Fig. 3), since, if SSB protein were required for the initial formation of a kinetically stable RecA protein–saturated single-stranded M13 DNA complex, then only a catalytic amount of SSB would be required. Since ATP is continuously hydrolyzed, the amounts of RecA protein and SSB protein bound to DNA at any instant must represent some steady-state average of their relevant kinetic and equilibrium properties.

Finally, in step (4), RecA protein forms a unique complex with the DNA that requires DNA homology and pairing, and results in a complex that is resistant to displacement by SSB protein. We know little regarding the details of this structure other than the fact that complementary sequences of DNA are required for its formation and that it only forms at elevated temperatures and magnesium concentrations, and we are not certain why this complex is resistant to displacement by SSB protein. Therefore, the structure of the RecA protein–single-stranded DNA complex depicted as the final product in Figure 12 should be viewed as schematic and, in that regard, two additional comments should be made. First, although the structure of the RecA protein–DNA complex within the hairpin regions is depicted with a RecA protein molecule bound to each DNA strand of the hairpin, it is also possible that both DNA strands are bound to only one RecA protein molecule *via* two separate DNA-binding sites within the protein. Studies of the duplex DNA-stimulated ATPase activity (Kowalczykowski, 1985; unpublished results) suggest that this may be the case and such an alternative view of the complex in Figure 12 may be more appropriate. Secondly, the complementary base-pairing in the RecA protein–DNA complex in Figure 12 is shown as being intramolecular; it is also possible that this final structure involves intermolecular pairing as well. In the case of the poly(dA)·poly(dT) pairing, this must clearly involve intermolecular pairing only.

The experiments involving the differing effects of SSB protein on the RecA protein ATPase activity stimulated by individual strands of poly(dT) or poly(dA) *versus* the renatured poly(dA)·poly(dT) duplex can also be understood within the general context of Figure 12. With either poly(dA) or poly(dT), RecA protein is able fully to saturate the DNA in the absence of SSB protein (step 1), since secondary structure is absent. However, when SSB protein is added, as in step (2), the outcome of competition for the limiting number of DNA sites is such that SSB protein displaces the RecA protein in a time-dependent reaction. This displacement results from the fact that SSB protein binds more tightly to these single-stranded DNA substrates *and* because, in the absence of secondary structure, RecA protein is unable to form the unique structure depicted in step (4). However, if the individual strands of poly(dA) and poly(dT) are allowed to renature in the presence of RecA protein *prior* to the addition of SSB protein, the resulting base-pairing produces a RecA protein–DNA complex that is resistant to inhibition by SSB protein. Clearly, homologous pairing is an important feature of this complex, since the only difference between these sets of experiments is the presence of complementary base-pairing.

Again, it should be emphasized that the steps in the above scheme are not under thermodynamic control but rather are under kinetic control. The very fact that the effect of SSB protein on ATPase activity depends on the order of addition demonstrates that the system is not at equilibrium. RecA protein is unable to denature duplex DNA (Casenave *et al.*, 1984; Kowalczykowski, unpublished results) and this appears to be the result of some kinetic limitation. In addition, there

is a dramatic lag in the rate of ATP hydrolysis when duplex DNA is used as a substrate (Kowalczykowski, 1985). This lag is more pronounced at higher magnesium concentrations and is possibly the kinetic reason for the inability of RecA protein to melt into the duplex regions of single-stranded M13 DNA at high magnesium concentrations, as described here. Thus, one role of SSB protein is to eliminate this kinetic limitation. In addition, the observation that, at low RecA protein concentrations, SSB inhibits the ATPase activity, while at high RecA protein concentrations SSB protein stimulates it, suggests that a competition between these two proteins is occurring and that the net outcome of this competition is likely to be determined by a combination of equilibrium and kinetic considerations.

The direct DNA-binding studies described in the accompanying paper (Kowalczykowski *et al.*, 1987) demonstrate that SSB protein will readily displace cofactor-free RecA protein from single-stranded DNA, but it is unable to displace the ATP-γ-S–RecA protein complex from DNA. Since we have demonstrated that both ATP-γ-S and ATP binding induce a high-affinity state of RecA protein for single-stranded DNA (Menetski & Kowalczykowski, 1985), we would propose that this form of the protein is responsible for the displacement of SSB protein. However, ATP hydrolysis results in the formation of a low affinity ADP-bound state of the protein, and the equilibrium studies demonstrate that SSB protein is capable of displacing this species of RecA protein unless it binds another ATP molecule. Thus, as stated above, the outcome of this competition is dependent upon the relative rates of the relevant association and dissociation events. All of these ideas are consistent with the ATP concentration dependence effects observed in the presence of SSB protein (Fig. 10). At the lower ATP concentrations, SSB protein is able completely to inhibit the ATPase activity of RecA protein (presumably as a result of its displacement from the DNA), whereas at higher ATP concentration, no inhibition is observed. These results also implicate the ATP-bound form of RecA protein as being important in the kinetic stability of this complex. Interestingly, the ATPase activity of RecA protein becomes more processive at higher ATP concentrations, with RecA protein hydrolyzing up to 10 to 20 ATP molecules prior to displacement from the DNA (Kowalczykowski & Krupp, unpublished results). This kinetic property may also be important to the steady-state outcome of the DNA binding competition between RecA and SSB proteins. Unfortunately, only a limited amount of information is available concerning the dynamics of these interactions and further studies will be required to understand them more completely.

(c) *Relationship to other studies*

Recently, Tsang *et al.* (1985) isolated and characterized presynaptic complexes of RecA protein and single-stranded M13 DNA that were formed under a variety of conditions. They found that in 1 mM-$MgCl_2$ or in 13 mM-$MgCl_2$ in the presence of SSB protein, the molar binding stoichiometry of RecA protein to DNA was 1 : 3·7. However, in 4 to 13 mM-$MgCl_2$ the binding stoichiometry was observed to be $\approx$1 : 12 when SSB protein was absent. These values are in excellent agreement with the stoichiometries obtained from the ATPase activity studies described here; i.e. 1 : 4 at 1 mM-$MgCl_2$, or at 4 mM and 10 mM-$MgCl_2$ in the presence of SSB protein, *versus* 1 : 12 at 4 mM and 10 mM-$MgCl_2$ in the absence of SSB protein. In addition, analysis of the binding data in their Table 3 shows that the saturation by SSB protein occurs at an approximate stoichiometry of 20 nucleotides per SSB protein monomer; this is in good agreement with the stoichiometry of 15 nucleotides per SSB protein monomer that we obtained from Figure 3. Finally, Tsang *et al.* (1985) demonstrated that at 1 mM-$MgCl_2$, SSB protein reduced the amount of RecA protein bound to the DNA by approximately 50%, which is in qualitative agreement with our binding studies and ATPase activity results that demonstrate almost complete inhibition and displacement of RecA protein by SSB protein.

Our data and that of Tsang *et al.* (1985) also provide a consistent explanation of the variation in binding stoichiometry for RecA protein that has been reported. These data demonstrate that the binding stoichiometry obtained using single-stranded M13 DNA will change by approximately threefold when measured at low *versus* high magnesium concentrations. A 2·5-fold difference in binding stoichiometry has been observed by Morrical & Cox (1985) and can be attributed solely to effects on secondary structure of the DNA. In agreement, our ATPase activity data demonstrate that there is no variation in the apparent RecA protein-binding stoichiometry with magnesium chloride concentration, when single-stranded DNA lacking secondary structure such as poly(dT), poly(dA) or etheno M13 DNA is used. Thus, the modified etheno M13 DNA is a more suitable substrate than is natural M13 single-stranded DNA for use in RecA protein–DNA-binding studies because it avoids the complications introduced by changes in secondary structure stability resulting from variations in parameters such as $MgCl_2$ and NaCl concentrations.

These results also provide insight into the DNA competition experiments described by Cox & Lehman (1982), which led to the proposal that SSB protein stabilizes the RecA protein–DNA complexes. In their experiments, RecA protein (0·9 μM) was added at a concentration sufficient to bind only the initial amount of single-stranded DNA present (3·3 μM). This concentration was determined using a stoichiometry of four nucleotides per RecA protein monomer and is correct provided that SSB protein is present. In the absence of SSB protein, however, only approximately one-third to one-half of the single-stranded M13 DNA is available to the RecA protein for

binding. Therefore, approximately one-half to two-thirds of the added RecA protein is free to bind rapidly to the challenge DNA and subsequently to participate in strand assimilation, as is experimentally observed. In the presence of SSB protein, however, most of the RecA protein will be associated (although only transiently) with the initial single-stranded DNA and, therefore, is unable to instantaneously react with the challenge DNA. Thus, transfer of RecA protein to the challenge DNA molecule will be limited by the rate of dissociation of RecA protein from the initial DNA, which has a half-time of approximately five minutes (Kowalczykowski *et al.*, 1987). Superimposed on this limitation is the likelihood that the free SSB protein would bind to the challenge DNA first, thereby resulting in a further kinetic inhibition of the association of RecA protein binding with the challenge DNA. These two effects would strongly favor the participation of the initial RecA protein–single-stranded DNA complex in the strand assimilation reaction in the presence of SSB protein, to the exclusion of the challenge DNA. Thus, in the absence of SSB protein, a rapid exchange between single-stranded DNA molecules would be observed, resulting in equal participation in strand assimilation, but in the presence of SSB, the transfer of RecA protein to the challenge DNA would be inhibited, resulting in the absence of participation of the challenge DNA in strand assimilation.

The interpretation of the stimulatory effects of SSB protein described here are not necessarily confined to the ATPase activity of RecA protein. Both Resnick & Sussman (1982) and Moreau & Roberts (1984) demonstrated that SSB protein stimulates the protease activity of RecA protein when the RecA protein concentration is in excess of the DNA concentration. Analysis of their data demonstrates that both the initial rate of repressor cleavage and the optimal rate of cleavage increases approximately 2 to 2·5-fold. These results are consistent with the interpretation that approximately one-half to one-third of the single-stranded DNA is inaccessible to RecA protein due to the presence of secondary structure, unless SSB protein is present. Therefore, it is possible that the stimulatory effect of SSB protein on protease activity has the same molecular basis that we have discussed regarding the ATPase activity.

The stimulatory effect of SSB protein on ATPase activity can be observed if another helix-destabilizing protein is substituted, i.e. gene *32* protein. Gene *32* protein stimulates both the ATPase activity of RecA protein, as well as the strand assimilation activity (Shibata *et al.*, 1980; Weinstock *et al.*, 1982). Thus, either helix-destabilizing protein can function in these reactions and the competition of either of these proteins with RecA protein for DNA binding is an important aspect of the proper function of this system. It appears that the outcome of the binding competition between RecA protein and a helix-destabilizing protein such as SSB or gene *32* protein is the result of a delicate balance between their relative DNA-binding affinities, association and dissociation rates, and protein concentrations. Alterations in this balance could have a profound impact on RecA protein function: for example, if the helix-destabilizing protein does not have a sufficiently high affinity for single-stranded DNA, then it may be unable to denature secondary structure (Fig. 12, step (2)) and, therefore, limit access of RecA protein to these regions, resulting in an inhibition of presynaptic complex formation. Alternatively, if the helix-destabilizing protein binds too tightly to single-stranded DNA, then RecA protein will be unable to displace it and inhibition would also result (Fig. 12, step (3)). If such a delicate balance does exist, then one might expect to find SSB protein mutants whose phenotypes are characterized by defects in SOS induction or recombinational repair as a consequence of either increased or decreased SSB protein DNA-binding affinity. Two such SSB protein mutants have, in fact, been described. Resnick & Sussman (1982) and Cohen *et al.* (1983) demonstrated that the SSB-113 protein, which is defective in SOS induction, inhibits the protease activity of RecA protein, and Chase *et al.* (1984) demonstrated that SSB-113 protein binds to single-stranded DNA more tightly than does the wild-type protein. Thus, the mutant phenotype of the SSB-113 protein may actually result, in part, from its *increased* DNA-binding affinity, which could result in the displacement of RecA protein from single-stranded DNA. The second SSB mutant protein, SSB-1, results in a protein with a lower affinity for single-stranded DNA (Williams *et al.*, 1984) and which is also defective in a variety of RecA protein-related functions (Chase *et al.*, 1983). The SSB-1 protein is much less effective in duplex DNA destabilization and therefore might be expected to be defective in step (3) of our model in Figure 12. Thus, our model can provide a molecular explanation for the effect of two very different SSB mutant proteins on RecA protein function. Biochemical studies such as those described here may be useful to further understand the molecular basis of these SSB protein defects.

The authors are grateful to Dr A. J. Clark (University of California at Berkeley) and Dr Roger McMackin (Johns Hopkins University) for sending strains, to Dr Charles Radding (Yale University) for communicating results prior to publication, and to Joseph Menetski and Linda Roman for critical reading of the manuscript. This work was supported in part by funds from the National Institutes of Health (grant AI-18987) and from the Earl M. Bane Biomedical Research Fund, and by an American Cancer Society Junior Faculty Research Award (JFRA-70).

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Edited by A. Klug